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Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

# CHARAKTERIZACE MULTIREZISTENTNÍCH IZOLÁTŮ *KLEBSIELLA PNEUMONIAE* A *ENTEROCOCCUS FAECIUM* SPEKTROSKOPICKÝMI A GENOTYPICKÝMI METODAMI

Diplomová práce

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Hradec Králové 2016

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# CHARACTERISATION OF MULTIDRUG RESISTANT *KLEBSIELLA PNEUMONIAE* AND *ENTEROCOCCUS FAECIUM* ISOLATES BY SPECTROSCOPIC AND GENOTYPIC METHODS

Diploma thesis


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**Declaration**

"I declare that this thesis is my original work. All literature and other sources that I used during my work are stated in the literature list and cited properly. This work has not been used to achieve same or another degree."

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# Abstract

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Title: Characterisation of multidrug resistant *Klebsiella pneumoniae* and *Enterococcus faecium* isolates by spectroscopic and genotypic methods.

Ever increasing antimicrobial resistance is currently a worldwide problem, traditionally addressed by DNA-based approaches. This study aimed to evaluate the potential of Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) for the characterization of multidrug resistant carbapenemase-producing *K. pneumoniae* and *E. faecium* isolates. We analysed 20 clinical *K. pneumoniae* isolates obtained from different community laboratories from Portugal between March 2014 and September 2015 and 143 previously characterized vancomycin-resistant *E. faecium* isolates obtained from humans, animals, and the environment in 26 countries between 1992 and 2015. Isolates were primarily characterized by genotypic methods including antimicrobial susceptibility testing, detection of carbapenemases and extended-spectrum β-lactamases (ESBLs), identification of antibiotic resistance coding transposons (Tn) and plasmids and genetic relatedness of isolates multi-locus sequence typing (MLST) and subsequently by FTIR and comparison of spectra by multivariate data analysis. *K. pneumoniae* isolates produced KPC-3 and ESBLs (SHV or CTX-M types) and were resistant to aminoglycosides (76 %), carbapenems (70 %) or nitrofurantoin (55 %). $Bla_{KPC-3}$ was identified within Tn *4401* variant "d" and IncFIA and IncN plasmids. Using FTIR analysis, we were able in less than 48 hours to distinguish five clones that perfectly matched results obtained by MLST identifying sequence types ST147, ST15, ST231, ST348, and ST109. *E. faecium* showed 24 sequence types belonging to 6 BAPS (Bayesian Analysis of Population Structure) subgroups (2.1a, 2.1b, 3.1, 3.2, 3.3a1, 3.3a2) which were discriminated by FTIR analysis. FTIR-ATR coupled with multivariate data analysis was shown to be a promising money and time saving alternative tool for fast assessments of clonal relationships among clinically relevant *K. pneumoniae* and *E. faecium* isolates.

# Abstrakt

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Stále se zvyšující antimikrobiální rezistence je v současné době celosvětovým problémem. Mezi významné metody charakterizace antimikrobiální rezistence patří tradičně genomické postupy. Cílem této studie bylo zhodnotit potenciál metody Fourier transform infrared spectroscopy se zeslabeným kompletním odrazem (FTIR-ATR) pro identifikaci multirezistentních karbapenemasu produkujících *K. pneumoniae* a *E. faecium* izolátů. Analyzovali jsme 20 klinických *K. pneumoniae* izolátů získaných z různých laboratoří v Portugalsku v období od března 2014 do září 2015 a 143 dříve popsané vankomycin-rezistentní *E. faecium* izoláty získaných od lidí, zvířat a životního prostředí v 26 zemích mezi lety 1992 a 2015. Izoláty byly primárně charakterizovány genotypovými metodami, včetně testování antimikrobiální citlivosti, detekce karbapenemáz a betalaktamáz s rozšířeným spektrem (ESBLs), identifikace odolnosti vůči antibiotikům transpozonů a plazmidů a genetické příbuznosti izolátů multilokusovou sekvenční typizací (MLST) a následně pomocí metody FTIR a porovnání spekter vícerozměrné analýzy dat. *K. pneumoniae* izoláty produkovaly KPC-3 a variabilně ESBL (SHV nebo CTX-M typy) a byly rezistentní k aminoglykosidům (76 %), karbapenemům (70 %) nebo nitrofurantoinu (55 %). $Bla_{KPC-3}$ byla identifikována v transposonu (Tn) 4401 varianty "d" a IncFIA and IncN plasmidech. Pomocí FTIR-ATR analýzy jsme byli schopni v době kratší než 48 hodin rozlišit pět klonů, což odpovídalo výsledkům získaným metodou MLST (sekvenční typy ST147, ST15, ST231, ST348, ST109). Bylo identifikováno 24 sekvenčních typů *E. faecium*, které byly přiřazeny do 6 BAPS (Bayesian analýzy populační struktury) podskupin (2.1a, 2.1b, 3.1, 3.2, 3.3a1, 3.3a2), které byly určeny FTIR analýzou. Bylo prokázáno, že FTIR-ATR spojená s MLST by mohly být levnou a časově nenáročnou alternativou pro rozpoznání klonálních vztahů mezi klinicky relevantními *K. pneumoniae* a *E. faecium* izoláty.

# Obsah

1. Abbreviations ........................................................................................10

2. Introduction ...........................................................................................12

3. Theoretical part......................................................................................14

3.1. Antimicrobial resistance .....................................................................14

3.1.1. Efflux pumps ....................................................................................15

3.1.2. Modification of target structures ......................................................16

3.1.3. Reduction of permeability .................................................................16

3.1.4. β-lactamases ....................................................................................17

3.1.5. Carbapenemases ..............................................................................19

3.2. Vehicles of bacterial resistance spreading...........................................20

3.2.1. Plasmids............................................................................................20

3.2.2. Transposons.......................................................................................21

3.2.3. Integrons and gene cassette ..............................................................22

3.2.4. Insertion sequences...........................................................................22

3.3. Antibiotics ...........................................................................................22

3.3.1. β-lactam antibiotics ...........................................................................23

3.3.2. Vancomycin.......................................................................................24

3.4. *Klebsiella pneumoniae* ..........................................................................25

3.4.1. Occurrence and pathogenicity of *K. pneumoniae*.............................25

3.4.2. Possible therapy options for resistant form of *K. pneumoniae*:.........26

3.5. The genus *Enterococcus*........................................................................27

3.5.1. Occurrence of Enterococci .................................................................27

3.5.2. Characterisation and epidemiology of vancomycin resistant *Enterococcus* .......................................................................................27

3.5.3. Mechanism of resistance in Enterococci ...........................................29

3.5.4 Population structure of *E. faecium* ... 29
3.5.5. Possible therapy options ... 31
3.6. Identification of mechanisms of antibiotic resistance ... 32
3.6.1. Antimicrobial susceptibility testing ... 32
3.6.2. Blue Carba test ... 32
3.7. Methods for bacterial typing ... 33
3.7.1. Multilocus sequence typing ... 33
3.7.2. Fourier transform infrared spectroscopy with attenuated total reflectance ... 34
4. Goals ... 35
5. Experimental part ... 36
5.1. Chemicals and preparation of solutions ... 36
5.1.1. Phenotypic methods ... 36
5.1.2. Genotypic methods ... 36
5.2. Origin of samples ... 37
5.2.1. *Klebsiella pneumoniae* ... 37
5.2.2. Enterococcus ... 37
5.3 Experimental procedures ... 42
5.3.1. Blue Carba ... 42
5.3.2. Antimicrobial susceptibility testing (disk diffusion and E-test) ... 43
5.3.3. Polymerase chain reaction ... 43
5.3.4. Multilocus sequence typing ... 44
5.3.5. Purification of DNA ... 44
5.3.6. DNA sequencing ... 44
5.3.7. Fourier transform infrared spectroscopy with attenuated total reflectance ... 45
6. Results ... 46
6.1. *Klebsiella pneumoniae* ... 46

6.1.1 Clonal relationships ........ 46
6.1.2. Antimicrobial susceptibility patterns and detection of carbapenemases or extended-spectrum β-lactamases ........ 46
6.1.3. Plasmids and transposons ........ 47
6.2. *Enterococcus* ........ 51
6.2.1. Discrimination by BAPS subgroups ........ 51
6.2.1. Discrimination by STs ........ 51
7. Discussion ........ 54
7.1. Characterisation of KPC-3-producing *K. pneumoniae* isolates ........ 54
7.2. FTIR is a suitable method for the rapid identification of isolates ........ 55
8. Conclusions ........ 57
9. References ........ 58

# 1. Abbreviations

| | |
|---|---|
| AST | Antimicrobial susceptibility testing |
| BAPS | Bayesian Analysis of Population Structure |
| *bla* gene | β-lactamase gene |
| C | Clone |
| CTX-M | Cefotaximase |
| ESBL | Extended-spectrum β-lactamase |
| EUCAST | European Committee for Antimicrobial Susceptibility Testing |
| FTIR | Fourier transform infrared spectroscopy |
| FTIR-ATR | Fourier transform infrared spectroscopy with attenuated total reflectance |
| HG | Housekeeping genes |
| Inc | Incompatibility group |
| K types | Capsular types |
| KPC | *Klebsiella pneumoniae* carbapenemase |
| LV | Locus variant |
| MDR | Multidrug resistance |
| MGE | Mobile genetic element |
| MHA | Mueller-Hinton agar |
| MIC | Minimum inhibitory concentration |
| MLST | Multilocus sequence typing |
| NDM | New Delhi metallo-β-lactamase |
| OXA | Oxacillinase |
| PCR | Polymerase chain reaction |
| PFGE | Pulsed-field gel electrophoresis |
| PLSDA | Partial least square discriminant analysis |
| SHV | Sulphydryl variable β-lactamase |
| ST | Sequence type |
| TEM | Temoniera β-lactamase |
| Tn | Transposon |

| | |
|---|---|
| URI | Urinary tract infection |
| VIM | Verona integron-encoded metallo-β-lactamase |
| VRE | Vancomycin resistant Enterococcus |

# 2. Introduction

Since introduction of antibiotic pharmacotherapy clinicians have had to face problems associated to bacterial resistance. However, since of 21st century antibiotic resistant bacteria with limited treatment options had escalated to a global health crisis *(Wright, 2011, Blair et al., 2015)*. Clinically relevant antibiotic non-sensitive strains in hospitals worldwide belong most often to the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*), whose name implies their ability to "escape" to antibiotic treatment *(Boucher et al., 2009, Rice, 2008).* These pathogens reveal multidrug resistance (MDR), extremely drug resistance or even pan drug resistance *(Woodford et al., 2011, Falagas and Bliziotis, 2007)*. Because *S. aureus*, *P. aeruginosa* and *A. baumannii* are beyond the scope of this diploma thesis, mechanisms of antibiotic resistance of *K. pneumoniae* and *Enterococcus* will be reviewed only.

Multidrug resistant and vancomycin resistant Enterococci (VRE) and multidrug resistant *K. pneumoniae* producing carbapenemases are both particularly resistant opportunistic nosocomial pathogens widely capable of horizontal transfer of antibiotics resistance genes even to other species *(Munoz-Price and Quinn, 2009, Podschun and Ullmann, 1998, Willems et al., 2012, Zirakzadeh and Patel, 2006)*. VRE *faecium* or extended-spectrum β-lactamase (ESBL) and/or *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* cause serious health complications such as urinary tract infections (UTI), bacterial pneumonia, intra-abdominal infections, bacteraemia, endocarditis and other severe infections occurring usually in immunocompromised patients in intensive care units or healthcare institutions. Moreover, the costs to treat complications caused by resistant bacteria are supposed to exceed €1.5 billion per year in Europe *(Blair et al., 2015, Podschun and Ullmann, 1998, Zirakzadeh and Patel, 2006, Struve and Krogfelt, 2003, Schembri et al., 2005)*.

The first case of KPC infection was reported in eastern USA in 1996 *(Nordmann and Poirel, 2014, Chen et al., 2014a)*. However, during last twenty years, outbreaks of KPC producing isolates have been increasingly evident worldwide, e.g. Colombia, France, China, Ireland, Puerto Rico, UK and many others *(Nordmann et al., 2009)*. *K. pneumoniae* sequence type (ST) 258 play key role in the KPC worldwide dissemination and in particularly high superiority of these enzymes in Israel, Italy, Greece and USA *(Munoz-Price et al., 2013, Villa et al., 2013)*. Carbapenemases are bacterial

enzymes capable to hydrolyse a wide spectrum of β-lactam ring derived compounds including first-, second- and third-generation of cephalosporins, monobactams and carbapenems, and also β-lactam inhibitors such as clavulanic acid *(Chen et al., 2014a, Shaikh et al., 2015)*. VRE forms in the presence of inducer (glycopeptide antibiotics) in the cell wall resistant precursors with different termini such as D-Ala-D-Lac or D-Ala-D-Ser instead of the original responsive D-Ala-D-Ala sequence *(Murray, 2000)*. Thus, early detection of these multidrug resistant pathogens in the nosocomial setting is crucial to optimize infection control and therapeutic decisions.

Methods for genetic and phenotypic characterization of *K. pneumoniae* isolates include polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism *(Woodford et al., 2011)*, multilocus sequence typing (MLST), double-disk synergy test, the E-test, and others reviewed by *Al-Bayssari et al*. (2015) or by *Chen at al.* (2014a). Currently spectrophotometry assay usually followed by PCR are gold standard methods for detection of KPC enzymes coding gene *(Hirsch and Tam, 2010)*. Furthermore, clinical microbiology laboratories frequently use automated methods for phenotyping, i.e. antibiotic susceptibility testing, chromogenic agar-based tests for routine analysis of surveillance, colonization, infection, and outbreaks of multidrug resistant bacteria isolated from patients *(Coburn et al., 2014a, Zirakzadeh and Patel, 2006)*.

All methods available commonly have certain limitations in terms of time-, money-, quality of the sample- demands and/or they are quite expensive, therefore **it is still of clinical and epidemiological importance to develop new methods to meet the criteria of high throughput and sensitive detection system while saving time and money** *(Shaikh et al., 2015)*. Therefore, in the present thesis we aimed to explore the potential of Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) alone or coupled with multivariate analysis data for discrimination of main multidrug resistant clones causing human infections.

# 3. Theoretical part

## 3.1. Antimicrobial resistance

World Health Organization defines antimicrobial resistance as resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it *(WHO, 2015)*. Therefore, increasing use of antibiotics leads to elevated tolerance of bacteria. Particular types of resistance are not uniformly defined. In this thesis we will use one term, i.e. MDR is crossresistance to three or more antibiotic classes *(Tenover, 2006, Woodford et al., 2011)*.

Resistance may be distinguished based on its origin. Intrinsic resistance is caused by natural features of bacteria (e.g. type of cell wall, production of β-lactamases, etc.) and acquired resistance that is obtained by horizontal or vertical transmission of mobile genetic elements (e.g. by plasmids) coding determinants of resistance during cell life *(Zhou et al., 2015, Ruppe et al., 2015, Alanis, 2005).*

Genetic information responsible for the resistance among bacteria can be transferred through several pathways, i.e. conjugation, transformation and transduction (via transposons). **Conjugation** is the most common approach of horizontal bacterial resistance transmission that is based on transfer of plasmids, containing resistant genes, between Gram-negative bacteria through a special elongated protein tool called a *pilus* and between Gram-positive bacteria thanks to sex pheromones. **Transduction** is the process whereby the genetic information of one cell is transported into the second cell via "vector", most often a bacterial virus (bacteriophage). This method transmits both bacterial and viral DNA. **Transformation** occurs when the bacteria adopt and incorporate the DNA segments from the surrounding environment ("naked DNA"), which is released from lysed cells there. Such DNA may carry resistance determinants and formerly susceptible bacterium becomes *(Tenover, 2006, Alanis, 2005)*. For instance *K. pneumoniae* resistant to carbapenems could use multiple mechanisms comprising hyperproduction of enzymes (e.g. ESBLs and carbapenemases) and alterations in outer membrane permeability together along with upregulation of efflux systems *(Chen et. al 2014a)*. Further types of resistance encountered by various bacteria are shown in Figure 1.

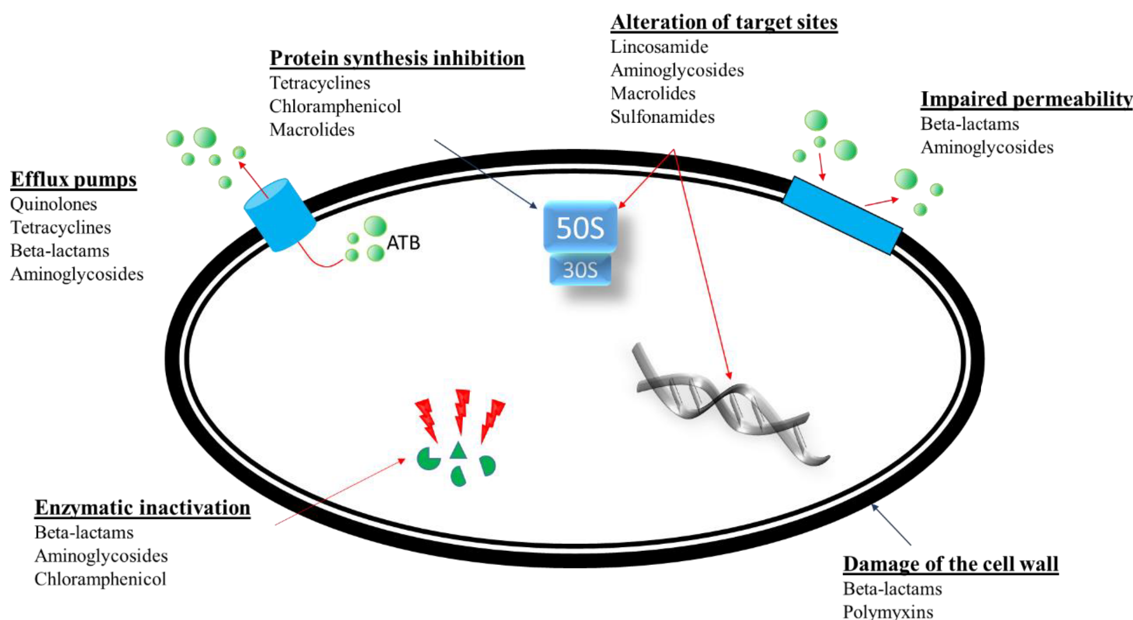


**Figure 1**: A few actions of antibiotics and mechanisms of antimicrobial resistance

Recent research from *Zhou et al.* (2015) newly suggests three types of mechanisms against antimicrobial agents; i) biofilms, which are responsible for limiting penetration of the antibiotics to the bacterial body (not shown), ii) structural, such as the cell wall, cytoplasmic membrane, encased efflux pumps, and iii) modifications of intracellular materials (target structures, impaired permeability) or capability to convert toxic compounds into nontoxic metabolites. Mechanisms of resistance can therefore be generally classified as non-enzymatic or antibiotic-inactivating enzymes dependent

Adapted from: *Shaikh et al. (2015), Zhou et al. (2015), Ruppe et al. (2015), Tenover (2006)*

### 3.1.1. Efflux pumps

Efflux process removes bacteria-threatening substances (e.g. antibiotics, toxins) from the interior of the cells. This process requires specific transport proteins, i.e. efflux pumps found in both Gram-positive (except of the resistance-nodulation-division family), Gram-negative bacteria, and even eukaryotic cells. Some efflux systems are drug-specific, whereas others may accommodate multiple drugs, and thus contribute to bacterial MDR *(Webber et Piddock, 2002, Sun et al., 2014, Butaye, 2003)*.

This type of mechanism was first described by *McMurry et al.* (1980) in *Escherichia coli* resistant against tetracycline. Nowadays, efflux mechanisms are disseminated across wide-range of bacteria populations *(Butaye, 2003)*.

According to several criteria such as energy dependence or localization bacterial efflux transporters in the prokaryotes are categorized into five major families; the major facilitator superfamily (MFS), the multidrug and toxin extrusion (MATE), the resistance-nodulation-division (RND), the small multidrug resistance (SMR) or ATP-binding cassette (ABC) superfamily *(Webber et Piddock, 2002, Sun et al., 2014, Butaye et al., 2003).*

### 3.1.2. Modification of target structures

Bacteria can increase their ability to survive by changing target structures of the antibiotic. If an antibiotic drug cannot bind properly due to a structural change in the target site, antibiotics lose their efficacy. These changes can occur quickly with minimal propensity to alter cellular functions (microbe fitness) or genes encoding target molecules *(Shaikh et al., 2015, Wright, 2011, Blair et al., 2015).*

In this way, bacteria have developed resistance to many clinically important antibiotics. For instance, the chloramphenicol-florfenicol resistance methyltransferase is responsible for posttranslational methylation in the 23S RNA subunit and foreclosures access of the target sites for a wide range of drugs such as oxazolidonones (including linezolid), pleuromutilins, phenicols, lincosamides and streptogramins. Another example is mutation of penicillin-binding proteins, resulting in resistance to β-lactams *(Blair et al., 2015, Alanis, 2005).*

### 3.1.3. Reduction of permeability

Gram-negative bacteria are naturally less permeable to most of antibiotics when compared with Gram-positive species. Gram-negative bacteria have an outer membrane that serves as an extra permeability barrier. Hydrophilic drugs are not able to pass through this lipophilic membrane without porin proteins built-in in the membrane. Most members of *Enterobacteriaceae* have main porins (e.g. OmpF and OmpC of *E. coli*) that function as non-specific channels. In order to prevent passage of antibiotics, bacteria reduce number of porin channels or replacing of non-selective porins with those more-selective *(Blair et al., 2015).*

### 3.1.4. β-lactamases

β-lactamases are highly divergent and are clustered into several families and variants. These enzymes are the most relevant mechanism of resistance since it is often plasmid-mediated and then transmissible while having powerful ability to hydrolyse the β-lactams antibiotics already in the periplasmic space. These enzymes constitute the main origin of β-lactams resistance in *Enterobacteriaceae* family *(Pitout 2006, Ruppe et al., 2015)*.

There are two basic schemes to cluster β-lactamases, i.e. Ambler classification and Bush–Jacoby–Medeiros functional system. Despite high diversity of β-lactamases, only a few of them are epidemiologically relevant. Ambler classification categorizes β-lactamases according to characterisation of molecular structure (amino acid sequence and conserved motifs) into 4 classes: A, B, C, or D. Bush–Jacoby–Medeiros functional system is used as additional characterisation of β-lactamases to substrate and inhibitor profiles *(Bush, 2013, Naas et al., 2008a, Shaikh et al., 2015)*.

Ambler classification (Figure 2): β-lactamases class A, C and D are serine-based enzymes, since a conserved serine in the active site is responsible for nucleophile attack on the β-lactam C–N bond. Class A includes the majority of ESBLs, i.e. Temoniera (TEM), suplhydryl variable (SHV) and cefotaximase (CTX-M) *(Jeon et al., 2015, Naas et al., 2008a, El Salabi et al., 2013)*. Enzymes belonging to class B are well-known as metallo-β-lactamases, since they require a zinc cofactor and water molecule to activate and split the β-lactam ring *(Jeon et al., 2015, Naas et al., 2008a).*

In the 1980s and 1990s, the most widespread ESBLs were mutants of parent enzymes with narrow spectrum of activity - TEM and SHV (derivatives of TEM-1, TEM-2 and SHV-1). First ESBL known as SHV-2 (derived by point mutation from SHV-1) was identified in 1983. However, CTX-M β-lactamases represent most β-lactamases in the present day *(Canton et al., 2012, Lal et al., 2007 Naas et al., 2008a, Paterson and Bonomo, 2005, Shaikh et al., 2015)*.

Usually, ESBLs hydrolyze oxy-imino-cephalosporins (third and fourth generation of cephalosporins) and monobactams. ESBLs are often found in large plasmids carrying other antibiotic resistance genes (e.g. against sulphonamides, trimethoprim, chloramphenicol). However, ESBL can be somewhat susceptible both to cephamycins such as cefoxitin and carbapenems and β-lactamase inhibitors (*Canton et al., 2012, Pitout, 2006)*.

*K. pneumoniae* and *E. coli* are the most common *Enterobacteriaceae* species producing ESBLs, although the presence of these enzymes was also observed in other *Enterobacteriaceae*. Currently, there are more than 200 different types of ESBLs characterized *(Canton et al., 2012, Paterson and Bonomo, 2005*, *Zaniani et al., 2012)*.

Enzymes are responsible for resistance to at least some members of 3rd generation cephalosporins and aztreonam. β-lactamase inhibitors may still be a potential treatment for a limited time *(Heritage et al., 1999, Lal et al., 2007, Paterson and Bonomo, 2005)*.

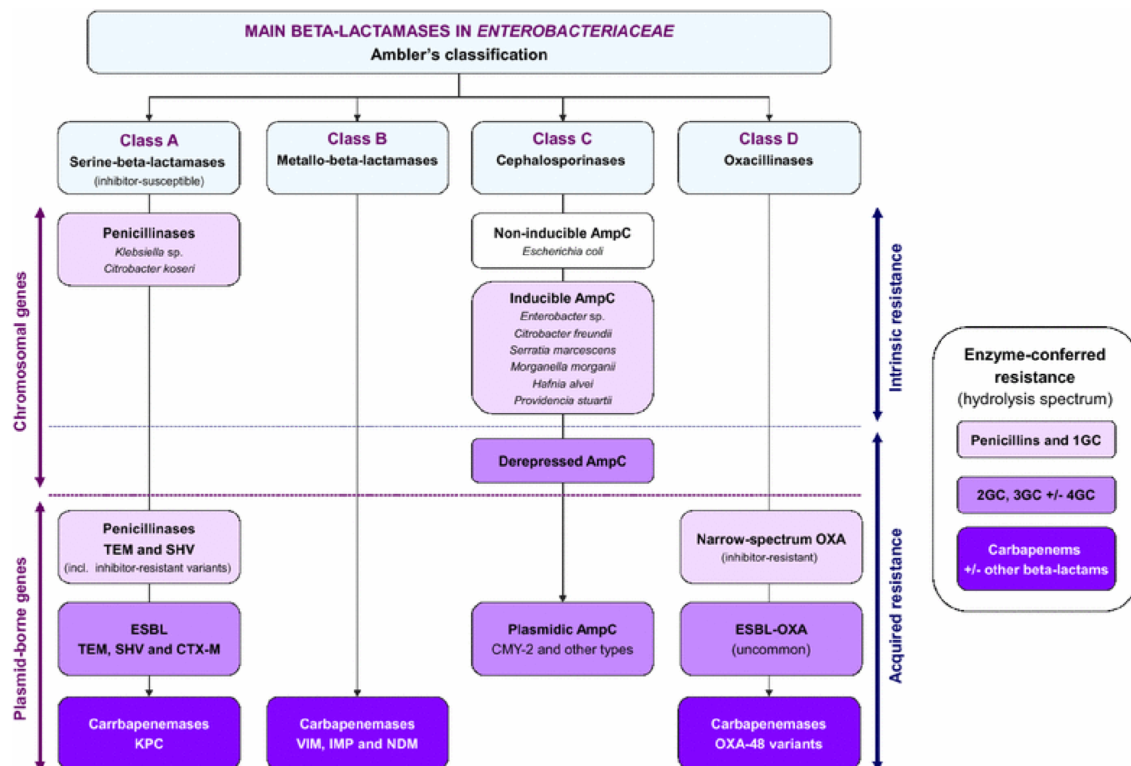


**Figure 2:** Individual classes of β-lactamases occurring in *Enterobacteriaceae*

Taken from: *Ruppe et al*. (2015)

CTX-M β-lactamases (and also TEM and SHV ESBLs) are capable to inactivate broad-spectrum of cephalosporines, but generally CTX-M enzymes they exhibit greater activity against cefotaxime (a β-lactam antibiotic of 3rd generation) than against ceftazidime (a β-lactam antibiotic of 3rd generation) by hydrolysis *(Paterson and Bonomo, 2005)*. CTX-M enzymes have a several fold higher sensitivity to tazobactam than to clavulanic acid (*Canton and Coque, 2006*, *Hartl et al., 2006, Paterson and Bonomo, 2005*). Origin of CTX-M enzymes is not bound on mutations of previous plasmid mediated enzymes, but is most likely linked with mobilization of chromosomal

β-lactamase genes (*bla)*, incorporated into mobile genetic elements (MGE), from *Kluyvera* sp. *(Canton et al., 2012, Hartl et al., 2006, Paterson and Bonomo, 2005, Poirel et al., 2012a, Tzouvelekis et al., 2000)*. Since the turn of the century, increased amounts of CTX-M enzymes have occurred in nosocomial and community settings worldwide. Global expansion has likely affected both, spreading out of multiple specific clones, and/or MGE. CTX-M-14 and CTX-M-15 are the most important members of CTX-M class dispersed in the environment, animals and people globally *(Canton and Coque, 2006, Canton et al., 2012)*.

### 3.1.5. Carbapenemases

Carbapenemases have high capability to hydrolyse both classical β-lactams (penicillins and cephalosporines) and carbapenems. Presence or absence of divalent cations are important for activation and therefore carbapenemases are divided into metallo-β-lactamases (zinc-dependent class B) and non-metallo-β-lactamases (zinc-independent classes A, C and D) *(Jeon et al., 2015, Ruppe et al., 2015)*. Classes A (KPC types), B Verona integron-encoded metallo-β-lactamase (VIM) and New Delhi metallo-β-lactamase types (NDM) and class D oxacillinases (OXA-48-like enzymes) are the most widespread *(Parisi et al., 2015)*.

Class A is divided into 6 distantly related branches: KPC, IMI (imipenem-hydrolyzing β-lactamase)/NMC-A (non-metallo carbapenemase of class A), SME (*Serratia marcescens* enzyme), GES (Guiana extended-spectrum β-lactamase), SFC-1 (*Serratia fonticola* carbapenemase) and SHV-38 enzyme. *Enterobacteriaceae* members are their primer originator, albeit for some of them it was not identified. A vast majority of these enzymes are inhibited by clavulanic acid or other β-lactam inhibitors. Hydrolysing profile is different for each enzyme and includes penicillins, narrow and extended-spectrum cephalosporins, monobactams, and carbapenems. This is important in the clinical setting since it prevents the use of β-lactams to treat infections caused by these bacteria *(Jeon et al., 2015, Walther-Rasmussen and Hoiby 2007)*. KPC β-lactamases hydrolyse all β-lactam antibiotics e.g. carbapenems, cephamycins, monobactams and clavulanic acid. Spread of these enzymes is most often linked with a specific clone, *K. pneumoniae* ST 258, which has been identified throughout the world with KPC-2 or KPC-3. In 2014, 22 different KPC enzymes were identified (*Chen et al., 2014a)*.

Class B is mainly composed of 4 subgroups: VIM, IMP (imipenem-resistant *Pseudomonas*), SPM-1 (Sao Paulo metallo-β-lactamase) families and emerging NDM group. They confer resistance to all β-lactam antibiotics except monobactams *(Jeon et al., 2015).*

Enzymes of the last mentioned class D, belong to the OXA family because they can hydrolyse oxacillin faster than benzylpenicilline. Clavulanic acid has insignificant effect against them. Class D was identified mainly in *Acinetobacter* family, but at least OXA-48 was identified in *Enterobacteriaceae* members *(Jeon et al., 2015)*.

## 3.2. Vehicles of bacterial resistance spreading

Vertical and horizontal gene transfer play a key role in the spread of resistance throughout a microbial population (adaptation for new environment, stressful conditions, and antibiotic resistance) and thus enhancing their survival and adaptation to different hosts and settings. Plasmids, transposons (Tn), insertion sequences and integrons constitute MGE responsible for that *(Alanis, 2005, Smillie et al., 2010, Bennett, 2008, Poirel et al., 2012a).*

### 3.2.1. Plasmids

Plasmids are circular extrachromosomal double-stranded DNA with autonomous replication and possess a *conserved region* with genes implicated in vital functions such as replication, maintenance and transfer and an *accessory region* comprising genes implicated in adaptive features such as antibiotic resistance, virulence or bacteriocin production. Plasmids encoding antibiotic resistance were first identified in *E. coli* in the 1950's, and soon after their ability to transfer between cells was recognized *(Smillie et al., 2010, Clewell, 2014, Taylor et al., 2004)*.

In Gram-negative bacteria, particularly in *Enterobacteriaceae*, they have been the main reason for the dissemination of β-lactamases (e.g. ESBLs and carbapenemases) and constitute a major obstacle to prevent the spread of antimicrobial resistance. The vast majority of the plasmids are transferred by conjugation *(Carattoli, 2013*, *Couturier et al., 1988, Clewell, 2014).*

Usually multidrug resistant plasmids are larger than 50 kb. They are self-conjugative and encode refined mechanisms, which control copy number of plasmids

in cells. Due to this strict control of replication, two plasmids cannot share the same replicon and be stably propagated in the same cell line. Therefore, this phenomenon is referred to as plasmid incompatibility, caused by the close relationship among the sequences controlling replication. This has been used to classify plasmids in homogenous groups (Incompatibility groups, Inc) in the case of *Enterobacteriaceae* *(Carattoli, 2013, Couturier et al., 1988).*

Standard method for identification of plasmids is PCR-based replicon typing, which is aimed at a different replicon plasmids of the main *Enterobacteriaceae* families *(Carattoli, 2013)*

Plasmid identification and characterization is important from a global perspective, because a better understanding of the spread of resistant bacterial features is necessary for its control *(Smillie et al., 2010)*.

### 3.2.2. Transposons

Tn are special bouncing elements transmitting and incorporating resistance genes from any site of a genomic element to another either within the same DNA molecule or between two different molecules e.g. plasmid and bacterial chromosome. Generally, Tn do not require homology of the DNA elements among which Tn spread, although some type preferences exist *(Bennett, 2008)*. Tn can be categorised into two different classes: a) the Tn3-type transposons (otherwise known as class II) include two terminal, inverted, repeated sequences: transposase, resolvase and recombinase b) other Tn known as composite (otherwise known as class I) consisted of two copies of the same or a closely related insertion sequences. Composite Tn can activate DNA fragments placed between insertion sequences *(Poirel et al., 2012a).*

Tn*4401* is a Tn*3*-based transposon with five isoforms (a-e). This Tn is the most common site of occurrence and frequent mobilization of the rapidly spreading *bla*$_{\mathbf{KPC}}$ gene *(Chen et al., 2014a, Chen et al., 2012, Cuzon et al., 2011, Munoz-Price and Quinn, 2009)*.

### 3.2.3. Integrons and gene cassette

Integrons serve as genetic units with the capability to capture, mobilize and express mobile gene cassettes comprising of the gene (usually a resistant gene) and an integrase-specific-recombination site, also known as 59-be element. Their key components consist of *int1* gene encoding recombination enzyme (integrase) and *attl* adjacent site recognized by the integrase and with functions as receptor site (*attc* insertion sequence) and expression promotor for gene cassettes *(Bennett, 2008, Poirel et al., 2012a)*.

Integrons are classified through the amino acid identity of integrase gene divided into three groups, of which the most common are Class 1 integrons. Class 1 is also known as multidrug resistance integron, widely represented among Gram-negative rods *(Bennett, 2008, Poirel et al., 2012a)*.

### 3.2.4. Insertion sequences

Insertion sequences are characterized by their largest expansion and the smallest size (<2.5 kb) among all transposable elements and capability of independent transposition. Transposase enzyme, whose activity controls these elements, is responsible for the integration process, recognizing and mobilizing of the inverted repeat sequences (at its extremities) causing integration mutations, genome rearrangements, increased transmission of resistance, and virulence factors within species (*Poirel et al., 2012a)*.

## 3.3. Antibiotics

The first use of sulfonamide antibiotics, followed by the discovery of newer agents such as penicillins, macrolides, tetracyclines, cephalosporins and many other antibiotics brought undisputed global benefit. Antibiotics reduced morbidity and mortality and therefore they allowed the creation of new medical disciplines associated with the high risk of infectious complications, e.g. organ transplants and hematooncology. However, more facts about the incidence of infections caused by bacteria resistant to antibiotics and other antibacterial agents have been spreading in the present day *(Nyč, 2007)*.

These strains, sometimes referred to as “superbugs”, are resistant to both older and the latest drugs *(Nyč, 2007, Alanis, 2005)*.

More worrisome is the fact that the so-called "golden age" of antibiotic development took relatively short time from 1940 to 1960. During which time a substantial part of our current repertoire of antibiotics from natural sources were discovered *(Spizek et al., 2010)*. Since 1962, when quinolones and streptogramins were introduced, it took another 38 years to introduce oxazolidinones in 2000, and since the turn of the millennium, three new antibiotic classes have been introduced *(Walsh and Wencewicz, 2014*, *Bassetti et al., 2013, Nyč, 2007)*. Hence, Infectious Diseases Society of America approved program titled the "10 x 20" initiative according to which it is scheduled to develop ten new, safe and effective antibiotics by 2020 both from new drug classes and also from classes already known *(Infectious Diseases Society of, 2010, Bassetti et al., 2013)*.

Although there are undoubted advantages of antibiotics, it should be remembered, that their inappropriate and indiscriminate use has probably initiated the beginning of a post antibiotic era *(Nyč, 2007, Alanis, 2005)*.

### 3.3.1. β-lactam antibiotics

In 1928 sir Alexander Fleming observed antibacterial effect of unknown substance produced by mildew *Penicillium notatum* on Petri dish with Staphylococci. Pure substance (penicillin, named by Fleming) was isolated in 1939 by a team around Florey and Chain. Subsequently, penicillin was successfully used in clinical practice with mass production starting in 1943 *(Hartl et al., 2006)*.

For β-lactam antibiotics is the hallmark of the presence of four-membered β-lactam ring (Figure 6), this essential component is responsible for their antibacterial activity. All antibiotics of this category have a common mechanism of action, which is based on the inhibition of the formation of bacterial cell wall. β-lactam antibiotics are divided to natural: penicillins and cephalosporins (isolated from *Cephalosporium sp.*) and semi-synthetic: carbapenem (imipenem, doripenem, ertapenem, meropenem and biapenem) and monobactam (aztreonam) antibiotics. Cephalosporins are divided into 5 groups ($1^{st}$–$5^{th}$ generation). Third generation (or extended-spectrum) cephalosporins include ceftriaxone, cefotaxime, and ceftazidime and is mainly used for eradication hospital acquired infections caused by *Enterobacteriaceae* family. Carbapenems are used for treatment of infections caused by bacteria resistant to extended-spectrum cephalosporins. The structure of semi-synthetic β-lactam antibiotics retain only β-lactam

ring, but the other portions of the structure are from a greater or lesser part completely different *(El Salabi et al., 2013, Hartl et al., 2006, Jeon et al., 2015)*.

β-lactam antibiotics are "vulnerable" to the action of β-lactamases (Figure 6), and therefore the first inhibitors of these enzymes were invented in 1975. The first compound was clavulanic acid and by the modification of this molecule further substances came into the being, e.g. sulbactam and tazobactam *(Hartl et al., 2006)*.

**Figure 3**: β-lactam ring and effect of β-lactamases

Adapted to: *Khan (2012)*

### 3.3.2. Vancomycin

Vancomycin is a metabolic product of *Streptomyces orientalis*, which is a heterodimeric polypeptide, since the main polypeptide skeleton has glycosidically bound different types of sugars (Figure 4) *(Hartl et al., 2006)*.

**Figure 4:** Vancomycin structure

It has a narrow spectrum of action against Gram-positive bacteria (*Streptococcus* spp., *E. faecalis* and *E. faecium*; only some isolates, *Listeria monocytogenes*, *Corynebacterium jeikeium* and *Clostridium difficile*), predominantly staphylococci, by inhibiting the biosynthesis of peptidoglycan mureins, which form the basis for the construction of bacterial cell walls *(Hartl et al., 2006, Suchopár and Valentová, 2009)*. Vancomycin has a lot of side effects and the most problematic include nephrotoxicity *(Suchopár and Valentová, 2009)*.

## 3.4. *Klebsiella pneumoniae*

*Klebsiella* genus belongs to the *Enterobacteriaceae* family. These encapsulating bacteria are motile, Gram-negative and rod shaped *(Podschun and Ullmann, 1998)*. The outer capsule (constituted mainly by polysaccharides) is an important feature for protection against phagocytosis and serum initiated death. This "shield" is produced by a majority of *K. pneumoniae* members. At least 78 capsular types (K types) have been identified within *K. pneumoniae*, established primarily by serological procedures that are technically demanding and time-consuming and because of that, progressively abandoned. Each capsule of *K. pneumoniae* contains *wzi* gene, whose sequencing is simple and rapid approach for the prediction of the K types of most *K. pneumoniae* clinical isolates *(Schembri et al., 2005, Podschun and Ullmann, 1998, Brisse et al., 2013)*.

Interestingly, urea produced in human body is used by *Klebsiella* as a source of nitrogen that is necessary for its growth. It likely forms highly alkaline ammonia, which can be the main reason for serious damage of tissues and subsequently lead to a persistent chronic infection in the organism *(Maroncle et al., 2006).*

### 3.4.1. Occurrence and pathogenicity of *K. pneumoniae*

*K. pneumoniae* occurs both in the environment sources such as soil, water, sewage and in physiological tissue such as mucosal surfaces of horses, swine and, importantly, in nasopharynx and intestinal flora in humans. Antibiotic sensitive *K. pneumoniae* can be quickly transformed, most frequently into ESBL- as well as carbapenemase-producing microorganisms. This bacterium has been the most difficult to eliminate from high-risk areas such as hospice, intensive care units, etc. Similar to genus *Enterococcus*, *Klebsiella* species, especially *K. pneumoniae*, are also opportunistic pathogens; they cause up to 10 % of nosocomial infections encompassing pneumonia (mainly among alcohol-addicted people), liver abscesses, septicaemia, urinary and soft tissue infection in immunocompromised and hospitalized patients *(Van Laar et al., 2015, Podschun and Ullmann, 1998, Lal et al., 2007*, *Eftekhar and Naseh, 2015).*

The first report about ESBL-producing *Enterobacteriaceae* organisms dates back to Germany in 1983. Soon after, followed by infection outbreaks caused by these organisms in other European countries (Figure 5). The first infection with KPC was described in North Carolina in 1996. Currently, KPC is considered to be endemic in parts

of New York and New Jersey, however, KPC enzymes are disseminated in many other countries including European ones *(Knothe et al., 1983, Lautenbach et al., 2001, Munoz-Price and Quinn, 2009, Nordmann et al., 2009, Parisi et al., 2015).*

*K. pneumoniae* has revealed the increasing tendency of bacterial resistance to new antibiotics (e.g. third generation cephalosporins, carbapenems). Especially the presence of β-lactamases producers has even fatal consequences for both individuals and public health as they are often resistant to other antibiotic classes currently used *(Livermore et al., 2011, Parisi et al., 2015).*

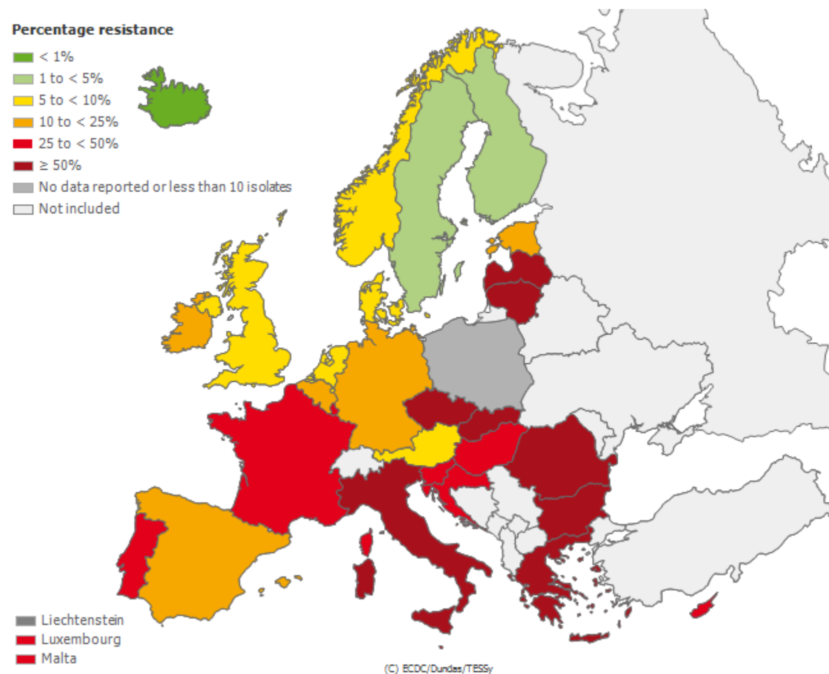


**Figure 5:** Resistant invasive isolates of *K. pneumoniae* to 3rd generation of cephalosporins in Europe. Taken from: *European centre for Disease Prevention and Control (2016a)*

### 3.4.2. Possible therapy options for resistant form of *K. pneumoniae*:

1) Infections caused by ESBL producers can be accordingly sensitive to **a**) amoxicillin-clavulanic acid or piperacillin-tazobactam, **b**) temocillin is stable against ESBLs and AmpC β-lactamases, **c**) carbapenems are preferred for septic or shock cases **d**) cephamycins have the potential to be used as an alternative for the treatment of infections due to ESBL producers *(Rodriguez-Bano, 2015).*

2) Treatment of infections caused by KPC producers remains in antibacterial agents: **a**) polymyxins (colistin) and carbapenems in particular in combination rate

of success increases rapidly, but colistin resistance has increased **b**) aminoglycosides exhibit good activity depending on the severity of the infection, **c**) tigecycline is still highly effective, **d**) cephalosporines and β-lactam/β-lactam inhibitors may also enable the effective treatment in non-systematic infections. However, none of these combination is ideal therapy for infections caused by KPC resistant isolates, although new drugs continue to be prepared *(Parisi et al., 2015, Hirsch and Tam, 2010*, *Nordmann et al., 2009).*

## 3.5. The genus *Enterococcus*

Enterococci are non-spore forming facultative anaerobic Gram-positive bacteria characterized by an enormous resistance against prolonged desiccation, oxidative stress, ionizing radiation, extreme temperatures, extreme pH (4.8–9.6), osmotic stress (up to 6.5 % NaCl) and importantly to vast majority of antibiotics. Enterococci may occur as single cell, in pairs or in chains of bacteria and many of them are of enteric origin as implied by their name. Nowadays, the genus Enterococcus includes 28 species *(Foulquie Moreno et al., 2006, Ramsey et al., 2014, Murray, 1990)*.

### 3.5.1. Occurrence of Enterococci

Enterococci occupy, due to their universal metabolic features on different niches, most frequently in the intestine, forming minor part of the normal intestinal microflora (<1 %). When a patient's health is compromised *E. faecalis* (80–90 %) and *E. faecium* might cause life threatening nosocomial infections *(Biavasco et al., 2007, Sghir et al., 1999).*

### 3.5.2. Characterisation and epidemiology of vancomycin resistant *Enterococcus*

VRE have been rising in different countries for the last three decades. They significantly contribute to the restriction of the using of glycopeptides in clinical settings and can even transmit vancomycin resistance to other bacterial genus. The first such case was documented in Michigan in 2003. It occurred between VRE and methicillin resistant *Staphylococcus aureus* within one patient who was infected with both bacteria. Since VRE (VanA phenotype) was first isolated in the United Kingdom in the late 1980´s, the incidence of resistant cases has steadily risen. Current (2014) occurrence of resistant

VRE *faecium* isolates in Europe is summarized in Figure 6 *(Thaker et al., 2015, Zirakzadeh and Patel, 2006, Xu et al., 2010)*.

Recently, enterococcal strains bearing a "silent" copy of vancomycin resistant genes have been described *(Coburn et al., 2014b)*. Such strains are potentially very dangerous as they phenotypically look like vancomycin sensitive enterococci and are therefore capable to evade detection tests using for drug sensitivity screening. Moreover, vancomycin sensitive strain can be transformed to VRE during antibiotic treatment. Hence, they have been termed vancomycin-variable enterococci (*Thaker et al., 2015)*.

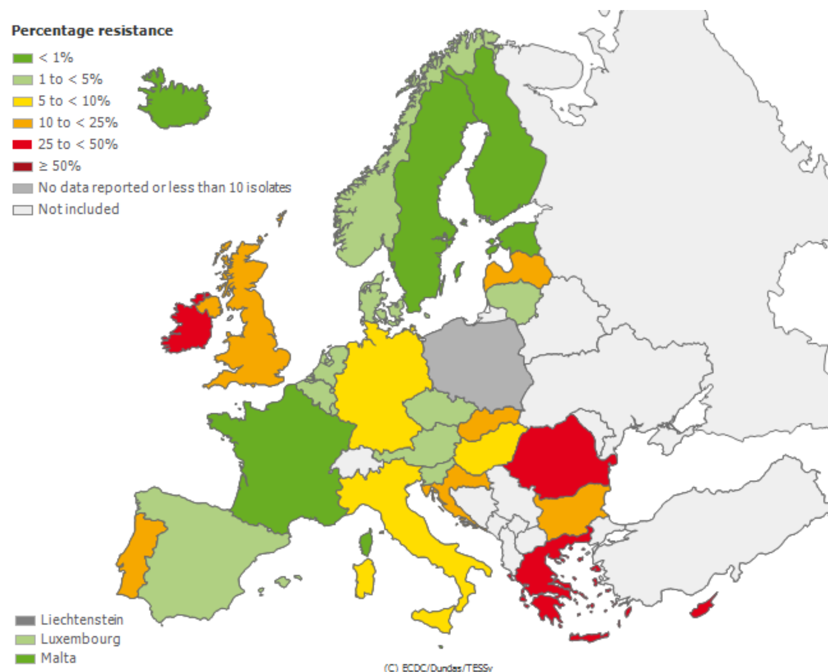


**Figure 6:** Resistant invasive isolates of *E. faecium* resistant to vancomycin in Europe.

Taken from: *European centre for Disease Prevention and Control (2016b)*

The most vulnerable groups of patients to VRE infection are placed in strictly pathogen free wards. VRE infection primarily appear in urinary tract (e.g. cystitis, pyelonephritis, prostatitis), wounds, intra-abdominally (e.g. biliary tree, gastrointestinal tract) and/or in form catheter mediated bacteraemia; one of the most serious complications *(Zirakzadeh and Patel, 2006)*.

### 3.5.3. Mechanism of resistance in Enterococci

There are 9 types of gene clusters labelled according to the designation of the ligase gene, which is expressed in code: either a D-Ala:D-Lac (*vanA, vanB, vanD* and *vanM*) or a D-Ala:D-Ser *(vanC, vanE, vanG, vanL* and *vanN*) ligase. These ligases causes resistance in enterococci by production of peptidoglycan precursors in original sequence D-alanyl-D-alanine (D-Ala–D-Ala) with a moderate affinity for glycopeptide antibiotics *(Xu et al., 2010, Lebreton et al., 2011)*.

The most worldwide disseminated vancomycin resistant phenotypes are VanA and VanB. Former is typically attributed to resistance to high concentrations of both vancomycin and teicoplanin induced by either vancomycin or teicoplanin treatment. Latter phenotype is induced by vancomycin treatment and reveals acquired resistance against to even low concentrations of vancomycin only. VanA and VanB are coded by genes carried on transposons *Tn1546* and *Tn1549/Tn15382*, respectively, which may be found both on plasmids or in chromosome *(Hegstad et al., 2010*, *Thaker et al., 2015, Xu et al., 2010)*.

Synthesis of resistant peptidoglycan precursors require three enzymes, i.e. VanH (dehydrogenase), VanA (ATP dependent ligase) and VanX (dipeptidase) *(Courvalin, 2006)*. Other proteins VanR and VanS belong to 2-element regulatory system directing transcription of the resistance mediating three-gene *van*HAX cluster. VanR is a cytoplasmic regulator responsible for transcriptional activation. VanS is histidine kinase anchored in membrane, involved in the phosphorylation and dephosphorylation processes, based on the either presence or absence of glycopeptides (Figure 7) *(Courvalin, 2006, Walsh et al., 1996)*.

### 3.5.4 Population structure of *E. faecium*

A study by *Willems et al.* (2012) showed significant differences between related hospital and farm animal *E. faecium* isolates due to Bayesian Analysis of Population Structure (BAPS) clustering of 491 STs of *E. faecium*. All STs were grouped to 13 BAPS groups with apparent predominance for groups 2 and 3 in humans containing 227 and 190 STs, respectively *(Willems et al., 2012)*. Two subgroups namely 2-1 and 3-3 contained mainly (80 %) nosocomial isolates from hospitalized patients but 2-1 subgroup also contained a large amount of animal-related isolates *(Willems et al., 2012)*.

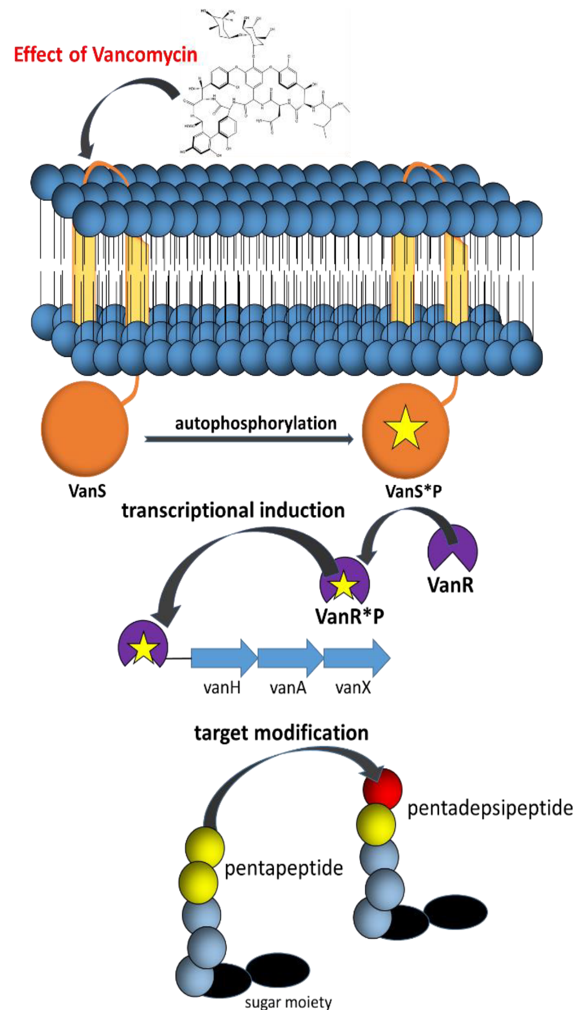


**Figure 7:** Mechanism of vancomycin resistance. VanS is a histidine kinase, located in the membrane and with ability ATP dependent autophosphorylation after vancomycin recognition (by direct binding in the case of VanSb). The phosphate (P) is subsequently transferred on first member of two-component regulatory system (VanR+VanS) in the cytoplasmic space. Phosphorylated VanR binds to the intergenic section upstream of the *vanHAX* operon and facilitates transcription drug resistance genes. Concerning cassette proteins, VanH provides D-Lac by the biochemical reduction of pyruvate. VanA is an ATP-dependent ligase synthesising the ester D-Ala-D-Lac. VanX is a dipeptidase removing D-Ala-D-Ala, which is expressed by chromosomal cell wall biosynthesis apparatus which is continuously produced. The resulting depsipeptide is incorporated into intracellular cell wall precursor biosynthesis, which is an important supplier of reagents required for the extracellular synthesis of peptidoglycan necessary for cell division and growth. The result is a 1000-fold lower affinity vancomycin and hence its ineffectiveness.

Adapted from: *Thaker et al. (2015)*

In 2015, 2402 *E. faecium* isolates consisted of 837 different STs, which were divided into 8 groups of BAPS. In groups BAPS 2 (39.7 %), BAPS 3 (31.5 %), BAPS 1 (15.1 %) and BAPS 7 (8.5 %) were represented most STs while the groups BAPS 5 (1.9 %), BAPS 4 (1.3 %), BAPS 8 (1.2 %), and BAPS 6 (0.8 %) were in a clear minority *(Tedim et al., 2015)*. According to *Tedim et al.* (2015), 150 isolates were divided into 75 different STs and among them were globally spread ST groups, like ST78, ST17, and ST18, and also ST102, ST22, ST94, ST9, and ST5. These 75 STs were partitioned into BAPS 1 (24 STs, 22.0 % of isolates), BAPS 2 (19 STs, 31.3 % of isolates), BAPS 3 (20 STs, 36.7 % of isolates), BAPS 7 (8 STs, 7.3 % of isolates), and BAPS 8 (3 STs, 2.7 % of isolates).

### 3.5.5. Possible therapy options

a) Quinupristin/dalfopristin is a parenteral combination of streptogramine type B (30 % quinupristin) and type A (70 % dalfopristin). This combination is able to manage the various VRE infection with efficiency of over 60 %. Due to the lack of bactericidal effect this combination is more suited as a support component *(O'Driscoll and Crank, 2015)*.

b) Oxazolidinones: Linezolid is the only antibiotic drug authorized by the Food and Drug Administration for the treatment of VRE infections. Resistance to this antibiotic is rare and usually linked with previous linezolid treatment. Tedizolid is new oxazolidinone antibiotic drug with spectrum including both VanA- and VanB-types VRE. As compared with linezolid, tedizolid has a greater potential to be a drug of first choice for serious VRE *(O'Driscoll and Crank, 2015)*.

c) Lipoglycopeptides: Telavancin has been used since 2009 for treatment of problematic skin and skin structure infections. Since 2013 it has been approved for hospital-acquired pneumonia and ventilator-associated pneumonia. Dalbavancin was approved for skin and skin structure infections indication in 2013 and oritavancin has the broadest spectrum against a very wide spectrum of resistant Gram-positive bacteria such as VanA and VanB expressing VRE *(O'Driscoll and Crank, 2015, Murray, 2000)*.

d) Daptomycin is a cyclic lipopeptide whose bactericidal activity depends on the used concentration. As compared with linezolid patients infected by VRE and treated by daptomycin showed higher mortality. Daptomycin together with linezolid are

recommended as drugs of first choice for the treatment of VRE bacteremia *(O'Driscoll and Crank, 2015)*.

e) Tigecycline is a glycylcycline with activity against tetracycline resistant VRE *(O'Driscoll and Crank, 2015)*. Unfortunately resistance to tigecycline was very recently reported *(Niebel et al., 2015)*.

## 3.6. Identification of mechanisms of antibiotic resistance

### 3.6.1. Antimicrobial susceptibility testing

Disk diffusion technique is the reference semi-quantitative method for antimicrobial susceptibility testing (AST) used in most clinical microbiology laboratories. This is due to its versatility, accuracy, reproducibility and minimal demands for special equipment *(Matuschek et al., 2014)*.

European Committee for Antimicrobial Susceptibility Testing (EUCAST) initiated alignment of differently evaluated minimum inhibitory concentration (MIC) breakpoints in Europe. For these purposes, it was developed standardized disk diffusion method with harmonized MIC breakpoints. Complete and annually updated approach of sample preparation for EUCAST disk diffusion technology is available on the EUCAST website (http://www.eucast.org) *(Matuschek et al., 2014)*.

E-test falls within the category of *in vitro* quantitative methods for ascertaining the MIC of various antibacterial substances and microorganisms on agar medium. This method is easily readable and provides in comparison with other phenotypic methods advantage in terms of precise therapeutic concentrations *(Sader and Pignatari, 1994)*.

Double-disk synergy test is a method based on the detection of the deformation zones of inhibition, which are produced by strains tested on Mueller-Hinton agar (MHA) discs between cephalosporins and aztreonam, and the disk with amoxicillin/clavulanic acid. Distance of discs is between 20–30 mm from the center, according to the type of test and laboratory experience *(Hrabak et al., 2008).*

### 3.6.2. Blue Carba test

This method is a rapid (less than 2 hours) detection tool for the Ambler class A, B and D carbapenemases (KPC, IMP, NDM, VIM, SPM, and OXA), which consists

of *in vitro* hydrolysis of imipenem antibacterial drug Gram-negative bacterial strains such as *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. *(Pires et al., 2013)*. Detection is signalled by the colour change of bromothymol blue indicator due to changes in pH values. Positive carbapenemases presence is confirmed when test/negative-control solution, respectively, turns **a**) green/blue **b**) yellow/blue or **c**) yellow/green. In the negative case both solutions will remain either blue or green (Figure 8) *(Pires et al., 2013, Pasteran et al., 2015)*.

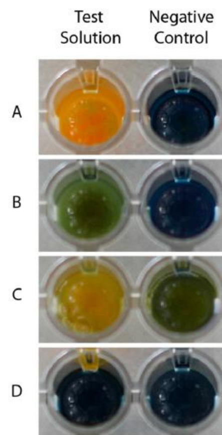


**Figure 8:** Representative results of BlueCarba test. The samples A, B and C represent carbapenemase producers and D represents non-carbapenemase producer. Test solutions are in left column and negative control in right column.

Taken from: *Pires et al. (2013)*

## 3.7. Methods for bacterial typing

### 3.7.1. Multilocus sequence typing

Bacterial isolates may be identified by the specific sequence of so-called "housekeeping" genes (HG) *(Spratt, 1999)*. Different allele and alleles of individual loci consist series of integers which determine ST or allelic profile. The relationships between the various STs and clonal complex or clonal group can conveniently be depicted by e-BURST or more recently BAPS software. Both methods process different information for the establishment of clonal relationships between isolates (*Feil et al., 2004*, *Woodford et al., 2011, Willems et al., 2012)*.

MLST is a method for identification of the genetic relationships among bacterial strains based on polymorphisms of 6–10 HG. In both *K. pneumoniae* and *E. faecium,* 7 HG have been defined for MLST purposes (Table 1).

The database for independent comparison of STs is available online (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html and http://pubmlst.org/efaecium/) and under continuous supervision of administrator for each species or group of species. A limiting factor is insufficient capability to point to the genetic changes across the isolates in outbreak situations unlike PFGE method *(Diancourt et al., 2005, Jolley and Maiden, 2010, Mathers et al., 2015, Spratt, 1999)*.

**Table 1: Housekeeping genes of *K. pneumoniae* and *E.faecium***

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *K. pneumoniae* | **gapA** | **infB** | **mdh** | **pgi** | **phoE** | **rpoB** | **tonB** |
| *E. faecium* | **atpA** | **Adk** | **ddl** | **gdh** | **gyd** | **purK** | **pstS** |

***K. pneumoniae:*** *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *infB* (translation initiation factor 2), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *rpoB* (β-subunit of RNA polymerase), *tonB* (periplasmic energy transducer)

***E. faecium:*** *atpA* (ATP synthase, alpha subunit), *adk (*adenylate kinase), *ddl* (D-alanine:D-alanine ligase), *gdh* (glucose-6-phosphate dehydrogenase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *purK* (phosphoribosylaminoimidazol carboxylase ATPase subunit), *pstS* (phosphate ATP-binding cassette transporter)

Adapted from: http://pubmlst.org/kpneumoniae/info/primers.shtml, *Homan et al.,* (2002)

### 3.7.2. Fourier transform infrared spectroscopy with attenuated total reflectance

Infrared spectroscopy is a method that uses infrared radiation. The principle is based on delivering energy quanta and changes in the vibrational and rotational behaviour of biomolecules *(Alvarez-Ordonez et al., 2011)*. FTIR-ATR is a method working with complete information about the chemical composition of the sample providing a fingerprint that in principle will be specific for each genera or bacterial species. The basis of FTIR spectroscopy is the interaction of infrared radiation with a sample, in our specific case with the bacterial isolate, providing a specific fingerprint that reflects the structure and composition of the whole cell. In the ATR mode, the infrared beam contact with the bacterial isolate and became attenuated. The magnitude of the attenuation depends on the bacteria in contact with the beam *(Naumann et. al, 1991)*. FTIR works with complete phonetic and genetic fingerprint studied cells and allows accurate differentiation of microorganisms even to the subspecies, strain and/or serotype/serogroup level *(Alvarez-Ordonez et al., 2011, Taha et al., 2014, Wenning and Scherer, 2013)*. FTIR application possibilities are wide e.g. pharmaceutical industry, microorganisms and in strains characterization, and medical applications *(Taha et al., 2014)*.

# 4. Goals

This study focused on characterisation of multidrug resistant *Klebsiella pneumoniae* and *Enterococcus faecium* isolates by spectroscopic and genotypic methods.

Techniques applied to address the goal follow:

a) molecular characterization of multidrug resistant *K. pneumoniae* by genotypic methods,
b) evaluation of FTIR-ATR in the differentiation of *E. faecium* and *K. pneumoniae* clones.

# 5. Experimental part

## 5.1. Chemicals and preparation of solutions

### 5.1.1. Phenotypic methods

*Reagents for Blue-Carba test*: Test solution was prepared from aqueous solutions 0.04 % bromothymol blue (Merck Millipore, Germany), 0.1 mM $ZnSO_4$ pH 6.0 (Merck Millipore, Germany) and 0.3 % imipenem (Tienam 500; Merck Sharp & Dohme, France). Final pH of test solution was 7.0. A negative-control solution consisted of 0.04 % bromothymol blue with pH 7.0.

*Reagents for EUCAST and E-test*: MHA-2 (bioMérieux, France) was dissolved in distilled water (15.2 g in 400 mL) and sterilised in autoclave model uniclave 88 (ajc, Portugal); sterilized MHA-2 was poured into Petri dishes (ø 60 mm). For AST disks with different antibiotics (OXOID, UK) were used and for E-test strips with carbapenems were used (Liofilchem, Italy).

### 5.1.2. Genotypic methods

*Reagents for PCR*: Go*Taq* G2 Flexi DNA Polymerase, $MgCl_2$, 5xGreen Go*Taq* (G2) Flexi Buffer, primers and dNTP´s were obtained from Promega (WI, USA). KAPA *Taq* HotStart PCR Kit with dNTP´s was purchased from Kapa Biosystems (South Africa). Conditions for preparations of single mixtures for PCR reactions are mentioned in Table 2. Primers were diluted in distilled water either in a ratio of 1:5 or 1:10.

*Reagents for gel electrophoresis*: GRS Ladder 50 µg, GRS Universal Ladder 50 µg and GRS Agarose LE were acquired from GRISP Research Solutions (Portugal). 50x TAE Tris/Acetic Acid/EDTA Buffer was bought from BIO-RAD Laboratories (Portugal). SYBR Safe DNA gel stain (10000x concentrate in DMSO) was purchased from Thermo Fisher Scientific (Portugal) and Bromophenol Blue (Sigma-Aldrich, Portugal). Distilled sterilized water was used for dilution purposes. The 1.5 % agarose gel was made by dissolving agarose in boiling 1x concentrated electrophoresis buffer Tris-AceticAcid-EDTA ("1xTAE") made of 50x concentrated TAE. To the warm mixture it was added SYBR Safe DNA gel stain for visualisation of DNA in the gel. Liquid solution was poured into a mold equipped with precision-planted comb for making of wells for sample application.

*Reagents for purification of DNA*: NZYGelpure set composed of Binding buffer, Wash buffer (concentrate) and Elution buffer (does not contain EDTA), NZYTech spin columns and collection tubes (2 mL) was obtained from NZYTech company (Portugal).

**Table 2:** Kappa polymerase mixture for 1 sample and GoTaq polymerase mixture for 1 sample (µL)

| Kappa polymerase mixture | | GoTaq polymerase mixture | |
|---|---|---|---|
| **d $H_2O$** | 15,4 | **d $H_2O$** | 12,9 |
| **Buffer 5X** | 5 | **Buffer 5X** | 4 |
| **$MgCl_2$** | 1,5 | **$MgCl_2$** | 1,2 |
| **dNTP´s** | 0,5 | **dNTP´s** | 0,4 |
| **Primer F** | 0,25 | **Primer F** | 0,2 |
| **Primer R** | 0,25 | **Primer R** | 0,2 |
| **Kappa (0,8U)** | 0,1 | **Taq (0,8U)** | 0,1 |
| **V. Final** | 23 | **V. Final** | 20 |

## 5.2. Origin of samples

### 5.2.1. *Klebsiella pneumoniae*

We screened 20 isolates of *K. pneumoniae* with reduced susceptibility to carbapenems that were obtained mainly from non-hospitalized patients (16 Female and 4 Male) with age range from 61 to 89 years in long-term care facilities (*n*=11), nursing homes (*n*=6), community health centres (*n*=2) and hospital (*n*=1) located in the North and Centre of Portugal between 2014 and 2015. 95 % of the samples consisted of isolates from urine and the remaining 5 % from pulmonary sputum. Detailed epidemiological data are summarized in Table 3.

They were identified in different Community Laboratories from the North of Portugal: i) Botelho Moniz Análises Clínicas, Santo Tirso, Portugal (n=17); ii) Misericordia Porto (*n*=2); iii) Laboratory Carlos Torres (*n*=1) (Table 3). Initial bacterial identification and antibiotic susceptibility testing were performed via automated VITEK (bioMérieux, Marcy l'Étoile, France) system.

### 5.2.2. Enterococcus

We observed 143 multi drug resistant *E. faecium* isolates that were obtained from hospitalized patients (*n*=105), hospital waste waters and river (*n*=8), pigs (*n*=25) and other different sources (n=5) from different countries (*n*=24) with Portugal being

predominant (*n*=65). The countries included Argentina, Australia, Brazil, Canada, Colombia, Denmark, Ecuador, Finland, Germany, Hungary, Italy, Paraguay, Poland, Portugal, UK, USA, Netherlands, Norway, Saudi Arabia, Serbia, Singapore, Spain, Switzerland and Tunisia (Table 4).

These isolates were inoculated on MHA-2 prior to the application on the FTIR-ATR device (PerkinElmer, USA) and subsequently categorized into different groups according to the chemometrics analysis of the obtained spectra.

**Table 3: Origin of *Klebsiella pneumoniae* samples**

| N. of Isolate | Source | Previous hospitalizations (hospital)[c] | Date of isolation | Service | Age | Gender | Product | Pathology |
|---|---|---|---|---|---|---|---|---|
| **K56** | NH C | UN | 03.10.2014 | LCT | 87 | M | U | UN |
| **K70** | LTCF E | Several previous | 11.02.2015 | LST | 86 | F | U | UTI |
| **K86** | LTCF A | UN | 23.06.2015 | LST | 89 | F | U | UTI |
| **K87** | LTCF A | Hospital B | 14.07.2015 | LST | 88 | F | U | UTI |
| **K88** | LTCF A | Hospital B | 20.07.2015 | LST | 83 | F | E | RI |
| **K89** | LTCF F | Hospital E | 12.08.2015 | LST | 81 | F | U | UTI |
| **K90** | LTCF B | Hospital C | 01.09.2015 | LST | 85 | F | U | UTI |
| **K91** | Hospital H | Hospital F | 03.09.2015 | LST | 72 | M | U | UTI |
| **K92** | LTCF B | UN | 24.09.2015 | LTCF B | 61 | F | U | UN |
| **K93** | LTCF B | UN | 24.09.2015 | LTCF B | 89 | F | U | UTI |
| **K95** | LTCF A | Hospital B | 23.09.2015 | LST | 88 | F | U | UTI |
| **K115** | LTCF C | Hospital G | 08.10.2015 | LST | 76 | M | U | UTI |
| **K116** | NH A | Hospital A | 06.10.2015 | LST | 82 | M | U | UTI |
| **K124** | LTCF F | Hospital C | 17.10.2015 | LST | 83 | F | U | UTI |
| **K125** | LTCF A | UN | 07.10.2015 | LST | 84 | F | U | UTI |
| **K126** | NH B | Hospital A | 23.10.2015 | LST | 77 | F | U | UTI |
| **K127** | NH B | Hospital A | 15.10.2015 | LST | 88 | F | U | UTI |
| **K132** | LTCF A | Hospital B | 03.11.2015 | LST | 83 | F | U | UTI |
| **K133** | NH A | Hospital A | 10.11.2015 | LST | 78 | F | U | UTI |
| **K134** | NH E | Hospital G | 28.10.2015 | LST | 89 | F | U | UTI |

| | | |
|---|---|---|
| Hospital A (Gaia) | LTCF A (UCC Vila Real) | NH A (Montepio Gaia) |
| Hospital B (Vila Real) | LTCF B (UCC Misericordia Porto) | NH B (CliHotel Gaia) |
| Hospital C (HUC) | LTCF C (UCC Povoa de Varzim) | NH C (Home) |
| Hospital D (Pedro Hispano) | LTCF D (UCC Sr Prazeres VR) | NH D (Meda, Guarda) |
| Hospital E (Cantanhede) | LTCF E (UCC Avanca) | NH E (Camarate) |
| Hospital F (Sta Maria) | LTCF F (UCC Mealhada) | |
| Hospital G (Beatriz Ângelo) | LCT=laboratory carlos torres | U=Urine |
| Hospital H (Algarve) | LST=laboratory santo tirso | E=Expectoration |
| | LTCFM= LTCF Misericordia | RI=Respiratory inf. |
| A, B, D - North | A. B, C, D - North | UN=Unknown |
| C, E, F, G - Center | E. F - Center | N=No |

**Table 4: Origin and identification of vancomycin resistant *Enterococcus faecium***

| STRAIN | BAPS | ST | Origin | Country | YEAR | ABR pattern |
|---|---|---|---|---|---|---|
| M260840 | 2.1a | ST412 | HP - CS | Portugal | 2012 | MDR-VRE-AmpiR |
| UW5905 | 2.1a | ST192 | HP - CS | Germany | 2004 | MDR-VRE-AmpiR |
| UW6337 | 2.1a | ST203 | HP - CS | Germany | 2005 | MDR-VRE-AmpiR |
| VRE2 | 2.1a | ST203 | HP - CS | Denmark | 2005-2008 | MDR-VRE-AmpiR |
| Lin1 | 2.1a | ST203 | HP - CS | USA | NK | MDR-VRE-AmpiR |
| E422 | 2.1a | ST203 | HP - CS | Ecuador | NK | MDR-VRE-AmpiR |
| Efm49 | 2.1a | ST203 | HP - CS | Spain | 2006 | MDR-AmpiR |
| Efm50 | 2.1a | ST203 | HP - CS | Spain | 2006 | MDR-AmpiR |
| Efm51 | 2.1a | ST203 | HP - CS | Spain | 2007 | MDR-AmpiR |
| E1644 | 2.1a | ST78 | HP - CS | Netherlands | 2002 | MDR-VRE-AmpiR |
| AE01 | 2.1a | ST78 | HP - CS | Italy | 2001-03 | MDR-VRE-AmpiR |
| 2348 | 2.1a | ST78 | HP - CS | Argentina | 1998 | MDR-VRE-AmpiR |
| Canada | 2.1a | ST412 | HP - CS | Canada | 2005 | MDR-VRE-AmpiR |
| M239495 | 2.1a | ST412 | HP - CS | Portugal | 2011 | MDR-VRE-AmpiR |
| M214602 | 2.1a | ST412 | HP - CS | Portugal | 2011 | MDR-VRE-AmpiR |
| E234 | 2.1a | ST80 | Sewage | Portugal | 2002 | MDR-VRE-AmpiR |
| 638466 | 2.1a | ST80 | HP - CS | Portugal | 1997 | MDR-VRE-AmpiR |
| E2365 | 2.1a | ST80 | HP - CS | Hungary | 2004 | MDR-VRE-AmpiR |
| UW1806 | 2.1a | ST117 | HP - CS | Germany | 1998 | MDR-VRE-AmpiR |
| HPH8b | 2.1a | ST117 | HP - CS | Portugal | 2014 | MDR-VRE-AmpiR-LinR |
| HPH9 | 2.1a | ST117 | HP - CS | Portugal | 2014 | MDR-VRE-AmpiR |
| HPH8a | 2.1a | ST117 | HP - CS | Portugal | 2014 | MDR-AmpR-LinR |
| HPH8d | 2.1a | ST117 | HP - CS | Portugal | 2014 | MDR-AmpR-LinR |
| UW11182 | 2.1a | ST117 | HP - CS | Germany | 2013 | MDR-LinR |
| UW10764 | 2.1a | ST117 | HP - CS | Germany | 2013 | MDR-LinR |
| UW10536 | 2.1a | ST117 | HP - CS | Germany | 2013 | MDR-LinR |
| HUB2862 97/12 | 2.1a | ST117 | HP - CS | Barcelona | 2010-2012 | MDR-LinR |
| HUB2154 39/11 | 2.1a | ST117 | HP - CS | Barcelona | 2010-2012 | MDR-LinR |
| Efm121 | 2.1a | ST117 | HP - CS | Spain | 2010 | MDR-LinR |
| Efm157 | 2.1a | ST117 | HP - CS | Spain | 2010 | MDR-LinR |
| Efm126 | 2.1a | ST117 | HP - CS | Spain | 2010 | MDR-LinR |
| Efm128 | 2.1a | ST117 | HP - CS | Spain | 2011 | MDR-LinR |
| 3339V05 | 2.1b | ST493 | HV | Denmark | 2005 | MDR-VRE |
| 841V03 | 2.1b | ST147 | HV | Denmark | 2003 | MDR-VRE |
| H305 | 2.1b | ST5 | HP - CS | Portugal | 2002 | NO MDR (VanR, EryR) |
| HF50104 | 2.1b | ST5 | Pig feces | USA | 2008 | MDR-VRE |
| 75302981 | 2.1b | ST5 | Pig feces | Denmark | 2003 | MDR-VRE |
| 77304631 | 2.1b | ST5 | Pig feces | Denmark | 2005 | MDR-VRE |
| 20067012 26 | 2.1b | ST5 | Pig feces | Denmark | 2006 | MDR-VRE |

| 75302474 | 2.1b | ST5 | Pig feces | Denmark | 2003 | MDR-VRE |
|---|---|---|---|---|---|---|
| **STRAIN (SN)** | **BAPS** | **ST** | **Origin** | **Country** | **YEAR** | **ABR pattern** |
| HF50215 | 2.1b | ST5 | Pig feces | USA | 2008 | MDR-VRE |
| 573 | 2.1b | ST185 | Piggery | Portugal | 2007 | MDR-VRE |
| HF50203 | 2.1b | ST185 | Pig feces | USA | 2008 | MDR-VRE |
| 558 | 2.1b | ST185 | Piggery | Portugal | 2007 | NO MDR (VanR, TetR) |
| 76307861 | 2.1b | ST185 | Pig feces | Denmark | 2004 | MDR-VRE |
| 73306141 | 2.1b | ST185 | Pig feces | Denmark | 2001 | NO MDR (VanR, TetR) |
| DB20546 | 3.1 | ST280 | HP - CS | Singapore | 2004 | MDR-VRE-AmpiR |
| OB19275 | 3.1 | ST280 | HP - CS | Singapore | 2004 | MDR-VRE-AmpiR |
| P575 | 3.1 | ST280 | HP - CS | Paraguay | NK | MDR-VRE-AmpiR |
| HST1 | 3.1 | ST280 | HP - CS | Portugal | 2006 | MDR-VRE-AmpiR |
| H323 | 3.1 | ST280 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| H352 | 3.1 | ST280 | HP - CS | Portugal | 2003 | MDR-VRE-AmpiR |
| H358 | 3.1 | ST280 | HP - CS | Portugal | 2003 | MDR-VRE-AmpiR |
| H207 | 3.1 | ST280 | HP - CS | Portugal | 2002 | MDR-AmpiR |
| 2 | 3.1 | ST1058 | HP (stool) | Portugal | 2015 | ND |
| B | 3.1 | ST1058 | HP (stool) | Portugal | 2015 | ND |
| 145 | 3.1 | ST1058 | cow milk | Tunisia | 2014 | MDR-LinR |
| 149 | 3.1 | ST1058 | cow milk | Tunisia | 2014 | MDR-LinR |
| 336 | 3.1 | ST1058 | cow milk | Tunisia | 2014 | MDR-LinR |
| TR52-40 | 3.1 | ST32 | trout | Portugal | 2012 | NO MDR (TetR, EryR) |
| 70411 | 3.1 | ST670 | HP - CS | Portugal | 1997 | MDR-VRE-AmpiR |
| HF50106 | 3.2 | ST6 | Pig feces | USA | 2008 | MDR-VRE |
| HF50204 | 3.2 | ST6 | Pig feces | USA | 2008 | MDR-VRE |
| HF50105 | 3.2 | ST6 | Pig feces | USA | 2008 | ND |
| 4D | 3.2 | ST6 | Pig feces | Switzerland | 1999 | MDR-VRE |
| 2S2 | 3.2 | ST6 | Pig feces | Portugal | NK | MDR-VRE |
| 7S4S | 3.2 | ST6 | SH | Portugal | 1997 | MDR-VRE |
| E8SV3 | 3.2 | ST6 | Pig feces | Denmark | 1995 | MDR-VRE |
| 3322V05 | 3.2 | ST6 | HV | Denmark | 2005 | MDR-VRE |
| 2006702204 | 3.2 | ST6 | Pig feces | Denmark | 2006 | MDR-VRE |
| 74306583 | 3.2 | ST6 | Pig feces | Denmark | 2002 | MDR-VRE |
| 76302875 | 3.2 | ST6 | Pig feces | Denmark | 2004 | MDR-VRE |
| 76304622 | 3.2 | ST6 | Pig feces | Denmark | 2004 | MDR-VRE |
| 77305523 | 3.2 | ST6 | Pig feces | Denmark | 2005 | NO MDR (VanR, TetR) |
| 2006701227 | 3.2 | ST6 | Pig feces | Denmark | 2006 | ND |
| 73305193 | 3.2 | ST6 | Pig feces | Denmark | 2001 | ND |
| E0013 | 3.3a1 | ST18 | HP - CS | UK | 1992 | MDR-VRE-AmpiR |
| 2227 | 3.3a1 | ST18 | HP - CS | Poland | 2005 | MDR-VRE-AmpiR |
| VRE101 | 3.3a1 | ST18 | HP - CS | Denmark | 2005-2008 | MDR-VRE-AmpiR |
| E0532 | 3.3a1 | ST18 | HP - CS | Australia | 1998 | MDR-VRE-AmpiR |

| STRAIN (SN) | BAPS | ST | Origin | Country | YEAR | ABR pattern |
|---|---|---|---|---|---|---|
| S30 | 3.3a1 | ST18 | HP - CS | Saudi Arabia | 2000-03 | MDR-VRE-AmpiR |
| C497 | 3.3a1 | ST18 | HP - CS | Colombia | NK | MDR-VRE-AmpiR |
| E2373 | 3.3a1 | ST18 | HP - CS | Serbia | 2005 | MDR-VRE-AmpiR |
| 484101 | 3.3a1 | ST18 | HP - CS | Portugal | 2000 | MDR-VRE-AmpiR |
| H312 | 3.3a1 | ST18 | HP - CS | Portugal | 2003 | MDR-VRE-AmpiR |
| H328 | 3.3a1 | ST18 | HP - CS | Portugal | 2003 | MDR-VRE-AmpiR |
| 446511 | 3.3a1 | ST18 | HP - CS | Portugal | 1996 | MDR-VRE-AmpiR |
| H37 | 3.3a1 | ST18 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |
| 48311 | 3.3a1 | ST18 | HP - CS | Portugal | NK | MDR-VRE-AmpiR |
| H174 | 3.3a1 | ST18 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| H315 | 3.3a1 | ST18 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| HPH1 | 3.3a1 | ST18 | HP - CS | Portugal | 2007 | MDR-VRE-AmpiR |
| HPH6 | 3.3a1 | ST18 | HP - CS | Portugal | 2007 | MDR-VRE-AmpiR |
| H88 | 3.3a1 | ST18 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |
| H142b | 3.3a1 | ST18 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |
| H176 | 3.3a1 | ST18 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| H182 | 3.3a1 | ST18 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| H239 | 3.3a1 | ST18 | HP - CS | Portugal | NK | MDR-VRE-AmpiR |
| H361 | 3.3a1 | ST18 | HP - CS | Portugal | 2003 | MDR-VRE-AmpiR |
| H362 | 3.3a1 | ST18 | HP - CS | Portugal | 2003 | MDR-VRE-AmpiR |
| 129600 | 3.3a1 | ST18 | HP - CS | Portugal | NK | MDR-VRE-AmpiR |
| H87 | 3.3a1 | ST18 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |
| H284 | 3.3a1 | ST18 | HP - CS | Portugal | NK | MDR-VRE-AmpiR |
| H175 | 3.3a1 | ST18 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| E1 | 3.3a1 | ST18 | Sewage | Portugal | 2001 | MDR-VRE-AmpiR |
| VG6 | 3.3a1 | ST18 | Urban sewage | Portugal | 2004 | MDR-VRE-AmpiR |
| H321 | 3.3a1 | ST132 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| 526660 | 3.3a1 | ST132 | HP - CS | Portugal | 2000 | MDR-VRE-AmpiR |
| 28798 | 3.3a1 | ST132 | HP - CS | Portugal | 1999 | MDR-VRE-AmpiR |
| H40 | 3.3a1 | ST132 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |
| H74 | 3.3a1 | ST132 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |
| H311 | 3.3a1 | ST132 | HP - CS | Portugal | 2002 | MDR-VRE-AmpiR |
| H71 | 3.3a1 | ST132 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |
| H363 | 3.3a1 | ST132 | HP - CS | Portugal | 2003 | MDR-VRE-AmpiR |
| E233 | 3.3a1 | ST132 | Sewage | Portugal | 2002 | MDR-VRE-AmpiR |
| E49 | 3.3a1 | ST132 | Sewage | Portugal | 2001 | MDR-VRE-AmpiR |
| E169 | 3.3a1 | ST132 | Sewage | Portugal | 2001 | MDR-AmpiR |
| 446 | 3.3a1 | ST132 | Piggery | Portugal | 2006 | MDR-VRE-AmpiR |
| VRE100 | 3.3a1 | ST173 | HP - CS | Australia | 2001 | MDR-VRE-AmpiR |
| E197 | 3.3a1 | ST368 | Sewage | Portugal | 2001 | MDR-VRE-AmpiR |

| STRAIN (SN) | BAPS | ST | Origin | Country | YEAR | ABR pattern |
|---|---|---|---|---|---|---|
| E417 | 3.3a2 | ST17 | HP - CS | Ecuador | NK | MDR-VRE-AmpiR |
| VREII | 3.3a2 | ST17 | HP - CS | Finland | 1996-98 | MDR-VRE-AmpiR |
| 604/06 | 3.3a2 | ST17 | HP - CS | Spain | 2006 | MDR-VRE-AmpiR |
| 135487 | 3.3a2 | ST17 | HP - CS | Spain | 2005 | MDR-VRE-AmpiR |
| 361400 | 3.3a2 | ST17 | HP - CS | Spain | 2004 | MDR-VRE-AmpiR |
| Ef80 | 3.3a2 | ST17 | HP - CS | USA | 1990-92 | MDR-VRE-AmpiR |
| Vri16 | 3.3a2 | ST17 | HP - CS | Brasil | NK | MDR-VRE-AmpiR |
| 2391 | 3.3a2 | ST17 | HP - CS | Argentina | 1998 | MDR-VRE-AmpiR |
| E1340 | 3.3a2 | ST17 | HP - CS | Norway | 1999 | MDR-AmpiR |
| E241 | 3.3a2 | ST17 | Sewage | Portugal | 2002 | MDR-AmpiR |
| E0481 | 3.3a2 | ST16 | HP - CS | Netherlands | 1999 | MDR-VRE-AmpiR |
| E0805 | 3.3a2 | ST16 | HP - CS | Netherlands | 2000 | MDR-VRE-AmpiR |
| E1651 | 3.3a2 | ST16 | HP - CS | Netherlands | 2002 | MDR-VRE-AmpiR |
| E0161 | 3.3a2 | ST16 | HP - CS | USA | NK | MDR-VRE |
| E2480 (C68) | 3.3a2 | ST16 | HP - CS | USA | 1996 | MDR-VRE-AmpiR |
| E1132 | 3.3a2 | ST16 | HP - CS | USA | 2001 | MDR-VRE-AmpiR |
| 529940 | 3.3a2 | ST16 | HP - CS | Portugal | 2000 | MDR-VRE-AmpiR |
| Efm39 | 3.3a2 | ST16 | HP - CS | Spain | 2001-04 | MDR-AmpiR |
| 2664 | 3.3a2 | ST16 | HP - CS | Argentina | 2000 | MDR-VRE-AmpiR |
| PAO2 | 3.3a2 | ST16 | HP - CS | Australia | 1996-99 | MDR-VRE-AmpiR |
| UW6511 | 3.3a2 | ST202 | HP - CS | Germany | 2006 | MDR-VRE-AmpiR |
| AE12 | 3.3a2 | ST209 | HP - CS | Italy | 2002 | MDR-VRE-AmpiR |
| H138 | 3.3a2 | ST719 | HP - CS | Portugal | 2001 | MDR-VRE-AmpiR |

Abbreviation: NK, not known; ND, not defined; HP-CS, hospitalised patient-clinical sample; SH, slaughterhouse; HV, healthy volunteers; ABR pattern, antimicrobial resistance pattern; TetR, tetracycline resistance; AmpiR, ampicillin resistance; EryR-erythromycin resistance; VanR, vancomycin resistance; MDR, multidrug resistace; ST, sequence type; BAPS, Bayesian Analysis of Population Structure

## 5.3 Experimental procedures

### 5.3.1. Blue Carba

One loop (approximately 5 µL) of a pure bacterial culture smeared on MHA-2 was resuspended in 100 µL of both test and negative-control solution in 96-well microplates and agitated for 2 hours at 37 °C and 150 rpm in Incubator ES-20 (Biosan, Latvia). Finally, we evaluated positivity/negativity of samples for carbapenemases.

### 5.3.2. Antimicrobial susceptibility testing (disk diffusion and E-test)

Part of grown colonies of each bacterial isolates were brought by loop and slurried in a test tube with sterile saline solution and homogenised by vortexing to reach a 0.5 MacFarland density (approximately corresponds to $10^6$ CFU/mL) by DEN-1 McFarland Densitometer (Liofilmchem, Italy). The bacterial suspension was smeared on a Petri dish (20 mL MHA-2) with a sterile cotton swab three times (always from a different direction). Subsequently, the discs with antibiotics; β-lactams (amoxicillin-clavulanic acid, mecillinam, cefoxitin, extended-spectrum cephalosporins, aztreonam, carbapenems), aminoglycosides (amikacin, gentamicin, netilmicin, kanamycin, tobramycin, streptomycin), quinolones (ciprofloxacin, nalidixic acid), folate pathway inhibitors (trimethoprim, trimethroprim-sulfamethoxazole), tetracyclines (minocycline, tetracycline), chloramphenicol, fosfomycin and colistin (OXOID, UK) were applied by Oxoid™ AST Disk Dispenser (Thermo Fisher Scientific**,** Portugal) and E-test strips with imipenem, meropenem and ertapenem were applied and performed 16–18 hours incubation at 37 °C in bacteriological incubator I Series (Raypa, Spain). Finally, inhibition zone diameters were determined and interpreted according to EUCAST 6.0 guidelines (http://www.eucast.org/eucast_susceptibility_testing/disk_diffusion_methodology) and Clinical and Laboratory Standards Institute (CLSI, 2014) guidelines and breakpoints.

### 5.3.3. Polymerase chain reaction

Tested DNA (2 µL) was mixed with prepared PCR mixture (18 µL GoTaq or 23 µL KAPA) inside eppendorf tubes and subjected to a PCR reaction in the C1000 Thermal Cycler (BIO-RAD Laboratories, California, USA). All reactions for identification of carbapenemases ($bla_{\mathrm{NDM}}$, $bla_{\mathrm{VIM}}$, $bla_{\mathrm{IMP}}$, $bla_{\mathrm{KPC}}$, $bla_{\mathrm{OXA\text{-}48}}$), and other *bla* genes ($bla_{\mathrm{CTX\text{-}M}}$, $bla_{\mathrm{SHV}}$, $bla_{\mathrm{TEM}}$, $bla_{\mathrm{OXA}}$) was performed according *Bogaerts et al*., (2013)*, Curiao et al.,* (2010) and *Rodrigues et al.,* (2014).

After the finished reaction, the 4 µL from each eppendorf was with 2 µL of marker (Quick-Load® 100 bp DNA Ladder), obtained from NEB (MA, USA), quantitatively transferred into the wells within the 1.5 % agarose gel and then the electrophoresis was performed (90 V, approximately 40 minutes) due to PowerPac™ HC High-Current Power Supply (BIO-RAD Laboratories, California, USA). Visualization of products was performed by Gel Doc™ XR+ System (BIO-RAD Laboratories, California, USA) via

ImageLab (BIO-RAD Laboratories, California, USA). When needed, PCR products were purified by commercial kits before sequencing (see below).

### 5.3.4. Multilocus sequence typing

The preparation was the same as for the PCR, including the used volumes (2 μL DNA and 23 μL PCR mix). Reactions were performed according to (http://bigsdb.web.pasteur.fr/klebsiella/primers_used.html) in representative isolates, as described *(Rodrigues et al., 2014)*. The resulting products were checked by Gel Doc™ XR+ System (BIO-RAD Laboratories, California, USA) then sent to the subsequent sequencing by using Sanger technology by outsourcing. Sequences from the seven different housekeeping genes were compared with those included in the *K. pneumoniae* MLST database and alleles and STs were assigned (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html).

### 5.3.5. Purification of DNA

To the volume of the PCR mixture (usually 20 μL) in NZYTech spin column was added a fivefold amount of Binding buffer. Columns stood 2 minutes and then were centrifuged for 1 minutes at 13.000 rpm in centrifuge MicroStar 17 (VWR International, PA, USA). Subsequently, the lowest part of the filtrate was discarded and put a new one, 600 μL of Wash buffer was added and again let 1 minute centrifuge at 13.000 rpm, the filtrate was discarded and the centrifugation was performed again in order to remove residual ethanol. The lowest part again removed and replaced by a clean 2 mL eppendorf. At the end, 50 μL Elution buffer was added and centrifuged at 13.000 rpm for 1 minute.

### 5.3.6. DNA sequencing

5 μL of investigated DNA was added together with 5 μL of corresponding diluted (1:10) primer to the 2 mL eppendorf, which was sent to sequencing by using Sanger technology by outsourcing.

### 5.3.7. Fourier transform infrared spectroscopy with attenuated total reflectance

Spectra were acquired using a PerkinElmer Spectrum BX FTIR System spectrophotometer in the ATR mode with a PIKE Technologies Gladi ATR accessory from 4000–400 $cm^{-1}$ and a resolution of 4 $cm^{-1}$ and 32 scan co-additions. Isolates were grown on MHA at 37 ºC for 18 hours and colonies were directly applied in the ATR crystal and dried in a thin film. For each isolate, 9 spectra were acquired corresponding to three biological replicates (obtained from the same agar plate) and three instrumental replicates (obtained in three independent days).

For FTIR-ATR, spectra were processed and analysed with Principal Component Analysis or partial least square discriminant analysis (PLSDA) considering the phospholipids/DNA/RNA and polysaccharide regions (1500-900 $cm^{-1}$) after pre-processing with standard normal variate followed by the application of a Savitzky-Golay filter. All chemometric models were performed in Matlab version 8.3 (MathWorks, MA, USA) and PLSToolbox version 7.5 (Eigenvector Research Inc., WA, USA) by experienced users.

# 6. Results

## 6.1. *Klebsiella pneumoniae*

### 6.1.1 Clonal relationships

Genetic relatedness among isolates was evaluated primarily by FTIR spectroscopy and confirmed by MLST in representative isolates. The studied isolates (20 *K. pneumoniae*) were according FTIR-ATR grouped in clusters associated with 6 presumptive clones arbitrarily designated as C1, C5, C6, C8, C9 and C10. The vast majority formed C1 (9 isolates), whereas C6 (3 isolates) and C10 (2 isolates) was rather sporadic and all remaining groups contained 1 isolate. Further identification by MLST showed that a correspondence was found between most clones assigned by FTIR-ATR and specific ST: C1 (ST147), C5 (ST348), C6 and C8 (ST15), C9 (ST 109) and C10 (ST231). Although, in the case of groups C6 and C8 ST values were identical, capsular typing revealed differences in these isolates: C8 (wzi93/K60) and C6 (wzi19/K19) and also among all isolates (Table 5).

### 6.1.2. Antimicrobial susceptibility patterns and detection of carbapenemases or extended-spectrum β-lactamases

All studied isolates were KPC-3 carbapenemase producers, resulting in resistance to whole spectrum of β-lactam antibiotics. The presence of other β-lactamases has also been detected. A majority were enzymes of the SHV family (*n*=19 isolates) as well as specific OXA-9 enzyme (*n*=11 isolates) that had usually common occurrence with SHV and after KPC-3 the most abundant enzyme, several isolates (*n*=5) also contain enzymes of TEM family and in isolates K56 and K91 we detected also CTX-M-15 ESBLs.

Interestingly, in different STs dominated other types of SHV enzymes, for example SHV-11 in ST147, SHV-28 in ST15 and SHV-1 in ST231. Moreover, K98 isolate belonging to ST109 contained a new SHV β-lactamase containing mutation N196S.

All isolates except of ST109 showed resistance profiles to aminoglycosides (76 %). Resistance rates to carbapenems was 70 %, to nitrofurantoin was 55 % and to fosfomycin was 15 %. All isolates were susceptible to colistin (Table 6).

However, some isolates (mainly K126 and K133) from ST147 were susceptible to aminoglycosides and nitrofurantoin. The ST109 was susceptible to most non-β-lactam antibiotics. Moreover, for some isolates the MIC values for meropenem and imipenem were interpreted as susceptible by the clinical breakpoints defined by CLSI or EUCAST, in all cases they were above the epidemiological cut-off values (ECOFFs) defined for *K. pneumoniae* for these antimicrobial agents (http://www.eucast.org/mic_distributions_and_ecoffs/) (Table 6).

Both instances mentioned above showed a high correspondence between the results of FTIR and MLST (80 %) and FTIR and *wzi*/K-typing (85 %). (Table 5).

### 6.1.3. Plasmids and transposons

Two types of plasmids (IncFIA and IncN) were characterized among isolates with apparent predominance (80 %) for IncFIA plasmids (~115 kb). Plasmids IncN were found only in K115, K116, K126 and K133 isolates (ST147). Furthermore, it was discovered using PCR method, that $bla_{KPC}$ was anchored within Tn *4401* variant "d" (Table 5).

**Table 5: Genotypic and spectroscopic characterisation of *Klebsiella pneumoniae* isolates.**

| N. of Isolate | FTIR | ST | Plasmids associated with *bla*KPC (size; Inc groups) | Tn variant | wzi/K-type | KPC-type | Other β-lactamases | Non β-lactam phenotype |
|---|---|---|---|---|---|---|---|---|
| **K70** | C1 | 147 | FIA (~115kb) | Tn4401d | wzi64/K14.K64 | KPC-3 | OXA-9, TEM-1, SHV-11 | (AMK), CIP, (GEN), (KAN), MEC, (MIN), NAL, (NET), (NIT), (STR), (SXT), (TMP), (TOB) |
| **K86** | C1 | 147 | FIA (~115kb) | Tn4401d | wzi64/K14.K64 | KPC-3 | OXA-9, SHV-12 | |
| **K87** | C1 | 147 | FIA (~115kb) | Tn4401d | wzi64/K14.K64 | KPC-3 | OXA-9, SHV-28 | |
| **K89** | C1 | 147 | FIA (~115kb) | Tn4401d | wzi64/K14.K64 | KPC-3 | OXA-9, SHV-12 | |
| **K90** | C7 | 147 | FIA (~115kb) | Tn4401d | wzi64/K14.K64 | KPC-3 | OXA-9, SHV-11 | |
| **K93** | C1 | 147 | FIA (~115kb) | Tn4401d | wzi64/K14.K64 | KPC-3 | OXA-9, SHV-12 | |
| **K115** | C1 | 147 | N | Tn4401d | wzi64/K14.K64 | KPC-3 | SHV-11 | |
| **K116** | C1 | 147 | N | Tn4401d | wzi64/K14.K64 | KPC-3 | SHV-12 | |
| **K126** | C1 | 147 | N | Tn4401d | wzi64/K14.K64 | KPC-3 | SHV-11 | |
| **K133** | C1 | 147 | N | Tn4401d | wzi64/K14.K64 | KPC-3 | SHV-11 | |
| **K88** | C6 | 15 | FIA (~115kb) | Tn4401d | wzi19/K19 | KPC-3 | OXA-9, SHV-28 | (AMK), CIP, (CHL), GEN, KAN, MEC, NAL, NET, STR, SXT, TMP, TOB |
| **K95** | C6 | 15 | FIA (~115kb) | Tn4401d | wzi19/K19 | KPC-3 | OXA-9, SHV-28 | |
| **K132** | C6 | 15 | FIA (~115kb) | Tn4401d | wzi19/K19 | KPC-3 | OXA-9, SHV-28, TEM-1 | |
| **K125** | C12 | 15 | FIA (~115kb) | Tn4401d | wzi19/K19 | KPC-3 | OXA-9, SHV-28, TEM-1 | |
| **K91** | C8 | 15 | FIA (~115kb) | Tn4401d | wzi93/K60 | KPC-3 | OXA-9, SHV-28, CTX-M-15 | CIP, CHL, KAN, MEC, MIN, NAL, NET, NIT, STR, SXT, TMP, TOB |

| N. of isolate | FTIR | ST | Plasmids associated with *bla*KPC (size; Inc groups) | Tn variant | wzi/K-type | KPC-type | Other β-lactamases | Non β-lactam phenotype |
|---|---|---|---|---|---|---|---|---|
| **K127** | C10 | 231 | FIA (~115kb) | Tn4401d | wzi104/K- | KPC-3 | SHV-1, TEM-1 | (AMK), CIP, CHL, (GEN), KAN, MEC, MIN, NAL, (NET), NIT, STR, SXT, (TET), TMP, TOB |
| **K134** | C10 | 231 | FIA (~115kb) | Tn4401d | wzi104/K- | KPC-3 | SHV-1 | |
| **K124** | C11 | 231 | FIA (~115kb) | Tn4401d | wzi104/K- | KPC-3 | SHV-1 | |
| **K92** | C9 | 109 | FIA (~115kb) | Tn4401d | wzi22/K22.37 | KPC-3 | SHVnew (N196S) | STR, SXT, TMP |
| **K56** | C5 | 348 | FIA (~115kb) | Tn4401d | wzi94/K- | KPC-3 | OXA-1, TEM-1, CTX-M-15 | CIP, GEN, KAN, MEC, NAL, NET, STR, SXT, TET, TMP, TOB |

The characterisation of plasmids and KPC-type by PFGE and wzi/K-typing was performed by Ângela Novais et al. Abbreviation: AMK, Amikacin; GEN, Gentamicin; NET, Netilmicin; TOB, Tobramycin; KAN, Kanamycin; STR, Streptomycin; CIP, Ciprofloxacin; NAL, Nalidixic acid; SXT, Sulfamethoxazole/trimethoprim; TMP, Trimethoprim; TET, Tetracycline; MIN, Minocycline; CHL, Chloramphenicol; NIT, Nitrofurantoin; KPC, *Klebsiella pneumoniae* carbapenemase; CTX-M, cefotaximase; SHV, sulphydryl variable β-lactamase; TEM, Temoneira β-lactamase; OXA, oxacillinase; FTIR, Fourier transform infrared spectroscopy; Inc group, Incompatibility group; Tn variant, transposon variant; C, clone

**Table 6: Antibiotic resistance profiles of *Klebsiella pneumoniae***

| β-lactams | Antimicrobial agent | ST147 (10) | ST15-Kp2 (4) | ST15-Kp3 (1) | ST231 (3) | ST348 (1) | ST109 (1) |
|---|---|---|---|---|---|---|---|
| | **Amoxicillin/clavulanate** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **Ceftazidime** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **Cefotaxime** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **Cefepime** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **Cefotaxime** | 80 | 100 | 100 | 100 | 100 | 0 |
| | **Mecillinam** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **Aztreonam** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **Ertapenem** | 100 | 100 | 100 | 100 | 100 | 100 |
| | **MIC (range, mg/L)** | 1-4 | 4-12 | 6 | 3-6 | 6 | 1 |
| | **Imipenem** | 100 | 25 | 100 | 33 | 100 | 100 |
| | **MIC (range, mg/L)** | 3-8 | 2-8 | 6 | 1.5-32 | 12 | 3 |
| | **Meropenem** | 10 | 25 | 100 | 67 | 100 | 100 |
| | **MIC (range, mg/L)** | 1-12 | 1.5-3 | 6 | 2-24 | 4 | 3 |
| Non-β-lactams | **Amikacin** | 60 | 75 | 0 | 33 | 0 | 0 |
| | **Gentamicin** | 70 | 100 | 0 | 67 | 100 | 0 |
| | **Netilmicin** | 70 | 100 | 100 | 67 | 100 | 0 |
| | **Tobramycin** | 70 | 100 | 100 | 100 | 100 | 0 |
| | **Kanamycin** | 70 | 100 | 100 | 100 | 100 | 0 |
| | **Streptomycin** | 70 | 100 | 100 | 100 | 100 | 100 |
| | **Ciprofloxacin** | 100 | 100 | 100 | 100 | 100 | 0 |
| | **Nalidixic acid** | 100 | 100 | 100 | 100 | 100 | 0 |
| | **Sulfamethoxazole/trimethoprim** | 80 | 100 | 100 | 100 | 100 | 100 |
| | **Trimethoprim** | 80 | 100 | 100 | 100 | 100 | 100 |
| | **Tetracycline** | 0 | 0 | 0 | 67 | 100 | 0 |
| | **Minocycline** | 10 | 0 | 100 | 100 | 0 | 0 |
| | **Chloramphenicol** | 0 | 75 | 100 | 100 | 0 | 0 |
| | **Nitrofurantoin** | 60 | 0 | 100 | 100 | 100 | 0 |
| | **Fosfomycin** | 10 | 0 | 100 | 0 | 100 | 0 |
| | **Colistin** | 0 | 0 | 0 | 0 | 0 | 0 |

ST15 contained two clones Kp2 and Kp3, which were detected by PFGE method performed by Ângela Novais et al. Clinical Breakpoints (Ertapenem - S≤0.5 mg/L; Imipenem and Meropenem - S≤2 mg/L) and ECOFF values (Ertapenem - WT≤0.064 mg/L; Imipenem - WT≤1 mg/L; Meropenem, WT≤0.125 mg/L) for minimum inhibitory concentration (MIC) defined by EUCAST for K. pneumoniae.

## 6.2. *Enterococcus*

### 6.2.1. Discrimination by BAPS subgroups

The PLSDA model (first two latent variables 79.1 % of the spectral variability) obtained with infrared spectra separated the isolates into five clusters with correspondence with the five subgroups delineated by BAPS (with the exception of some isolates; Figure 9).

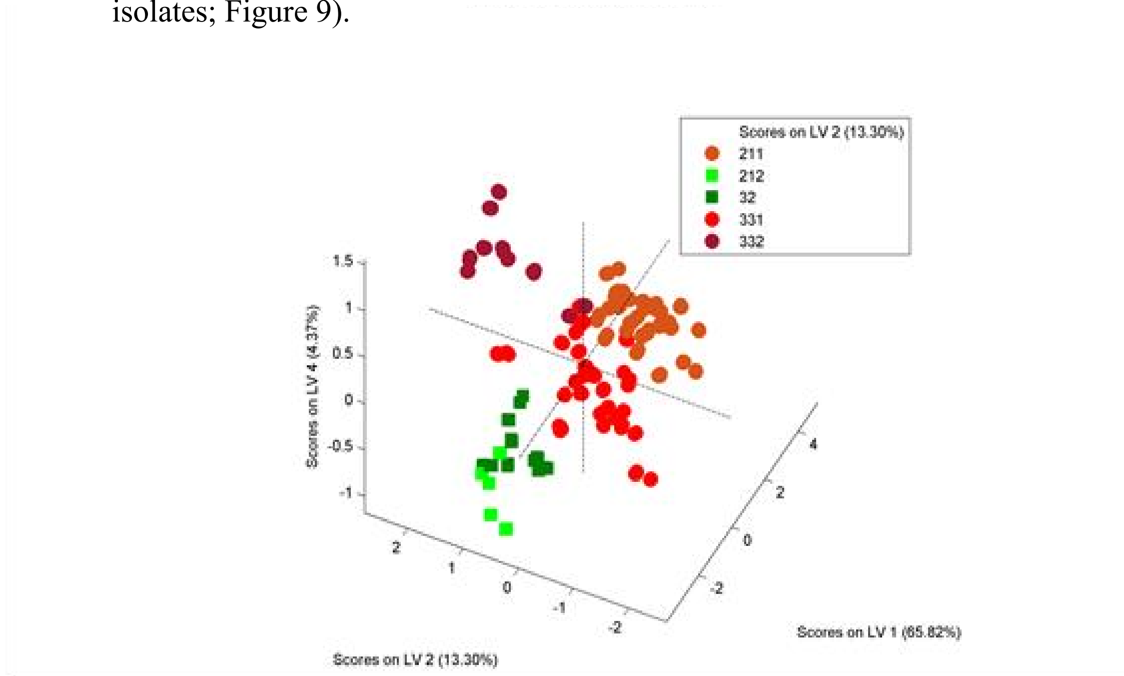


**Figure 9:** Score plot corresponding to the four locus variants (LVs) of the PLSDA regression model including all *E. faecium* isolates using the 1500-900 $cm^{-1}$spectral region.

### 6.2.1. Discrimination by STs

FTIR results allowed the categorization of isolates into different BAPS subgroups (*n*=6): BAPS 2.1a (38 % - ST78, ST80, ST117, ST192, ST203, ST412), BAPS 2.1b (9.79 % - ST5, ST147, ST185, ST280, ST493), BAPS 3.3a1 (30.77 % - ST18, ST132, ST173, ST368), BAPS 3.2 (10.49 % - ST6) and BAPS 3.3a2 (16.08 % - ST16, ST17, ST202, ST209, ST719)

The PLSDA model of mass spectra grouped isolates by STs exactly in the same way they clustered by BAPS subgroups (Figure 10). The model based on infrared spectra showed a high separation of STs belonging to BAPS subgroups 3.3a2 (ST16 and ST17) (Figure 10).

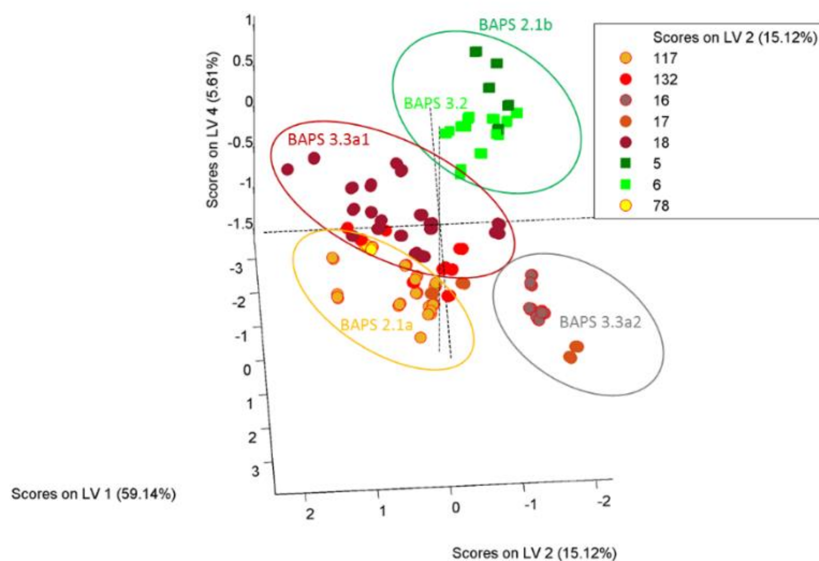


**Figure 10:** Score plot corresponding to the four LVs of the PLSDA regression model including all *E. faecium* isolates using the 1500–900 $cm^{-1}$ spectral region in B.

Discrimination of single LVs from the same BAPS subgroup: example of ST18 and ST132 (3.3a1). A second PLSDA model comprising spectra from ST18 and ST132 (single LVs from BAPS 3.3a1 not separated in the general model) showed a clear separation of the two groups with a high resolution by FTIR (first two latent variables encompassing 74.4 % of the spectral variability; Figure 11)

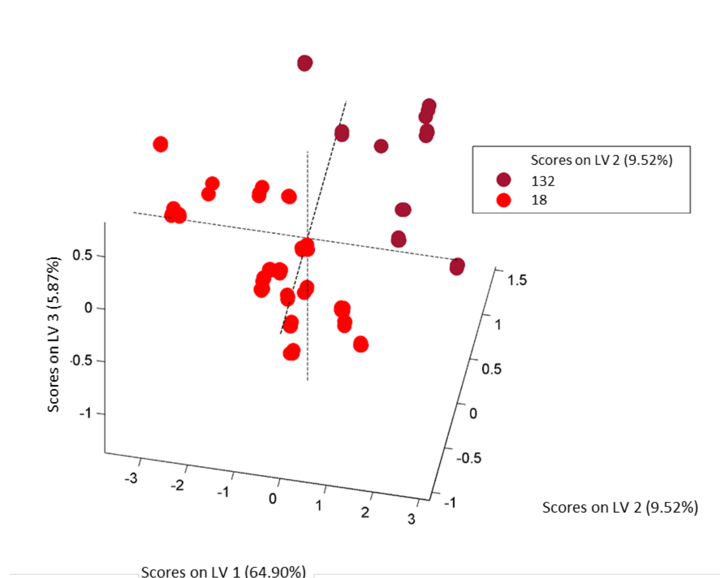


**Figure 11:** Score plot corresponding to the first three LVs of the PLSDA regression model including ST18 and ST132 *E. faecium* isolates.

Discrimination of single LVs from different BAPS subgroups ST5 (BAPS 2.1b) and ST6 (BAPS 3.2) showed a better separation also in a second model of infrared spectra (discriminated by two latent variables; Figure 12).

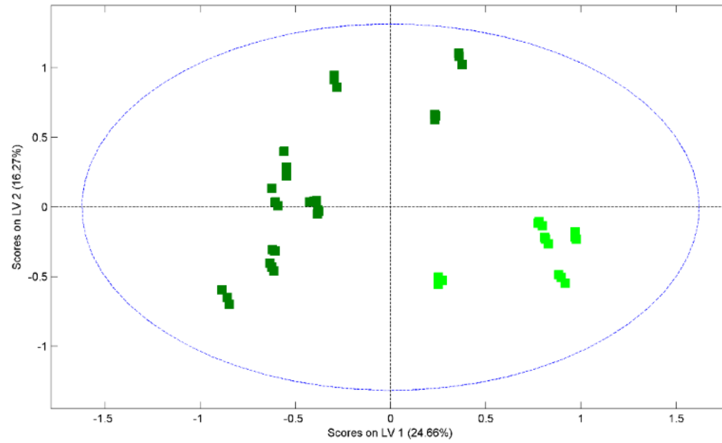


**Figure 12:** Score plot corresponding to the first two LVs of the PLSDA regression model including ST5 and ST6 *E. faecium* isolates. All spectra were evaluated by Ana Freitas et al.

# 7. Discussion

## 7.1. Characterisation of KPC-3-producing *K. pneumoniae* isolates

Our results show that KPC-3-producing *K. pneumoniae* isolates are besides Portugal hospitals currently spread in in normal disease symptoms free population, thus representing potential thread for public health. We used genotypic techniques to identify the determining factors that drive the dissemination of multidrug resistant KPC-3 producing *K. pneumoniae* isolates and the relationship of $bla_{KPC-3}$ with specific mobile genetic elements and/or respective clones. The occurrence of KPC enzymes is associated mainly with *K. pneumoniae*, although these enzymes have been also identified in many Gram-negative species *(Munoz-Price et al., 2013)*. In 2011, Portugal registered a case of KPC-2 producing *E. coli* in river samples from Santo Tirso. However, there were no known cases associated with human infections at that time *(Poirel et al., 2012b).* This trend of the probably negligible incidence of infection by KPC-producing isolates continued until 2013, when they (specifically KPC-3 producing bacteria) were identified in different hospitals in Portugal (*Glasner et al., 2013, Canton et al., 2012, Manageiro et al. 2015*).

Interestingly, in the Czech Republic, the first cases of KPC-producing *K. pneumoniae* occurred in patients repatriated from hospitals in Italy (KPC-3, ST512) and Greece (KPC-2, ST258), followed by a hospital outbreak caused by ST512 isolate in Prague in 2011. PFGE patterns showed a consensus between ST258 and ST512 isolates resistant to colistin *(Hrabak et al., 2013)*. While in the Czech Republic the situation with KPC-producing *Enterobacteriaceae* seems to be stabilized, in Portugal latest data shows a constantly deteriorating trend *(Albiger et al., 2015).*

In our study we found that the isolates tested were resistant to all *K. pneumoniae* isolates to ertapenem, 75 % resistant (mainly ST15 and ST231 isolates), to imipenem and 35 % resistant to meropenem (mainly ST15 and ST147). However, isolates were also not sensitive to other antibiotics but not colistin, thus fundamentally complicating to find safe and effective antibiotic treatment options.

Among the *K. pneumoniae* isolates, phenotypic resistance to β-lactams was predominantly mediated by production of KPC-3, CTX-M-15, SHV and TEM type β-lactamases. $Bla_{KPC-3}$ genes were borne on transposon Tn*4401* “d” isoform in two different plasmid´s types (IncFIA and IncN). $Bla_{KPC}$ genes dissemination is frequently

associated with different variants of this Tn*3* based transposon (Tn4401) *(Naas et al., 2008b)*. Tn*4401*d variant has been predominantly described in isolates from the USA to date. This fact is confirmed by *Chen et al., (2014b)*, who described that 20 % of 491 *K. pneumoniae* isolates contained KPC-3 within Tn*4401*d transposon in IncFIA plasmids (pBK30661 and pBK30683). In our study, we found that 80 % of all 20 isolates were positive for IncFIA plasmids carrying $bla_{KPC-3}$ genes and on the other hand linked to Tn*4401d* transposons. However, there have been cases from China and Argentina where $bla_{KPC}$ also occurred among non-Tn*4401* mobile elements *(Shen et al., 2009, Gomez et al., 2011)*. In the study by *Manageiro et al.* (2015), all identified $bla_{KPC-3}$ genes were carried on Tn*4401* transposons isoform "d", suggesting this mobile element as a crucial source of wide spectrum resistance.

In our study, using MLST we divided 20 *K. pneumoniae* carbapenemase-producing isolates into 5 STs: that is, ST15, ST109, ST147, ST231 and ST348. However, in the study *Manageiro et al.* (2015) different ST´s were found, i.e. among 29 *K. pneumoniae* isolates, a higher genetic diversity was described (namely ST14, ST15, ST34, ST59, ST147, ST416, ST698 and 2 new: ST 960 and ST 1138). By April 2014, a total of 1536 STs have been classified in the *K. pneumoniae* MLST online database (http://www.pasteur.fr/mlst) *(Chen et al., 2014a)* and up to 2014 various KPC enzymes have been found in more than 115 different STs *(Chen et al., 2014a)*. Worldwide, KPC enzymes were more frequently linked to clonal group 258 with two predominant STs; ST258, ST11 and less frequently ST340, ST437 and ST512 *(Chen et al., 2014a)*, and ST258 is considered as a key factor in the spread of KPC carbapenemase enzyme. On the contrary, in Portugal, the emergence of KPC-3 enzymes was linked to other clones (ST147 followed by ST15, ST109 and ST231) and ST 258 was not detected either in our study or in study of *Manageiro et al.,* (2015). ST147 isolates (the most abundant in our study) revealed the presence of KPC, OXA, TEM and SHV enzymes and we found a new SHV enzyme.

## 7.2. FTIR is a suitable method for the rapid identification of isolates

FTIR has been previously suggested as a promising time and money saving method for bacterial isolates identification (*Sousa et al., 2014*, *Taha et al.*, *2014*, *Criste et al.*, *2011)*. First this method was applied by *Sousa et al.,* (2014) on another nosocomial pathogen *Acinetobacter baumannii*. They compared their results obtained by FTIR with

the available classification (distribution into distinct groups with four modeled STs; ST98, ST103, ST208 and ST218) showing higher than 95 % reliability of the model prediction. Another study by *Taha et al.*, (2014) on VRE showed that FTIR has higher discrimination ability when compared with AST. Finally, *Criste et al.*, (2011) was able to distinguish with 100% accuracy more than 5 bacterial and fungi kinds including *K. pneumoniae*, *Escherichia coli*, *Staphylococcus aureus or Candida albicans)*. However, FTIR method is widely used not only in the field of bacteriology (*Rehman and Che Man, 2010*).

We optimized and tested FTIR on other types of bacteria further confirming potential of this method for purpose of isolates identification while it still seems to be a cheaper and quicker alternative than genotypic methods to distinguish clones of *K. pneumoniae* and *E. faecium* species. FTIR ranked isolates of *E. faecium* into BAPS groups with more than 70% accuracy and FTIR results for *K. pneumoniae* isolates in comparison with the MLST resulted in 80 % identification accuracy. We did not achieve so high accuracy as was reported on other bacteria. However, this might be caused by testing of other types of bacteria on one hand, and on the other hand we are also aware of lower number of tested isolates of *K. pneumoniae* that limits us to provide conclusive outcomes.

# 8. Conclusions

As the number of resistant bacteria is constantly growing, global interest is to find fast and cheap while accurate way for identification of resistant isolates that would enable individualized effective treatment. However, there are only limited options to quickly detect multidrug resistant *K. pneumoniae* and *E. faecium*.

We screened 20 isolates of *K. pneumoniae* with reduced susceptibility to carbapenems and 143 VRE *faecium*. All *K. pneumoniae* isolates were KPC-3 producers. Genetic relatedness among representative isolates was evaluated primarily by FTIR spectroscopy and confirmed by MLST results. The conformity was 80 %. The presence of different β-lactamases was also detected among single isolates with majority of enzymes belonging to SHV family, while identifying new SHV N196S. Furthermore, it was discovered using PCR method, that genes coding for KPC enzymes were anchored within transposon Tn*4401* variant "d" and mostly on IncFIA plasmids or IncN plasmids. All sequence types were resistant to several antibiotic classes, while remaining susceptible to colistin which was the most active antibiotic for all the strains tested.

FTIR showed a good discrimination capability between vancomycin resistant *E. faecium* major clones obtained from human infections and from commensal origins. FTIR results showed a high identification capability compared with genotypic methods, but it is necessary to perform additional tests with more isolates. Currently, FTIR method is not able determine the type of antibiotic treatment without other identification methods.

# 9. References

Abu Taha, M.I. Eideh, H.K., Saed, S.M. and Jaber, H. (2014) Fourier Transform Infrared Spectroscopy Typing an *Enterococcus* sp. *Journal of Modern Physics*, **5**, 1698-1707.

Al-Bayssari C, Dabboussi F, Hamze M, Rolain JM (2015) Detection of expanded-spectrum beta-lactamases in Gram-negative bacteria in the 21st century. Expert Rev Anti Infect Ther 13:1139-1158.

Alanis AJ (2005) Resistance to antibiotics: are we in the post-antibiotic era? Arch Med Res 36:697-705.

Albiger B, Glasner C, Struelens MJ, Grundmann H, Monnet DL, European Survey of Carbapenemase-Producing Enterobacteriaceae working g (2015) Carbapenemase-producing Enterobacteriaceae in Europe: assessment by national experts from 38 countries, May 2015. Euro Surveill 20.

Alvarez-Ordonez A, Mouwen DJ, Lopez M, Prieto M (2011) Fourier transform infrared spectroscopy as a tool to characterize molecular composition and stress response in foodborne pathogenic bacteria. J Microbiol Methods 84:369-378.

Bassetti M, Merelli M, Temperoni C, Astilean A (2013) New antibiotics for bad bugs: where are we? Ann Clin Microbiol Antimicrob 12:22.

Bennett PM (2008) Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol 153 Suppl 1:S347-357.

Biavasco F, Foglia G, Paoletti C, Zandri G, Magi G, Guaglianone E, Sundsfjord A, Pruzzo C, Donelli G, Facinelli B (2007) VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. Appl Environ Microbiol 73:3307-3319.

Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ (2015) Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42-51.

Bogaerts, P., Rezende de Castro, R., de Mendonça, R., Huang, T.-D., Denis, O., and Glupczynski, Y. (2013). Validation of carbapenemase and extended-spectrum β-lactamase multiplex endpoint PCR assays according to ISO 15189. J. Antimicrob. Chemother. 68, 1576–82. doi:10.1093/jac/dkt065.

Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48:1-12.

Brisse S, Passet V, Haugaard AB, Babosan A, Kassis-Chikhani N, Struve C, Decre D (2013) wzi Gene sequencing, a rapid method for determination of capsular type for Klebsiella strains. J Clin Microbiol 51:4073-4078.

Bush K (2013) The ABCD's of beta-lactamase nomenclature. J Infect Chemother 19:549-559.

Butaye P (2003) Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. International Journal of Antimicrobial Agents 22:205-210.

Canton R, Coque TM (2006) The CTX-M beta-lactamase pandemic. Curr Opin Microbiol 9:466-475.

Canton R, Gonzalez-Alba JM, Galan JC (2012) CTX-M Enzymes: Origin and Diffusion. Front Microbiol 3:110.

Clewell, Don B (February 2014) Antibiotic Resistance Plasmids in Bacteria. In: eLS. John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0001491.pub3

Coburn B, Low DE, Patel SN, Poutanen SM, Shahinas D, Eshaghi A, Willey BM, McGeer A (2014a) Vancomycin-variable Enterococcus faecium: in vivo emergence of vancomycin resistance in a vancomycin-susceptible isolate. J Clin Microbiol 52:1766-1767.

Coburn B, Low DE, Patel SN, Poutanen SM, Shahinas D, Eshaghi A, Willey BM, McGeer A (2014b) Vancomycin-variable Enterococcus faecium: in vivo emergence of vancomycin resistance in a vancomycin-susceptible isolate. J Clin Microbiol 52:1766-1767.

Courvalin P (2006) Vancomycin resistance in gram-positive cocci. Clin Infect Dis 42 Suppl 1:S25-34.

Couturier M, Bex F, Bergquist PL, Maas WK (1988) Identification and classification of bacterial plasmids. Microbiol Rev 52:375-395.

Criste, A., Cosmin, P. O. P., Vioara, M. I. R. E., & Nicodim, F. I. (2011). Use of FT-IR Technique as a Method for Differentiation of Bacterial and Fungal Strains. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Veterinary Medicine*, *1*(68).

Curiao, T., Morosini, M. I., Ruiz-Garbajosa, P., Robustillo, A., Baquero, F., Coque, T. M., et al. (2010). Emergence of bla KPC-3-Tn4401a associated with a pKPN3/4-like plasmid within ST384 and ST388 Klebsiella pneumoniae clones in Spain. J. Antimicrob. Chemother. 65, 1608–14. doi:10.1093/jac/dkq174.

Cuzon G, Naas T, Nordmann P (2011) Functional characterization of Tn4401, a Tn3-based transposon involved in blaKPC gene mobilization. Antimicrob Agents Chemother 55:5370-5373.

Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S (2005) Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. J Clin Microbiol 43:4178-4182.

Eftekhar F, Naseh Z (2015) Extended-spectrum beta-lactamase and carbapenemase production among burn and non-burn clinical isolates of Klebsiella pneumoniae. Iran J Microbiol 7:144-149.

El Salabi A, Walsh TR, Chouchani C (2013) Extended spectrum beta-lactamases, carbapenemases and mobile genetic elements responsible for antibiotics resistance in Gram-negative bacteria. Crit Rev Microbiol 39:113-122.

European centre for Disease Prevention and Control (2016a) Proportion of 3rd gen. cephalosporins Resistant (R) Klebsiella pneumoniae Isolates in Participating Countries in 2014. Available at URL: http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx#sthash.ZP64ewbF.dpuf Access: 12th of May 2016

European centre for Disease Prevention and Control (2016b) Proportion of Vancomycin Resistant (R) Enterococcus faecium Isolates in Participating Countries in 2014. Available at URL: http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx#sthash.N6eJiiVi.dpuf Access: 12th of May 2016

Falagas ME, Bliziotis IA (2007) Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? Int J Antimicrob Agents 29:630-636.

Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 186:1518-1530.

Foulquie Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L (2006) The role and application of enterococci in food and health. Int J Food Microbiol 106:1-24.

Glasner C, Albiger B, Buist G, Tambic Andrasevic A, Canton R, Carmeli Y, Friedrich AW, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Nordmann P, Poirel L, Rossolini GM, Seifert H, Vatopoulos A, Walsh T, Woodford N, Donker T, Monnet DL, Grundmann H, European Survey on Carbapenemase-Producing Enterobacteriaceae Working G (2013) Carbapenemase-producing Enterobacteriaceae in Europe: a survey among national experts from 39 countries, February 2013. Euro Surveill 18.

Gomez SA, Pasteran FG, Faccone D, Tijet N, Rapoport M, Lucero C, Lastovetska O, Albornoz E, Galas M, Group KPC, Melano RG, Corso A, Petroni A (2011) Clonal dissemination of Klebsiella pneumoniae ST258 harbouring KPC-2 in Argentina. Clin Microbiol Infect 17:1520-1524.

Hartl J (2006) *Farmaceutická chemie IV*. 1. vyd. Praha: Karolinum, ISBN 80-246-1169-4.

Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A (2010) Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. Clin Microbiol Infect 16:541-554.

Heritage J, M'Zali FH, Gascoyne-Binzi D, Hawkey PM (1999) Evolution and spread of SHV extended-spectrum beta-lactamases in gram-negative bacteria. J Antimicrob Chemother 44:309-318.

Hirsch EB, Tam VH (2010) Detection and treatment options for Klebsiella pneumoniae carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. J Antimicrob Chemother 65:1119-1125.

Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, van Embden JDA, Willems RJL (2002) Multilocus Sequence Typing Scheme for Enterococcus faecium. Journal of Clinical Microbiology 40:1963-1971.

Hrabak J, Vanis V, Bergerova T, Urbaskova P (2008) Průkaz beta-laktamáz širokého spektra (ESBL) a typu AmpC u enterobakterií. Available at URL: www.szu.cz/prukaz-beta-laktamaz-sirokeho-spektra-esbl-a-typu-ampc-u. Access: 9th of June 2016

Hrabak J, Papagiannitsis CC, Studentova V, Jakubu V, Fridrichova M, Zemlickova H, Czech Participants of European Antimicrobial Resistance Surveillance N (2013) Carbapenemase-producing Klebsiella pneumoniae in the Czech Republic in 2011. Euro Surveill 18:20626.

Chen L Chavda KD, Mediavilla JR, Jacobs MR, Levi MH, Bonomo RA, Kreiswirth BN (2012) Partial excision of blaKPC from Tn4401 in carbapenem-resistant Klebsiella pneumoniae. Antimicrob Agents Chemother 56:1635-1638.

Chen L, Mathema B, Chavda KD, DeLeo FR, Bonomo RA, Kreiswirth BN (2014a) Carbapenemase-producing Klebsiella pneumoniae: molecular and genetic decoding. Trends Microbiol 22:686-696.

Chen L, Chavda KD, Melano RG, Hong T, Rojtman AD, Jacobs MR, Bonomo RA, Kreiswirth BN (2014b) Molecular survey of the dissemination of two blaKPC-harboring IncFIA plasmids in New Jersey and New York hospitals. Antimicrob Agents Chemother 58:2289-2294.

Infectious Diseases Society of A (2010) The 10 x '20 Initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. Clin Infect Dis 50:1081-1083.

Jeon JH, Lee JH, Lee JJ, Park KS, Karim AM, Lee CR, Jeong BC, Lee SH (2015) Structural basis for carbapenem-hydrolyzing mechanisms of carbapenemases conferring antibiotic resistance. Int J Mol Sci 16:9654-9692.

Jolley KA, Maiden MC (2010) BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11:595.

Khan AU (2012) BLAD B-Lactamase Database. Available at URL: http://www.blad.co.in/index.html. Access: 9th of May 2016

Knothe H, Shah P, Krcmery V, Antal M, Mitsuhashi S (1983) Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of Klebsiella pneumoniae and Serratia marcescens. Infection 11:315-317.

Lal P, Kapil A, Das BK, Sood S (2007) Occurrence of TEM & SHV gene in extended spectrum beta-lactamases (ESBLs) producing Klebsiella sp. isolated from a tertiary care hospital. Indian J Med Res 125:173-178.

Lautenbach E, Patel JB, Bilker WB, Edelstein PH, Fishman NO (2001) Extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae: risk factors for infection and impact of resistance on outcomes. Clin Infect Dis 32:1162-1171.

Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, Leclercq R, Courvalin P, Cattoir V (2011) D-Ala-d-Ser VanN-type transferable vancomycin resistance in Enterococcus faecium. Antimicrob Agents Chemother 55:4606-4612.

Livermore DM, British Society for Antimicrobial Chemotherapy Working Party on The Urgent Need: Regenerating Antibacterial Drug D, Development (2011) Discovery research: the scientific challenge of finding new antibiotics. J Antimicrob Chemother 66:1941-1944.

Maroncle N, Rich C, Forestier C (2006) The role of Klebsiella pneumoniae urease in intestinal colonization and resistance to gastrointestinal stress. Res Microbiol 157:184-193.

Mathers AJ, Peirano G, Pitout JD (2015) The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clin Microbiol Rev 28:565-591.

Matushek E, Brown DF, Kahlmeter G (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect 20:O255-266.

Manageiro V, Ferreira E, Almeida J, Barbosa S, Simoes C, Antibiotic Resistance Surveillance Program in P, Bonomo RA, Canica M (2015) Predominance of KPC-3 in a survey for carbapenemase-producing Enterobacteriaceae in Portugal. Antimicrob Agents Chemother 59:3588-3592.

McMurry L, Petrucci RE, Jr., Levy SB (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli. Proc Natl Acad Sci U S A 77:3974-3977.

Munoz-Price LS, Quinn JP (2009) The spread of Klebsiella pneumoniae carbapenemases: a tale of strains, plasmids, and transposons. Clin Infect Dis 49:1739-1741.

Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP (2013) Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. The Lancet Infectious Diseases 13:785-796.

Murray BE (2000) Vancomycin-resistant enterococcal infections. N Engl J Med 342:710-721.

Murray BE (1990) The life and times of the Enterococcus. Clin Microbiol Rev 3:46-65.

Naas T, Poirel L, Nordmann P (2008a) Minor extended-spectrum beta-lactamases. Clin Microbiol Infect 14 Suppl 1:42-52.

Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P (2008b) Genetic structures at the origin of acquisition of the beta-lactamase bla KPC gene. Antimicrob Agents Chemother 52:1257-1263.

Naumann, D., Helm, D., & Labischinski, H. (1991). Microbiological characterizations by FT-IR spectroscopy. *Nature*, *351*(6321), 81-82.

Niebel M, Quick J, Prieto AM, Hill RL, Pike R, Huber D, David M, Hornsey M, Wareham D, Oppenheim B, Woodford N, van Schaik W, Loman N (2015) Deletions in a ribosomal protein-coding gene are associated with tigecycline resistance in Enterococcus faecium. Int J Antimicrob Agents 46:572-575.

Nordmann P, Cuzon G, Naas T (2009) The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect Dis 9:228-236.

Nordmann P, Poirel L (2014) The difficult-to-control spread of carbapenemase producers among Enterobacteriaceae worldwide. Clin Microbiol Infect 20:821-830.

Nyč O (2007) Potřeba a perspektivy nových antibiotik. Remedia; 17: 476–480.

O'Driscoll T, Crank CW (2015) Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. Infect Drug Resist 8:217-230.

Parisi SG, Bartolini A, Santacatterina E, Castellani E, Ghirardo R, Berto A, Franchin E, Menegotto N, De Canale E, Tommasini T, Rinaldi R, Basso M, Stefani S, Palu G (2015) Prevalence of Klebsiella pneumoniae strains producing carbapenemases and increase of resistance to colistin in an Italian teaching hospital from January 2012 To December 2014. BMC Infect Dis 15:244.

Pasteran F, Veliz O, Ceriana P, Lucero C, Rapoport M, Albornoz E, Gomez S, Corso A, Re LNG (2015) Evaluation of the Blue-Carba test for rapid detection of carbapenemases in gram-negative bacilli. J Clin Microbiol 53:1996-1998.

Paterson DL, Bonomo RA (2005) Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 18:657-686.

Pires J, Novais A, Peixe L (2013) Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. J Clin Microbiol 51:4281-4283.

Pitout, D., & Johann, D. (2006). Molecular detection of bacteria producing newer types of β-lactamases. *Current Genomics*, *7*(3), 171-177.

Podschun R, Ullmann U (1998) Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11:589-603.

Poirel L, Bonnin RA, Nordmann P (2012a) Genetic support and diversity of acquired extended-spectrum beta-lactamases in Gram-negative rods. Infect Genet Evol 12:883-893.

Poirel L, Barbosa-Vasconcelos A, Simoes RR, Da Costa PM, Liu W, Nordmann P (2012b) Environmental KPC-producing Escherichia coli isolates in Portugal. Antimicrob Agents Chemother 56:1662-1663.

Ramsey M, Hartke A, Huycke M (2014) The Physiology and Metabolism of Enterococci. In: Enterococci: From Commensals to Leading Causes of Drug Resistant Infection (Gilmore, M. S. et al., eds) Boston.

Rice LB (2008) Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis 197:1079-1081.

Rodrigues C, Machado E, Ramos H, Peixe L, Novais A (2014) Expansion of ESBL-producing Klebsiella pneumoniae in hospitalized patients: a successful story of international clones (ST15, ST147, ST336) and epidemic plasmids (IncR, IncFIIK). Int J Med Microbiol 304:1100-1108.

Rodriguez-Bano J (2015) The times they are a-changin': carbapenems for extended-spectrum-beta-lactamase-producing bacteria. Antimicrob Agents Chemother 59:5095-5096.

Rohman A, Man YBC (2010) Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. Food Research International 43:886-892.

Ruppe E, Woerther PL, Barbier F (2015) Mechanisms of antimicrobial resistance in Gram-negative bacilli. Ann Intensive Care 5:61.

Sader HS, Pignatari AC (1994) E test: a novel technique for antimicrobial susceptibility testing. Sao Paulo Med J 112:635-638.

Sghir A, Gramet G, Suau A, Rochet V, Pochart P, Dore J (2000) Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl Environ Microbiol 66:2263-2266.Shaikh S, Fatima J, Shakil S, Rizvi SM, Kamal MA (2015) Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J Biol Sci 22:90-101.

Shaikh S, Fatima J, Shakil S, Rizvi SM, Kamal MA (2015) Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. Saudi J Biol Sci 22:90-101.

Shen P, Wei Z, Jiang Y, Du X, Ji S, Yu Y, Li L (2009) Novel genetic environment of the carbapenem-hydrolyzing beta-lactamase KPC-2 among Enterobacteriaceae in China. Antimicrob Agents Chemother 53:4333-4338.

Schembri MA, Blom J, Krogfelt KA, Klemm P (2005) Capsule and fimbria interaction in Klebsiella pneumoniae. Infect Immun 73:4626-4633.

Smillie C, Garcillan-Barcia MP, Francia MV, Rocha EP, de la Cruz F (2010) Mobility of plasmids. Microbiol Mol Biol Rev 74:434-452.

Sousa C, Silva L, Grosso F, Lopes J, Peixe L (2014) Development of a FTIR-ATR based model for typing clinically relevant Acinetobacter baumannii clones belonging to ST98, ST103, ST208 and ST218. J Photochem Photobiol B 133:108-114.

Spizek J, Novotna J, Rezanka T, Demain AL (2010) Do we need new antibiotics? The search for new targets and new compounds. J Ind Microbiol Biotechnol 37:1241-1248.

Spratt BG (1999) Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. Curr Opin Microbiol 2:312-316.

Struve C, Krogfelt KA (2003) Role of capsule in Klebsiella pneumoniae virulence: lack of correlation between in vitro and in vivo studies. FEMS Microbiol Lett 218:149-154.

Suchopár, J, Valentová, Š (ed.). *Remedia compendium*. 4. vyd. Praha: Panax, 2009. ISBN 978-80-902806-4-9.

Sun J, Deng Z, Yan A (2014) Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem Biophys Res Commun 453:254-267.

Taylor, D. E., A. Gribeel, T. D. Lawley y D. M. Tracz. 2004. Antibiotic resistance plasmids. In: Plasmid Biology. Funnell BE, Phillips GJ (eds). American Societyfor Microbiology, Washington D.C.

Tedim AP, Ruiz-Garbajosa P, Corander J, Rodriguez CM, Canton R, Willems RJ, Baquero F, Coque TM (2015) Population biology of intestinal enterococcus isolates from hospitalized and nonhospitalized individuals in different age groups. Appl Environ Microbiol 81:1820-1831.

Tenover FC (2006) Mechanisms of antimicrobial resistance in bacteria. Am J Med 119:S3-10; discussion S62-70.

Thaker MN, Kalan L, Waglechner N, Eshaghi A, Patel SN, Poutanen S, Willey B, Coburn B, McGeer A, Low DE, Wright GD (2015) Vancomycin-variable enterococci can give rise to constitutive resistance during antibiotic therapy. Antimicrob Agents Chemother 59:1405-1410.

Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ (2000) CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. Int J Antimicrob Agents 14:137-142.

Van Laar TA, Chen T, You T, Leung KP (2015) Sublethal concentrations of carbapenems alter cell morphology and genomic expression of Klebsiella pneumoniae biofilms. Antimicrob Agents Chemother 59:1707-1717.

Villa L, Capone A, Fortini D, Dolejska M, Rodriguez I, Taglietti F, De Paolis P, Petrosillo N, Carattoli A (2013) Reversion to susceptibility of a carbapenem-resistant clinical isolate of Klebsiella pneumoniae producing KPC-3. J Antimicrob Chemother 68:2482-2486.

Walsh CT, Fisher SL, Park IS, Prahalad M, Wu Z (1996) Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. Chem Biol 3:21-28.

Walsh CT, Wencewicz TA (2014) Prospects for new antibiotics: a molecule-centered perspective. J Antibiot (Tokyo) 67:7-22.

Walther-Rasmussen J, Hoiby N (2007) Class A carbapenemases. J Antimicrob Chemother 60:470-482.

Webber MA (2002) The importance of efflux pumps in bacterial antibiotic resistance. Journal of Antimicrobial Chemotherapy 51:9-11.

Wenning M, Scherer S (2013) Identification of microorganisms by FTIR spectroscopy: perspectives and limitations of the method. Appl Microbiol Biotechnol 97:7111-7120.

WHO (2015) Antimicrobial resistence. Available at URL: http://www.who.int/mediacentre/factsheets/fs194/en/. Access: 7th of May 2016

Willems RJ, Top J, van Schaik W, Leavis H, Bonten M, Siren J, Hanage WP, Corander J (2012) Restricted gene flow among hospital subpopulations of Enterococcus faecium. MBio 3:e00151-00112.

Woodford N, Turton JF, Livermore DM (2011) Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev 35:736-755.

Wright GD (2011) Molecular mechanisms of antibiotic resistance. Chem Commun (Camb) 47:4055-4061.

Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA, Wang M (2010) vanM, a New Glycopeptide Resistance Gene Cluster Found in Enterococcus faecium. Antimicrobial Agents and Chemotherapy 54:4643-4647.

Zaniani FR, Meshkat Z, Naderi Nasab M, Khaje-Karamadini M, Ghazvini K, Rezaee A, Esmaily H, Nabavinia MS, Darban Hoseini M (2012) The Prevalence of TEM and SHV Genes among Extended-Spectrum Beta-Lactamases Producing Escherichia Coli and Klebsiella Pneumoniae. Iran J Basic Med Sci 15:654-660.

Zhou G, Shi QS, Huang XM, Xie XB (2015) The Three Bacterial Lines of Defense against Antimicrobial Agents. Int J Mol Sci 16:21711-21733.

Zirakzadeh A, Patel R (2006) Vancomycin-resistant enterococci: colonization, infection, detection, and treatment. Mayo Clin Proc 81:529-536.