Development of a Screening Algorithm for the Phenotypic Detection of Resistance Mechanisms in Multi-Drug Resistant Gram-Negative Bacteria

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CONTENT

PREFACE

This work is the result of a nine month internship in the clinical laboratory of the Virga Jesse hospital. The project was outlined by dr Reinoud Cartuyvels who I'd like to thank for guiding me and answering all my questions. Sita and Marijke, thank you for the practical support of my internship. Martine and Rita, you and the other laboratory workers of the microbiology and molecular biology lab too were also glad to help, thanks for that. Dr Koen Magerman, thank you for your advice and help in obtaining the necessary control strains. Sara, we spend many hours together working, thank you for being there for me and helping me out whenever you could. Carmen and Severina, I'd like to thank you for the enjoyable lunch and coffee breaks, for the chats and cheer-ups, for the time we spent together this year.

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ABBREVIATIONS

AG	aminoglycoside
AN	amikacin
AMC	amoxicillin clavulanic acid
APB	3-aminophenyl boronic acid
ASC	advanced-spectrum cephalosporines
ATCC	American type culture collection
ATM	aztreonam
CAZ	ceftazidime
CFT	ceftriaxone
CHDL	carbapenem hydrolyzing oxacillinases
CIP	ciprofloxacin
CLA	clavulanic acid
CLOXA	cloxacillin monohydrate sodium salt
CLSI	clinical and laboratory standards institute
CTX	cefotaxime
CTX-M	cefotaxime hydrolizing
DDST	double disk synergy test
EARSS	European antimicrobial resistance surveillance system
EDTA	ethylenediaminetetraacetic acid
ESBL	extended spectrum β-lactamases
FEP	cefepime
FOX	cefoxitin
FQ	fluoroquinolone
GES	Guiana extended spectrum
GIM	German imipenemase
GM	gentamicin
IMI	imipenem hydrolizing β-lactamase
IMP	active on imipenem
KPC	*Klebsiella pneumoniae* carbapenemase
MEM	meropenem
MBL	metallo-β-lactamase
MDR	multi-drug resistant
MH	Mueller-Hinton
MHT	modified Hodge test
MIC	minimal inhibitory concentration
NaCl	physiologic saline
NMC	not metalloenzyme carbapenemase
OXA	oxacillin-hydrolizing
PBP	penicillin-binding-protein
SHV	sulfhydryl variable
SIM	Seoul imipenemase
SME	*Serratia marcescens* enzyme
SPM	Sao Paulo metallo-β-lactamase
T/C	ticarcillin/clavulanate
TEM	Temoneira
UTI	urinary tract infections
VIM	Verona integron-encoded metallo-β-lactamase
VJH	Virga Jesse hospital

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Background: Gram-negative bacteria such as *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter spp* cause serious infections in hospitalized patients (e.g. urinary tract infections, pneumonia and sepsis). Treatment of these infections is often complicated due to the increasing bacterial resistance against different classes of antibiotics. The main consequences of MDR are higher morbidity and mortality, prolonged hospital stay, rising health care costs and an increased use of antibiotics.

Objectives: Worldwide, the emergence of MDR Gram-negative bacteria is described in literature. Within these bacteria, resistance enzymes contribute to the observed resistance. It is hypothesized that MDR Gram-negative strains in the VJH are increasing. In order to investigate this statement, epidemiology of these micro-organisms in the VJH was studied and a screening algorithm for the laboratory detection of the most common resistance enzymes was developed.

Methodology: Epidemiological analysis of data derived from the MOLIS laboratory information system was performed by the software programme Infectio. Belgian and European data were obtained from literature and databases (EARSS, ESAC). Phenotypic tests used to detect AmpC, ESBL, carbapenemases and MBL were performed as described in literature and CLSI guidelines. ESBL were molecularly detected by amplifying bla_{TEM}, bla_{TEM-24}, bla_{SHV} *and* bla_{CTX-M} using consensus primers for the different genes and identified by sequencing of the PCR product.

Results: In the VJH, ESBL were detected in 40/56 (71%) and AmpC in 18/54 (33%) MDR *Enterobacteriaceae*. Molecularly, ESBL was found in 44/60 (73%) MDR *Enterobacteriaceae* and *Acinetobacter spp*, 17 (28%) of which were positive for 2 genes. Most ESBL were TEM-24 (33/60, 55%), CTX-M 15 (13/60, 22%) or SHV-12 (5/60, 8%). In 15 MDR *Enterobacteriaceae,* 1 (7%) carbapenemase was detected. MBL were found in 4/12 (33%) MDR *P. aeruginosa*.

Conclusion: The hypothesis is rejected as epidemiology graphs (2005-2008) showed a decrease of most MDR species since 2007. Although increasing steeply, absolute numbers of *E. coli* and *K. pneumoniae* remained low in the study period. However, conclusions should be drawn carefully as data of only one year past 2007 are available. The screening algorithm seems to be a useful tool for the detection of resistance enzymes in the daily practice of a clinical laboratory, especially in *Enterobacteriaceae*. However, before it can be implemented, phenotypic detection tests of AmpC, carbapenemase and MBL need to be confirmed molecularly.

1 INTRODUCTION

This research project focuses on Gram-negative bacteria, well-known pathogens which are able to cause serious infections such as urinary tract infections (UTI), pneumonia and sepsis in hospitalized patients[1]. European data shows that treatment of these infections is often complicated due to the increased antibiotic resistance of bacteria[2, 3]. This problem is elucidated below.

1.1 Bacteria

Bacteria are unicellular prokaryotes whose structure and functions permit them to survive in possible adverse conditions[4].

1.1.1 Bacterial ultrastructure

Bacterial cytoplasm contains a nucleoid and many 70S ribosomes (but no other organelles). The nucleoid consists of a circular supercoiled chromosome and plasmids. Although not necessary for survival, these plasmids provide a selective advantage as they often confer resistance to antibiotics. The cytoplasmic membrane carries out many metabolic functions such as transport of metabolites, oxidative metabolism, biosynthesis and the separation of daughter chromosomes during mitosis. Gram-negative cells have an additional outer membrane (see 1.3.2). Peptidoglycan in the cell wall provides rigidity and shape to the cell. External structures give bacteria additional advantages. Examples are the glycocalyx (adherence and survival), flagella (motility), pili (adherence and chromosomal transfer between bacteria) and a capsule (factor of virulence, impedes antibiotic uptake)[4-6].

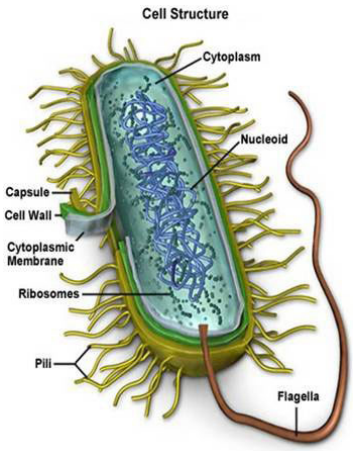


Figure 1: Bacterial ultrastructure. [http://www.ict-science-to-society.org]

1.1.2 Gram-negative bacteria

Based on their cell wall structure, bacteria are classified as Gram-positive or Gram-negative. Bacteria coloured blue after the Gram stain are called **Gram-positive**. Their cell wall consists of a plasma membrane and a thick peptidoglycan layer. The cell wall of **Gram-negative bacteria** on the contrary consists of a plasma membrane, a periplasmasic space and an outer membrane[7]. The outer membrane contains lipopolysacharides (LPS) and porins. LPS, which are unique for Gram-negative bacteria, stimulate the immune system and protect the bacterium from hydrophobic substances. Porines transport hydrophilic molecules (e.g. nutrients) into the periplasmic space[5].

The *Enterobacteriaceae* are a big and heterogeneous group of clinical important Gram-negative bacteria. Species such as *Escherichia coli, Citrobacter freundii, Enterobacter aerogenes* and *Klebsiella pneumoniae* are part of the normal flora of humans. They are responsible for 30-35% of all septicaemias, more than 70% of UTI and for many other infections[4].

Pseudomonas aeruginosa, an invasive Gram-negative bacterial pathogen found throughout the hospital environment, is an important cause of severe, often life-threatening, nosocomial infections including pneumonia, UTI and septicaemias. Because the low permeability of its cell wall, this pathogen is intrinsically

susceptible to only a few antibiotics. Furthermore, *P. aeruginosa* acquires resistance via β-lactamases, efflux pumps and a reduction of porin channels. Consequently, *P. aeruginosa* infections are often difficult to treat[3, 4].

Acinetobacter species are Gram-negative coccobacilli. *Acinetobacter baumannii,* found in various environmental sources such as soil and foods, is the species primarily associated with human disease, especially in critical ill hospitalized patients. Main clinical syndromes reported are pneumoniae and septicaemia. During the past decades, the incidence of *A. baumannii* infections increased, possibly due to advancements in medical support which have raised the proportion of the susceptible population[8].

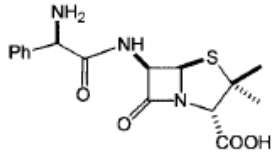
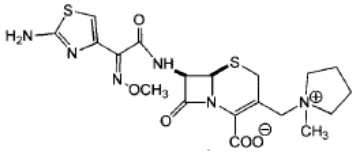
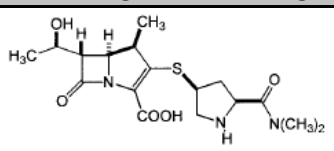
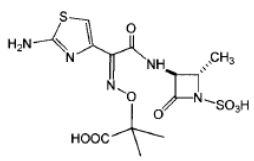
1.2 Infections: to treat or not to treat

Since World War II, bacterial infections have been treated with antibiotics. The availability of these drugs improved patient outcome considerably[1]. Unfortunately treatment of infections has become more difficult nowadays due to increasing antibiotic resistance[9]. In this section, both the mechanisms of action and possible causes of resistance are discussed for three major antimicrobial classes.

1.2.1 β-lactam antibiotics

β-lactam antibiotics (Table 1) **interfere with bacterial cell wall synthesis** by binding penicillin-binding proteins (PBPs). These proteins are regulatory enzymes, which catalyze the building and the cross-linking of peptidoglycan layers. When this cross-linking is prevented, the activation of autolysins causes the degradation of the cell wall and the bacterium dies[4].

Table 1: Overview of the different (sub)classes of β-lactam antibiotics. [http://www.bcfi.be/, Hujer *et al* AAC 2005 49(2)]

PENICILLINS	CEPHALOSPORINS
	
Penicillinase sensitive small spectrum penicillins	**First generation**
Penicillin G	Cefazoline
Penicillin V	
Penicillinase resistant small spectrum penicillins	**Second generation**
Fluxacillin	Cefamandol
Oxacillin	Cefuroxim
Cloxacillin	
Aminopenicillins	**Third generation**
Ampicillin (structure)	Cefotaxim
Amoxicillin	Ceftriaxone
Carboxypenicillins	Ceftazidim
Temocillin	
Combinations	**Fourth generation**
Amoxicillin + clavulanic acid	Cefepime (structure)
Piperacillin + tazobactam	
CARBAPENEMS	**MONOBACTAMS**
	
Meropenem (structure)	
Imipenem	Aztreonam (structure)

Bacteria can acquire **resistance** to β-lactam antibiotics by three general mechanisms. First, the interaction between the drug and its target (PBPs) can be prevented by changing size or charge of porins in the outer membrane. Second, PBPs can be modified in three ways: overproduction, acquisition of a structurally different protein with the same function or modification (recombination or point mutation) of the existing proteins[4,5]. Finally, β-lactamases such as AmpC, extended-spectrum-β-lactamases (ESBL), carbapenemases and metallo-β-lactamases (MBL) can hydrolyse the antibiotic[4]. Hydrolysis of antibiotics by β-lactamases is the most important resistance mechanism in Gram-negative pathogens[10]. β-lactamases can be classified functionally (Bush-Jacoby-Medeiros system) and molecularly (Ambler system)[11]. An overview of both systems is shown in Table 2. Class A, C and D β-lactamases posses an active-site serine, whereas class B β-lactamases usually require a zinc molecule for their catalytic activities[12, 13].

Table 2: Classification of β-lactamases according to the Bush-Jacoby-Medeiros (B-J-M) and the Ambler system. Substrate(s) and inhibitor of the enzymes are shown. XX: preferred hydrolysis. X*: hydrolysis of this substrate needs additional mechanisms. CLA: clavulanic acid. EDTA: ethylenediaminetetraacetic acid. +/-: weak inhibition. ND: not determined. [modified from Bush *et al* 1995 AAC 39(6)]

B-J-M	Subgroups	Ambler	Substrate				Inhibitor
			cephalosporin	penicillin	carbapenem	other	
1	-	C	x				-
2	a	A		x			CLA
	b	A	x	x			CLA
	be	A	x	x		monobactam	CLA
	br	A		x			CLA (+/-)
	c	A		x		carbenicillin	CLA
	d	D		x	x*	cloxacillin	CLA (+/-)
	e	A	x				CLA
	f	A	x	x	x		CLA
3	B1	B	x	x	x	most β-lactams	EDTA
	B2	B	x	x	xx		EDTA
	B3	B	xx	x	x		EDTA
4	-	ND		x			EDTA?

AmpC

"AmpC" refers not to a specific protein, but to a family of related enzymes produced in different members of *Enterobacteriaceae.* It turned out to be the first bacterial enzyme destroying penicillin in 1940[13]. When overexpressed, these enzymes are active on penicillins and even more on cephalosporins. Besides, AmpC can hydrolize cephamycins (e.g. cefoxitin), oxyiminocephalosporins (e.g. ceftazidim, cefotaxime and ceftriaxone) and monobactams (e.g. aztreonam). Extended-spectrum AmpC β-lactamases confer resistance to all cephalosporins, including the fourth generation (e.g. cefepime)[14]. In combination with porin loss, they may also mediate resistance to carbapenems. β-lactamase inhibitors have only a minor effect on most AmpC enzymes[13].

Various Gram-negative species express AmpC chromosomally. In *Enterobacter spp, C. freundii, Serratia marcescens* and *Morganella morganii* chromosomal AmpC expression is low but inducible in response to β-lactams such as ampicillin, amoxicillin, cephalosporins. A mutation in their *ampD* or *ampR* gene evokes AmpC hyperinducibility or constitutive hyperproduction. *A. baumannii* lacks the *ampR* gene so its AmpC β-lactamase is non-inducible. *P. aeruginosa* has three *ampD* genes, whose inactivation induces an upregulated AmpC production[13].

Since their first description in 1989, AmpC enzymes disseminated worldwide by moving to transmissible plasmids, which often carry multiple other resistance traits[12, 13, 15]. This way, bacteria such as

K. pneumoniae, E. coli, Salmonella and *Proteus mirabilis* were able to acquire class C enzymes[13]. *E. coli* does express chromosomal AmpC but due to the lack of strong promoter activity, its expression is only clinically important if it is plasmid-encoded[12].

AmpC is able to spread to other organisms within an hospital or geographic region. Besides, it impedes detection of ESBL and thus may evoke false positive susceptibility results. Hence, recognition of AmpC is important for infection control and adequate therapy[12, 13].

Extended-Spectrum-β-Lactamases

In the early 1980s, the introduction of third generation cephalosporins into clinical practice was a very important step in the fight against β-lactamase-mediated resistance to antibiotics. As soon as in 1983 however, plasmid-encoded β-lactamases, capable of hydrolyzing this new antibiotic class, were described in Western Europe[16, 17]. These enzymes are called ESBL and belong to subgroup 2be of B-J-M. They are capable of hydrolyzing not only the 1st, 2nd and 3rd generation cephalosporins but also penicillins and ATM (but not cephamycins or carbapenems)[16]. Hence, an isolate is reported as resistant to all penicillins, cephalosporins and ATM, once the presence of ESBL is phenotypic confirmed[18]. ESBL are inhibited by β-lactamase inhibitors such as CLA[16].

At the beginning, Temoneira (TEM) and Sulphydryl variable (SHV) enzymes were common β-lactamases but nowadays, cefotaxime hydrolizing (CTX-M) ESBL are most prevalent in clinical isolates, particularly in European countries[10, 17]. *E. coli* and *K. pneumoniae* are the major ESBL-producing organisms but currently ESBL are present in most genera of *Enterobacteriaceae* and in every region of the world[10, 17]. The fast dissemination of ESBL can be explained by their presence on large mobile genetic elements. Besides, plasmids often carry additional resistance genes (e.g. to aminoglycosides)[16,17]. PER- and oxacillin hydrolyzing (OXA)-type ESBL are more common in *P. aeruginosa* and *Acinetobacter spp*[17].

ESBL are an important reason for failure of empiric therapy and have serious consequences for infection control. Carbapenems are considered as the drugs of choice to treat serious infections caused by ESBL-producing *Enterobacteriaceae*: they are highly stable to β-lactamase hydrolysis and porin penetration is facilitated by their general size and structure[19].

Carbapenemases

Recently however, carbapenem resistance is described. This resistance can be caused by multiple mechanisms such as the combination of porin loss and plasmid-mediated AmpC or class A (TEM, SHV-2) β-lactamases, carbapenem-hydrolyzing β-lactamases (carbapenemases), reduced affinity of PBP for carbapenems, increased efflux and decreased permeability of the outer membrane[16, 19-21]. Generally, carbapenemases only confer carbapenem resistance if low permeability or high efflux is present naturally *(P. aeruginosa* and *Acinetobacter spp*) or acquired (*Enterobacteriaceae*)[22].

Carbapenemases can be divided into three classes. First, class A serine carbapenemases which belong to group 2f in B-J-M, hydrolyze a broad variety of β-lactams, including carbapenems, cephalosporins, penicillins and ATM. They can be subdivided into two groups: the relatively rare chromosomally encoded '*Serratia marcescens* enzyme' (SME), 'imipenem-hydrolyzing β-lactamase' (IMI) and 'not metalloenzyme carbapenemase' (NMC) and the plasmid-encoded '*Klebsiella pneumoniae* carbapenemases' (KPC) and 'Guiana extended spectrum' (GES) enzymes. Although divided over the two

subgroups, IMI and NMC deviate by only eight amino acids. KPC is the carbapenemase most likely to spread as their plasmids are mostly found in *K. pneumoniae*, which is known for accumulation and transfer of resistance determinants. KPC was discovered in the U.S.A in 1996 and reported worldwide since 2005[24]. GES is the largest group of the class A carbapenemases, but unlike KPC, they do not all hydrolyze carbapenems[23].

The second class mediating carbapenem resistance is class B MBL which hydrolyze carbapenems, cephalosporins and penicillins but not ATM[1]. However, presence of MBL does not necessarily induce carbapenem resistance. These enzymes will be discussed further on in this chapter[24].

The last class, class D serine-carbapenemases or OXA β-lactamases belong to the B-J-M subgroup 2d and can be divided into nine subgroups. The vast majority of these enzymes has been found in non-enterobacterial species such as *A. baumannii* and is inhibited by natrium chloride[21]. OXA β-lactamases hydrolyze penicillins, some cephalosporins and, although weak, the carbapenem imipenem[11]. In order to turn bacterial strains fully resistant to carbapenems, OXA β-lactamases may require additional mechanisms such as increased efflux, decreased influx or reduced affinity of PBPs for carbapenems[21, 23].

Metallo-β-lactamases

MBL belong to Ambler's class B based on their amino acid sequence homology and to the third group of B-J-M because of their substrate profiles (IPM hydrolysis), universal inhibition by metal ion chelator EDTA and lack of inhibition by serine β-lactamase inhibitors (e.g. CLA)[25, 26].

MBL mediate resistance to β-lactam antibiotics, just like serine β-lactamases, by cleaving the amide bound from the β-lactam ring. However the way to achieve this goal differs between both enzyme groups. In MBL, a distinct set of amino acids defines the active site that coordinates the zinc ion(s). These ions direct two water molecules necessary for hydrolysis. Because of their wide plastic active site groove, MBL have a broad spectrum of activity[25]. Clinically, MBL are the main carbapenemases since they hydrolyze virtually all β-lactams except ATM[26]. In addition, MBL genes are often located on plasmids together with genes encoding other resistances like aminoglycoside resistance genes[25].

The first MBL described were chromosomal encoded, but now the most common MBL families (VIM, IMP, GIM and SIM) are transferable[1]. MBL have spread from *P. aeruginosa* to *Enterobacteriaceae*. The first transferable MBL belonged to the 'active on imipenem' (IMP) family and was detected in Japan in 1988. Ten years later, these mobile MBL appeared in Southern Europe[25]. Other transferable MBL are 'Verona integron encoded MBL' (VIM), 'Sao Paulo MBL' (SPM-1), 'German imipenemase' (GIM-1) and 'Seoul imipenemase' (SIM). VIM and IMP are spreading all around the world[1, 25, 26]. IMP and VIM in *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* are of particular concern as they can confer resistance to all β-lactam classes (due to cephalosporinases, efflux pumps and low intrinsic outer membrane permeability)[1, 25].

1.2.2 Aminoglycosides

Aminoglycosides (AG) **inhibit protein synthesis** by binding irreversibly to the highly conserved A site of the 16S rRNA of the bacterial 30S ribosomal subunit (Figure 2). This evokes two effects: the production of aberrant proteins as the result of misreading the mRNA and the premature release of the ribosome from the mRNA, leading to incomplete proteins[4, 27].

Resistance to AG can arise in four different ways: one affecting the target of the antibiotic (mutation of the ribosomal binding site) and three influencing the drug itself (decreased uptake, increased efflux or enzymatic modification). The most common mechanism of resistance is the enzymatic modification of the antibiotic: amino- and hydroxyl groups of AG can be acetylated, adenylated or phosphorylated[4]. Recently, plasmid-encoded 16S rRNA methylases, enzymes which confer high-level resistance to all clinically important AG (except streptomycin), have emerged. Methylases are often associated with ESBL, mostly CTX-M [27].

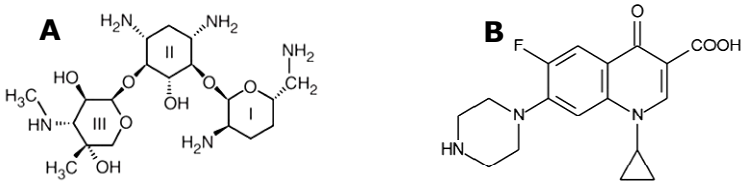


Figure 2: Structure of the aminoglycoside gentamicin (A) and a fluoroquinolone (B) [www.nature.com]

1.2.3 Fluoroquinolones

Fluoroquinolones (FQ) such as ciprofloxacin are bactericidal agents which **inhibit DNA replication, recombination and repair** by interfering the bacterial DNA gyrase. As a result, this enzyme no longer nicks the DNA strand to coil or uncoil a part of it. Uncoiling is important for replication and coiling permits the large molecule into the limited intracellular space. Besides, in order to translate the genes, different parts of the molecule must be changed sequentially to single stranded, uncoiled and coiled[4, 7].

Resistance to the FQ is mediated by accumulation of mutations in bacterial enzymes targeted by the FQ. In Gram-negative bacteria, the primary target is DNA gyrase, the secondary target DNA topoisomerase IV[28]. A decreased permeability of the membrane (due to mutations in the regulatory genes) and an increased efflux are possible too. Both are primarily chromosomally mediated[4].

1.3 The problem of resistance

Progress of medicine (ageing of patient populations, frequent surgical operations and a high number of immunosuppressed patients due to organ transplantation, chemotherapy…) has caused an intense use of antibiotics which has favoured selection for both inherent and acquired resistance mechanisms[1, 29]. Moreover, antibiotics are used in animals for treatment, prophylaxis and growth promotion, so resistant bacteria can also be passed to humans via the food chain[29]. As a result, the prevalence of resistance mechanisms mentioned above has increased over time. Nowadays treatment options are shrinking.

1.3.1 Inherent versus acquired resistance

Resistance mechanisms can be both inherent and acquired. **Inherent** resistance of bacteria has always been present, even before the creation of the first antimicrobial drug. It is a defence mechanism against antibiotics, chemicals produced by micro-organisms in the neighbourhood, in order to conquer space and food. Inherent resistance can be the result of decreased penetration of antibiotics, modification of the bacterial target and/or enzymes which inactivate the antimicrobial drug[5].

Since the discovery of pharmaceutical antibiotics, microbial pathogens have **acquired** resistance mechanisms to these drugs by selecting a spontaneous mutation or taking up new DNA. In the presence of an antibiotic, all micro-organisms will die except those who became less sensitive to the drug because of an

earlier spontaneous *mutation*. The second option is the exchange of chromosomal or plasmid-encoded resistance genes between bacteria by means of *transformation, transduction, conjugation or transposons*[5]. Transformation is the incorporation of naked DNA in the bacterial chromosome. Bacteria will generally restrict this kind of uptake to DNA of similar or familiar species. Conjugation is the most frequent type of resistance transfer in Gram-negative bacteria. After the DNA-donor and DNA-acceptor connect to each other by means of a sex-pilus, conjugative DNA (plasmid encoded or chromosomal integrated) is transferred from the donor to the acceptor. In case of transduction, bacteriophages transport chromosomal or plasmid-encoded resistance genes by infecting a new bacterial cell. Resistance genes can also be part of a transposon, a genetic element which can move from one bacterial strain to another, between plasmids and between plasmids and the bacterial chromosome[5, 7]. Transposons accelerate the spread of resistance mechanisms. Transfer of resistance is possible between Gram-negative and between Gram-positive bacteria. Besides, Gram-positives can pass on resistance to Gram-negative bacteria but transfer in the opposite direction is very rare[5].

1.3.2 Multi-drug resistance

A number of resistance **mechanisms** such as acquisition of plasmid or transposon, decreased permeability and upregulated broad-spectrum efflux pumps, create resistance to multiple antibiotic classes. Besides, mutations in the DNA mismatch repair system may select for hypermutability, which facilitates the emergence of further resistance[29, 30]. Multi-drug resistance in pathogens is ever-increasing and is now recognized as a major health problem: it does not only raise morbidity and mortality, it also causes an increased use of antibiotics, prolonged hospital stay and rising health care costs[3, 31].

In this study, multi-drug resistance (Table 3) is **defined** as resistance to cefotaxim, ceftazidime or aztreonam (β-lactam antibiotics) and to amikacin, gentamicin (aminoglycosides) or ciprofloxacin (FQ). The inclusion criterium is modified from the definition of multi-drug resistant (MDR) *E. aerogenes* used in the surveillance study of the Belgian Scientific Institute of Public Health[32].

At present, **alarming MDR Gram-negative bacteria** are *Acinetobacter species, P. aeruginosa* and *Enterobacteriaceae* (e.g. ESBL-producing *E. coli, Enterobacter* and *Klebsiella species*)[3].

Table 3: Definition of multi-drug resistance: a bacterium resistant to at least two different antibiotics, one from each column, is considered to be multi-drug resistant.

ceftazidime (CAZ)		ciprofloxacin (CIP)
OR		OR
cefotaxim (CTX)	AND	amikacin (AN)
OR		OR
aztreonam (ATM)		gentamicin (GM)

1.3.3 Health care-associated infections

Multi-drug resistance is of particular concern in **health care-associated infections** (HAI). According to the Centers for Disease Control and prevention criteria, an HAI is “a localized or systemic condition which results from an adverse reaction to the presence of an infectious agent(s) or its toxin(s) during hospital stay”. So, the infection was not present or incubating at time of admission[33] . Generally, infections arising 48 hours or more after the admission are considered health care-associated, but both the incubation time of the pathogen and the underlying condition of the patient can influence this time period[34].

HAI are the most prevalent complications in hospitalized patients[34]. Numerous factors present in the

hospital environment (indwelling devices, intense use of antibiotics, artificial ventilation, anaesthesia, surgery, intravenous therapy, immunosuppressed patients…) contribute to this kind of infections. Besides, pathogens adapted to the spatial environment could spread from patient-to-patient, possibly via health care workers[7].

In **Belgium**, 6.2% (95% confidence interval 5.9% - 6.5%) of the hospitalized patients acquires an HAI. This prevalence rate is similar to other European countries. The median age of patients acquiring HAI is 72 of which 58% are male. The hospital wards most prone to HAI are the intensive care unit and revalidation and treatment unit. UTI (23.9%) and lower respiratory tract infections (20.1%) are the most prevalent HAI, followed by surgical site infections (14.6%), blood stream infections (13.6%) and gastrointestinal infections (12.5%). *E. coli* is the pathogen causing most (15.46%) HAI. Other Gram-negative bacteria important in HAI are *P. aeruginosa* (4.49%), *K. pneumoniae* (3.61%), *K. oxytoca* (2.59%) and *A. baumannii* (2.08%). HAI prolong the mean stay in hospital with one weak and cause an additional cost of almost € 400 million per year. The biggest cost is the extra time in the hospital (mean cost of one day in a Belgian hospital is € 371). Other additional costs are lab tests, pharmaceutical products and medical fees. In Belgium, each year 2625 people die due to HAI[34, 35].

1.4 How to deal with multi-drug resistance?

In the last decade, concern about resistance has increased. Strategies proposed to deal with this problem are less and better antibiotic use (tailored antibiotics) and the prevention of cross-infection through high quality infection control measures such as hand washing, patient isolation and surveillance of MDR bacteria in the hospital [29].

1.4.1 Tailored antibiotics

Apart from discouraging unnecessary use, choosing the best antibiotic from the present agents is very important as there are marked differences in the ability to select new resistances both between and within drug classes[30]. In the treatment of serious bacterial infections, it is extremely important that the initial antimicrobial **therapy** is active against the causing organism(s). Inadequate empiric therapy is associated with poor clinical outcome, longer hospital stays and higher health care costs[3]. Initial antibiotic therapy of serious infections is therefore often broad-spectrum, covering the most important pathogens, including the resistant strains. The empirical therapy of choice should be based on the hospital's own antibiograms, which tend to differ from site to site[10]. Once the microbiological laboratory results are known, the empiric antibiotic treatment should be adapted to the identification and susceptibility of the pathogen. In this directed therapy, broad-spectrum antibiotics should be replaced if possible by antibiotics with a smaller spectrum. Tailored antibiotics are defined as right drug, right dose and right duration. There is a growing consensus that high-dose, short-duration therapy is clinically effective and less selective for resistance than long, low-dose regimens[29].

In order to guarantee the treatment of infections in the future, it is very important to develop **new antibiotics**. Despite the obvious need, pharmaceutical companies do not invest sufficiently in research and development of antibiotics which are active against MDR Gram-negative pathogens at present. Several factors explain this paradox. First, developing a new antibiotic is technical challenging (certainly an antibiotic active against Gram-negative bacteria) and very pricy (ca. €800 million per drug) while this kind of therapy is given to patients only for a short period of time. Second, antimicrobial drugs require a long

developing time which leads to a remaining patent life that might be insufficient to recover the investment. Finally, authorisation regulations are stricter for new antibiotics than for other therapeutic drugs[1]. The only new agent with anti-Gram-negative activity (except against *P. aeruginosa* and the *Proteae*) is Tigecycline which targets the 30S ribosome subunit. Although its efficacy in severe single pathogen infections is questioned, it is a step forward in the fight against resistance[29].

1.4.2 Infection control measures

Hand cleansing is the primary action to reduce HAI and cross-transmission of antimicrobial-resistant pathogens. It is important to realize that gloves do not fully protect from bacterial contamination, that alcohol-based handrub cleans the hands better than soap and that wearing rings and artificial fingernails increases the frequency of hand contamination. Besides, cross-transmission increases when hands are wet. As hand hygiene compliance takes time, understaffing or overcrowding is associated with the spread of micro-organisms[36].

It is estimated that some 30% or more of the HAI may be preventable by means of **surveillance** efforts and preventive strategies. Surveillance is the routinely and orderly collection of data based on a standard definition of cases. Two types of surveillance are possible: continuously or by means of prevalence studies. The first option is often used in high risk areas such as intensive care, renal dialysis and oncology units or in vulnerable patients (e.g. immunocompromised patients). It provides very precise information on incidence rates of infections, but it is time consuming and limited to the studied wards or patients. The second option, prevalence surveys, measures the proportion of the patients infected during the time period of the survey. Annual surveys can provide useful data on infection trends and effects of infection prevention, but the value of the results is more limited than in case of continuously surveillance. As both assessments provide complementary data, the combination of both systems is advised[34].

1.5 Hypothesis and objectives

Epidemiological information about MDR bacteria in hospitals as well as knowledge of their resistance mechanisms is indispensable for high quality infection control and antibiotic management[37]. This project focuses on the epidemiology and resistance mechanisms of MDR Gram-negative bacteria collected in the Virga Jesse hospital (VJH). As it is not feasible to study all mechanisms of resistance in detail, the focus will be on β-lactamases (AmpC, ESBL, carbapenemases and MBL). By means of the software programme Infectio (Info Partner, Nancy, France), the problem of MDR Gram-negative bacteria in the VJH will be mapped. In parallel, the collected strains will be phenotypical investigated in order to detect the four above mentioned enzymes. Next, we will look for genes causing the observed resistance by means of PCR. Sequencing is performed when the PCR yields positive results. This study aims to develop a screening algorithm for the laboratory detection of the most common enzymes causing resistance in MDR Gram-negative bacteria. It is hypothesized that the number of MDR Gram-negative strains in the VJH is increasing. Routinely detection of resistance enzymes in Gram-negative bacteria in the clinical laboratory of the VJH is the long term goal of this study. The developed screening algorithm will allow an high quality infection control and antibiotic management in the hospital

2 METHODOLOGY

The study consists of an epidemiological and an experimental part. In the epidemiology section, data on MDR Gram-negative bacteria in the VJH were derived from MOLIS, data on antibiotic use, resistance enzymes and antibiotic resistance from literature and databases (EARSS, ESAC). In the second part of the study, AmpC, ESBL, carbapenemases and MBL were detected by means of phenotypic tests. The ESBL detection test was molecularly confirmed.

2.1 Epidemiological analysis

Epidemiological analysis of Gram-negative bacteria resistant to CTX, CAZ or ATM and to AN, GM or CIP (MDR), identified in the VJH between 2005 and 2008 was performed with the software programme 'Infectio' (Info Partner, Nancy, France). Data were derived from the MOLIS laboratory information system (vision4health Laufenberg & co, Freienbach, Switzerland). Information on antibiotic consumption is routinely collected by the pharmacy in the VJH. Belgian and European data were derived from literature and databases (EARSS, ESAC). In the epidemiology section, descriptive statistics is used to indicate principal trends in the data[38].

2.2 Bacterial strains

Gram-negative isolates were identified by means of routine biochemical tests such as fermentation of glucose and lactose, alkalinisation of Simmons citrate medium, urease hydrolysis, indole production and motility[39]. Ambiguous identifications were confirmed by means of the API (20E & NE) biochemical identification system (bioMérieux, Brussels, Belgium) or sequencing of the 16S RNA gene. Antibiotic susceptibility testing was performed by disk diffusion according to Clinical and Laboratory Standards Institute (CLSI) guidelines on square Mueller Hinton agar II (MH) plates (BD, Erembodegem, Belgium) inoculated with 0.5 McFarland of the test strain. Antimicrobial agents used were CTX (30 µg), CAZ (30 µg), ATM (30 µg), AN (30 µg), GM (10 µg) and CIP (5 µg) (BD, Erembodegem, Belgium). Based on the zone diameter, bacteria are classified as sensitive, intermediate or resistant according to the CLSI guidelines[18].

Of the 111 MDR *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter spp* isolated in the clinical laboratory of the VJH from October 2008 the 15th of April 2009, 72 were collected. From each patient, only one (the most resistant) strain per species was used in the study. All selected strains were stored at -80°C (Microbank™, Pro-Lab Diagnostics, Geraardsbergen, Belgium).

2.3 Resistance enzymes in the VJH

2.3.1 Disk screening

MDR *Enterobacteriaceae* were screened for the presence of AmpC, ESBL, carbapenemases and MBL. Colonies were suspended in cation adjusted MH broth (BD, Erembodegem, Belgium) to a turbidity of 0.5 McFarland. This suspension was spread on a square MH plate using a cotton swab and CAZ, CTX, ceftriaxone (CFT, 30 µg), cefoxitin (FOX, 30 µg) and ticarcillin/clavulanate (T/C, 75+10 µg) disks were added (BD, Erembodegem, Belgium). After 16-20 hours incubation at 35 ± 2°C, the inhibition zones were evaluated. The criteria, shown in figure 3, are derived from the CLSI, Franklin and Jacoby[18, 40, 41]. Strains for which these criteria were fulfilled, were tested for the corresponding enzyme(s). Before the presence of a carbapenemase was tested, a second screening step was performed (cfr. 2.3.4). ATCC strains *K.*

pneumonia 700603 and *E. coli* 25922 were used as positive and negative control. In case of *Pseudomonas spp* and *Acinetobacter spp*, no screening was performed.

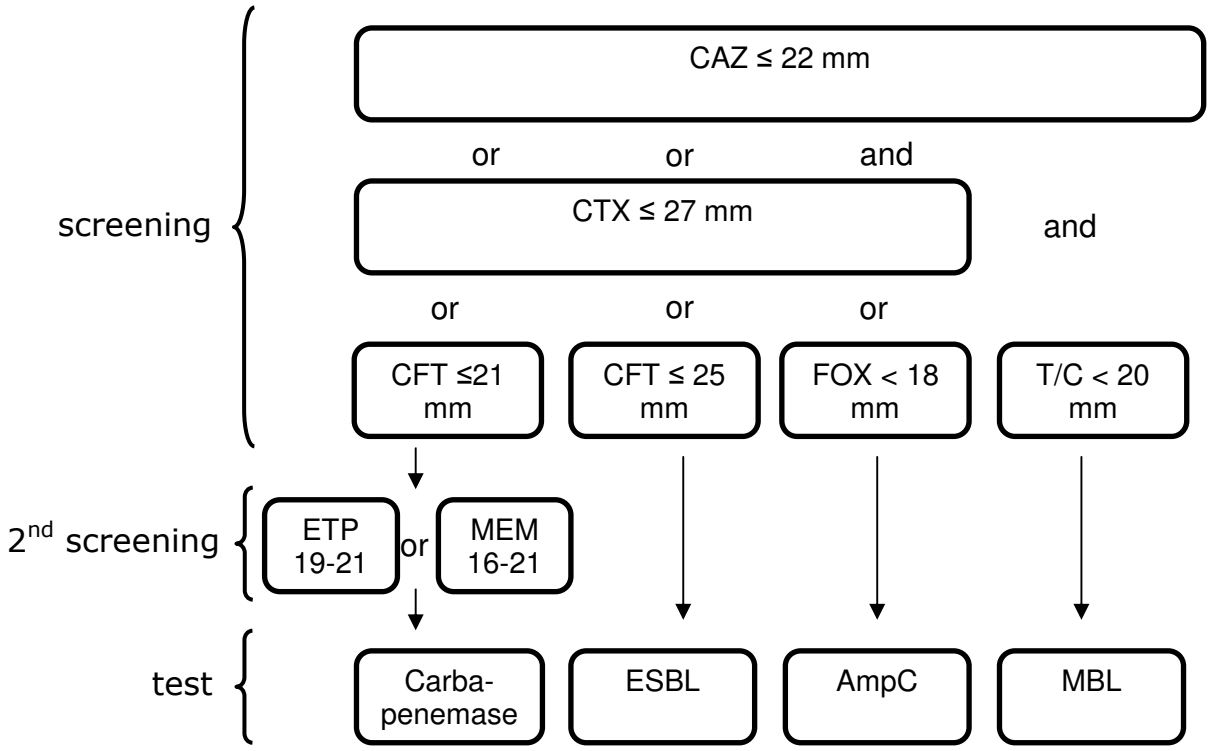


Figure 3: Screening algorithim to detect carbapenemases, ESBL, AmpC and MBL. Depending on the inhibition zone diameters, one or multiple of the above mentioned enzymes were tested. In case of the carbapenemases, a second screening is included. ESBL: extended-spectrum-β-lactamases, MBL: metallo-β-lactamases, CAZ: ceftazidime, CTX: cefotaxime, CFT: ceftriaxone, FOX: cefoxitin, T/C: ticarcillin/clavulanate.

2.3.2 Phenotypic detection of AmpC

According to the species, different tests were used to detect AmpC. For all species, a 0.5 McFarland suspension was prepared in MH-broth and spread onto a MH-plate (BD, Erembodegem, Belgium) using a cotton swab. For all *Enterobacteriaceae*, the external quality control strain *E. aerogenes* M5112 (Belgian Scientific Institute of Public Health, microbiology report 2004/02) was used as positive control, ATCC *E. coli* 25922 as negative control.

Enterobacteriaceae

CAZ disks were loaded with 600 µg 3-aminophenyl boronic acid (APB, Sigma Aldrich, Bornem, Belgium) and dried for 30 minutes. Disks containing CAZ and CAZ + 600 µg APB were placed at a centre-to-centre distance of 30 mm on the MH plate (Figure 4). Plates were incubated for 18 to 20 hours in ambient air at 35 ± 2°C. In the species *E. coli* and *K. pneumoniae*, zone diameter differences of at least 5 mm between the CAZ disks with and without APB are considered positive for AmpC[42]. For the other species, differences of at least 2 mm are considered positive (personal communication Glupczynski).

Figure 4: AmpC detection in *Enterobacteriaceae*: two CAZ disks at 30 mm, one with (I) and one without (II) ABP. The diameters differ > 5 mm so the test result is positive. CAZ: 30 µg ceftazidime, APB: 600 µg 3-aminophenyl boronic acid.

P. aeruginosa

Chromosomal AmpC in *P. aeruginosa* was detected by placing CAZ on MH (BD, Erembodegem, Belgium) with and without 500 mg/l cloxacillin (cloxa, Sigma, Bornem, Belgium). If the inhibition zone of CAZ increases with at least 10 mm in the presence of cloxa after overnight incubation, overproduction of AmpC is present (Figure 5)[43].

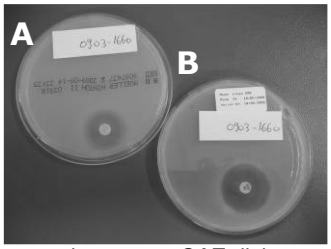


Figure 5: AmpC detection in *Pseudomonas aeruginosa*: one CAZ disk was placed on a MH plate (A) and one CAZ disk on MH enriched with 500 mg/ml cloxacillin (B). The diameters differ 10 mm so the test result is positive. CAZ: 30 µg ceftazidime.

2.3.3 Phenotypic detection of ESBL

Selected isolates are divided in three groups: *Enterobacteriaceae* without AmpC expression, *Enterobacteriaceae* with AmpC expression and *Acinetobacter spp* and *P. aeruginosa*. All strains were suspended to 0.5 McFarland in 0.9% w/v physiologic saline (NaCl) and spread on a MH plate with a cotton swab. The efficacy of all tests was controlled with the positive control *K. pneumoniae* ATCC 700603 (positive if ≥ 5 mm increase in CAZ-CLA zone diameter, ≥ 3 mm increase in CTX-CLA zone diameter) and the negative control ATCC *E. coli* 25922 (negative if ≤ 2 mm increase in zone diameter for antimicrobial agent tested alone versus its zone when tested in combination with CLA)[18].

Enterobacteriaceae

Two double-disk synergy tests (DDST) are performed on a MH plate to detect ESBL. In case of *K. pneumoniae*, *K. oxytoca* and *E. coli*, CTX (30 µg) ± CLA (10 µg) and CAZ (30 µg) ± CLA (10 µg) were placed centre-to-centre at 30 mm (BD, Erembodegem, Belgium). In case of *Enterobacter spp* and *Citrobacter spp*, CTX ± CLA is replaced by cefepime (FEP, 30 µg) ± CLA (10µg) (Neo-Sensitabs™ Rosco Diagnostics, Brussels, Belgium)[44].The agars were incubated at 35 ± 2° C in ambient air for 16-20 hours. A ≥ 5 mm increase in zone diameter for either antimicrobial agent tested with CLA compared to the agent alone, is reported as ESBL positive (Figure 6)[18].

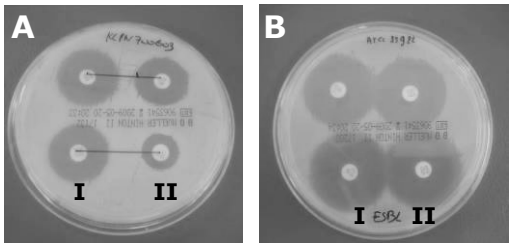


Figure 6: Positive (A) and negative (B) control of the ESBL test for *Klebsiella species* and *Escherichia coli*. A: disks containing 30 µg antibiotic + 10 µg clavulanic acid (I) increase inhibition zone diameters of disks, containing only 30 µg antibiotic (II) with at least 5 mm. B: no difference in inhibition zone diameter of disks, containing only 30 µg antibiotic (II) and disks containing both 30 µg antibiotic and 10 µg clavulanic acid.

Enterobacteriaceae expressing AmpC

If AmpC is present (cfr 2.3.2) in the tested *Enterobacteriaceae* or if AmpC is chromosomally expressed (*Enterobacter spp, C. freundii, M. morganii, Providencia stuartii or S. marcescens)*, ESBL is detected by a DDST in which FEP (30 µg) was placed centre-to-centre 20 mm from amoxicillin clavulanic acid (AMC, 20+10 µg) disk on a cloxa (250 mg/l) containing MH-plate (discs from BD, Erembodegem, Belgium). When a decreased susceptibility to FEP is combined with an enhanced inhibition zone of FEP near the AMC disk (synergy), the test is considered positive (Figure 7)[45] (personal communication Glupczynski).

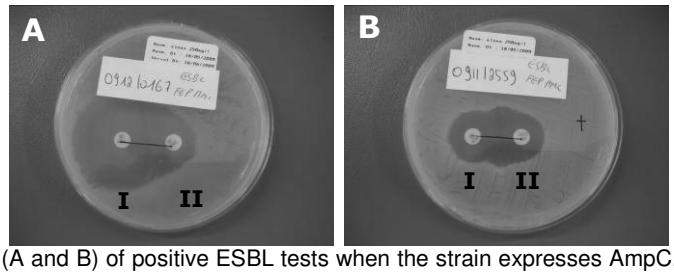


Figure 7: Two examples (A and B) of positive ESBL tests when the strain expresses AmpC. I: FEP(30 µg cefepime). II: AMC (20+10 µg amoxicillin clavulanic acid).

Acinetobacter spp and *P. aeruginosa*

Acinetobacter spp are tested with FEP, CAZ and T/C disks on a cloxa (200 mg/l) containing MH-plate; *P. aeruginosa* require FEP and T/C disks on a cloxa (250 mg/l) MH-plate. The distance for the discs was experimentally determined using the reference strain ATCC *K. pneumoniae* 700603 at edge-to-edge distance 10 mm. In both species, synergy (Figure 8) between FEP (or CAZ) and T/C indicate a positive test[46, 47].

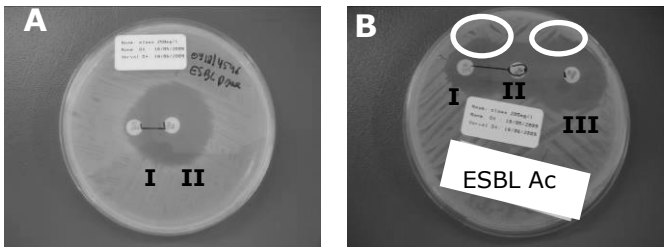


Figure 8: Positive results of the ESBL detection test in *Pseudomonas aeruginosa* (A) and *Acinetobacter species* (B). A: T/C (II) increases the inhibition zone diameter of FEP (I). B: the 'triangels' (white circles) between FEP (I) and T/C (II) and between CAZ (III) and T/C (II) indicate a positive result. CAZ: 30 µg ceftazidime. FEP: 30 µg cefepime. T/C: 75 µg ticarcillin + 10 µg clavulanic acid.

2.3.4 Phenotypic detection of carbapenem resistance

Selected strains (*Enterobacteriaceae* of which CAZ ≤ 22 mm or CTX ≤ 27 mm or CFT ≤ 21 mm) were tested for ETP (10 µg, OXOID, Ghent, Belgium) and MEM (10 µg, BD, Erembodegem, Belgium) susceptibility. After inoculation with a bacterial suspension in MH-broth with a turbidity of 0.5 McFarland, the plate was incubated at 35 ± 2°C for 16-20 hours. For strains with an inhibition zone between 19 and 21 mm for ETP or 16 and 21 mm for MEM (Figure 3, second screening), the modified Hodge test is performed according to the CLSI guidelines[18]. For this test, a MH plate was inoculated with a 1:10 dilution of a 0.5 McFarland suspension of ATCC *E. coli* 25922. After drying (~10 minutes), an ETP disc was put in the centre of the plate, and 3-5 colonies of test isolate were inoculated in a straight line (at least 20-25 mm) from the edge of the disc to the periphery of the plate using a thin loop. The presence of enhanced growth around the sample streak in the inhibition zone (Figure 9) after overnight incubation is interpreted as a positive test. ATCC *K. pneumoniae* BAA 1705 was used as positive control[18, 48].

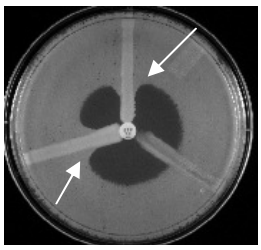


Figure 9: Modified Hodge test: the presence of a distorted inhibition zone (arrows) indicates the carbapenem-hydrolyzing activity of the test strain[18]

2.3.5 Phenotypic detection of MBL

Enterobacteriaceae of with CAZ ≤22 mm and T/C ≤ 20 mm, *P. aeruginosa* and *Acinetobacter spp* are tested for MBL by means of an EDTA DDST. The suspension (0.5 McFarland in 0.9% saline) of an overnight culture of the test strain was inoculated on a MH plate. After drying, 10 µg imipenem disks (IPM, OXOID, Ghent, Belgium) and blank paper disks (BD, Erembodegem, Belgium) were placed at both edge-to-edge distances of 5 and 10 mm[49]. On the blank disk, 10 µl of 0.5 M EDTA solution (Sigma, Bornem, Belgium) was applied. After overnight incubation at 35 ± 2°C, the agar plates were evaluated. An enlarged zone of inhibition (synergy) is considered as MBL positive (Figure 10)[50, 51]. The external quality control strain M9375 (Belgian Scientific Institute of Public Health, 2009) was used as positive control for MBL detection, ATCC 25922 as negative control.

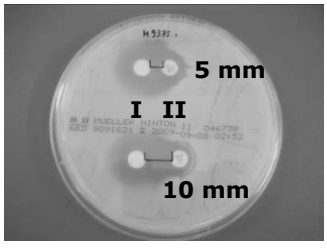


Figure 10: Positive metallo-β-Lactamase test of the reference M9375, which shows the enlarged inhibition zone of 30 µg imipenem (II) towards the blank disk containing 10 µl 0.5M EDTA (I), at 5 and 10 mm.

2.3.6 Molecular detection of resistance enzymes

ESBL

All MDR Gram-negative strains were tested for the presence of the bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes with simultaneous amplification of the gene coding for 16S as inhibition and amplification control. Amplification

was performed on a DNA Peltier Thermal Cycler 200 (MJ Research, Nevada, USA), followed by detection on a 1.5% agarose gel (NuSieve 3:1 agarose, VWR international, Leuven, Belgium). The sequences of the primers are listed in table 4. In order to detect all bla_{TEM} isoforms, two sets of primers (TEM and TEM') are necessary. Because bla_{TEM-24} is frequently described in Belgium, bla_{TEM} positive strains were examined for the presence of bla_{TEM-24}[52].

Table 4: Sequences and melting temperature (T_m) of primers used to detect extended spectrum β-lactamase genes *bla_{TEM}, bla_{CTX-M} and bla_{SHV}* and the 16S RNA gene

	Forward primer	T_m (°C)	Reverse primer	T_m (°C)	Ref.
bla_{TEM}	ATG AGT ATT MAA CAT TTC CG	46.6	CCA AWG CTT AAT CAG TGA GG	51	[53]
$bla_{TEM'}$	ATG GAT CCT CAA CAT TTC CG	52.6	CCA AWG CTT AAT CAG TGA GG	51	[53]
bla_{TEM-24}	GGG CAA GAG CAA CTC GGT	57.4	AGA CCC ACG CTT ACC GGT	56.6	[54]
bla_{SHV}	ATG CGT TWT DTT CGC CTG TG	58.1	AGC GTT GCC AGT GCT CGA TC	60.5	[53]
bla_{CTX-M}	SCV ATG TGC AGY ACC AGT AA	55.7	ACC AGA AYV AGC GGB GC	58.1	[53]
16S	AGA GTT TGA TCC TGG YTC AG	53.1	CTT TAC GCC CAR TAA WTC CG	52.6	[55]

The PCR mixture contains 1x buffer II, 1.5 mM $MgCl_2$, 200 nM of each dNTP, 200 nM of each primer and 0.04 units Amplitaq Gold DNA polymerase (Applied Biosystems, Lennik, Belgium). DNA extraction was performed by boiling the strains for 10 minutes. The end volume of the PCR reaction is 50 µl (5 µl sample + 45 µl mix). Positive and negative controls were included. The CTX-M positive control is kindly provided by Glupczynski, the other control strains are derived from the Belgian Scientific Institute of Public Health. The cycling conditions are shown in table 5.

Table 5: Cycling conditions of bla_{TEM}, $bla_{TEM'}$, bla_{TEM-24}, bla_{SHV}, bla_{CTX-M} and 16S

	TEM, TEM' and 16S			SHV, CTX-M and TEM-24		
	T (°C)	time (min)		T (°C)	time (min)	
hotstart activation	95	5		95	5	
denaturation	95	1		95	1	
annealing	50	1	35x	58	1	35x
elongation	72	1		72	1	
final extension	72	10		72	10	
	10	∞		10	∞	

PCR products showing bla_{TEM}, bla_{SHV} or bla_{CTX-M}, but no bla_{TEM-24} were sequenced. These amplicons were purified by means of the QIAquick PCR purification kit (QIAGEN, Venlo, The Netherlands) according to the manufacturers instructions. Dye terminator cycle sequencing was performed by adding 1 µl purified amplicon to a mixture containing 250 nM forward primer and 8 µl DTCS quick start master mix (Beckman Coulter, Woerden, The Netherlands), resulting in a total volume of 20 µl. The plasmid pUC18 was used as a positive control. The cycling conditions are shown in table 6.

Table 6: Cycling conditions of dye terminator cycle sequencing

	T (°C)	time (s)	
denaturation	96	20	
annealing	50	20	30x
extension	60	240	
	10	∞	

Sequencing products were purified by ethanol precipitation according to the manufacturers instructions and separated on a CEQ 8000 (Beckman Coulter, Woerden, The Netherlands). The obtained sequences were compared with sequences in Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and those on the website on ESBL nomenclature (http://www.lahey.org/studies/).

Carbapenemases and MBL

Primers for the detection of genes encoding ESBL, carbapenemases and MBL (Table 7) were blasted (http://www.ncbi.nlm.nih.gov/) against accession numbers listed on the website of ESBL (www.lahey.org/Studies/) and were completed by means of the review Queenan *et al*[11]. In order to detect more isoforms and/or to standardize the melting temperatures, primers for OXA-, NMC-, IMI-, VEB-, KPC-, SME-, SIM-, SPM-, GIM-, BEL-, PER-, GES-, VIM- and IMP-genes (Table 8) were adapted from literature. Because of feasibility reasons, MDR strains included in this study could not be tested with these primers.

Table 7: Enzyme isoforms detected by the different primers and the class of resistance enzymes to which they belong.

BEL	BEL-1	*ESBL*	**IMI**	IMI 1, 2	*carbapenemase*
GES	GES 1-9	*ESBL*	**KPC**	KPC 2-8	*carbapenemase*
PER	PER 1-3	*ESBL*	**NMC**	NMC	*carbapenemase*
VEB	VEB 1-6	*ESBL*	**OXA-23 like**	OXA 23, 27, 49, 73	*carbapenemase*
GIM	GIM-1	*MBL*	**OXA-24 like**	OXA 24-26, 40, 72	*carbapenemase*
IMP	IMP 1-22	*MBL*	**OXA-48**	OXA 48	*carbapenemase*
SIM	SIM-1	*MBL*	**OXA-58 like**	OXA 58, 96-97	*carbapenemase*
SPM	SPM-1	*MBL*	**SME**	SME 1-3	*carbapenemase*
VIM	VIM 1-18	*MBL*			
OXA-10	OXA 10, 11, 13, 14, 16, 17, 19, 28, 35, 54, 56, 101				*carbapenemase*
OXA-51 like	OXA 51, 64-69, 71, 76-83, 86-90, 92, 94-99, 106-112				*carbapenemase*

Table 8: Primers and their melting temperatures (T_m). Adaptations of the primers as described in the reference is marked in bolt. # nt: lenth of the amplicon. Polymorphism S: G or C; R: A or G; Y: C or T; K: G or T.

Gene	Forward primer	T_m (°C)	Reverse primer	T_m (°C)	# nt	Reference
BEL	GAC AAT GCC GCA GCT AAC C	57,9	CAG AAG CAA TTA ATA ACG CCC	55,4	409	[56]
GES	GTT TTG CAA TGT GCT CAA CG	56,5	TGC CAT AGC AAT AGG CGT AG	55,4	373	[57]
GIM	AGA ACC TTG ACC GAA CGC AG	58,9	ACT CAT GAC TCC TCA CGA GG**C A**	60,8	747	[58]
IMI	ATA GCC A**TC T**TG TTT AGC TC	47,8	TCT GCG ATT ACT TTA TCC TC	48,5	807	[59]
IMP	GGA ATA G**RR** TGG CTT AAY TCT C	54,1	GCA ACC AAA CYA CTA SGT TAT CT	54,3	188	[60]
KPC	ATG TCA CTG TAT CGC CGT C	52,9	TTA CTG CCC GTT GAC GC	55,2	881	[61]
NMC	GCA TTG ATA TAC CTT TAG CAG AGA **CTA GG**	59,3	CGG TGA TAA AAT CAC ACT GAG CAT A	59,1	2057	[62]
OXA-10	GTC TTT C**A**A GTA CGG CAT TA	49,4	ATT TTC TTA GCG GCA ACT TAC	52,2	719	[63]
OXA-23 like	GAT CGG ATT GGA GAA CCA GA	56,1	ATT TCT GAC CGC ATT TCC AT	55,7	500	[64]
OXA-24 like	GGT TAG TTG GCC CCC TTA AA	56,8	AGT TGA GCG AAA AGG GGA TT	56,5	249	[64]
OXA-48	TTG GTG GCA TCG ATT ATC GG	60,2	GAG CAC TTC TTT TGT GAT GGC	56,2	745	[65]
OXA-51 like	TAA TGC TTT GAT CGG CCT TG	57,5	TGG ATT GCA CTT CAT CTT GG	55,7	352	[64]
OXA-58 like	AAG TAT TGG GGC TTG TGC TG	56,5	CCC CTC TGC GCT CTA CAT AC	56,2	598	[64]
PER	GGA CAR TCS KAT GAA TGT CA	51,7	GGY SGC TTA GAT AGT GCT GAT	50,3	959	[66]
SIM	TAC AAG GGA TTC GGC ATC G	58,1	TAA TGG CCT GTT CCC ATG TG	57,9	570	[67]
SME	AAC GGC TTC ATT TTT GTT TAG	52,9	GCT TCC GCA ATA GTT TTA TCA	53,7	830	[68]
SPM	CCT ACA ATC TAA CGG CGA CC	56,0	TCG CCG TGT CCA GGT ATA AC	57,2	648	[58]
VEB	ACG AAG AAC AAA TGC ACA AGG	56,5	GAA CAG AAT CAG TTC CTC CG	53,4	88	[69]
VIM	**C**GA TGG YGT TTG GTY GCA TA	59,0	CGA ATG CGC AGC ACC AG	59,6	355	[60]

3 RESULTS

The goal of this research project is to study the epidemiology of MDR Gram-negative strains in the VJH and to develop a screening algorithm to detect the main enzymes causing antimicrobial resistance in the VJH. In the epidemiology section, MDR strains isolated in the VJH between 2005 and 2008 are discussed. In addition, data on antibiotic use, resistance enzymes and antibiotic resistance is documented. In the second part of the study, enzymes detected in MDR Gram-negative strains are presented.

3.1 MDR Gram-negative bacteria in the VJH

Between 2005 and 2008, 22171 Gram-negative strains were identified in the VJH. Of these, 3200 were MDR. In figure 11, the number of isolated MDR Gram-negative strains per species is shown. *E. coli* and *P. aeruginosa* were the species most frequently isolated in the VJH (2005-2008). *E. aerogenes* (1275/1762, 72%) and *Stenotrophomonas maltophilia* (463/942, 49%) are Gram-negative bacteria of which the largest part was MDR. These species were followed by *Acinetobacter spp* (75/399, 19%) and *P. aeruginosa* (609/3758, 16%) (Figure 11). Since *S. maltophilia* is intrinsically resistant to β-lactam antibiotics and AG, this germ was excluded from the study[70].

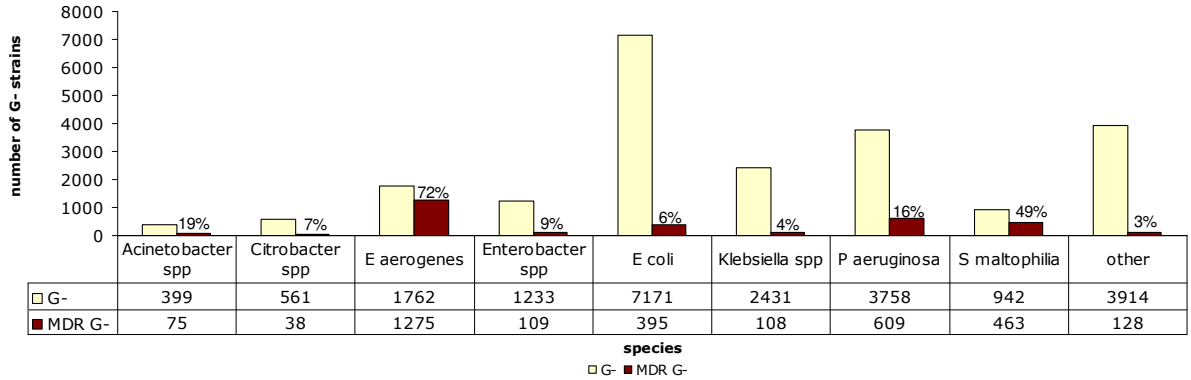


Figure 11 : Number of Gram-negative (G-) and MDR G- strains isolated and identified in the Virga Jesse hospital in the period 2005-2008. Percentage of MDR Gram-negative strains per species is shown. Infectio run 22-06-2009

In figure 12, the evolution of MDR Gram-negative bacteria in the VJH from 2005 till 2008 is shown. *E. aerogenes,* the most prevalent MDR species, is decreasing since 2005. Isolation of MDR Gram-negative bacteria is decreasing overall since 2007, with the exception of *E. coli* and *Klebsiella spp.*

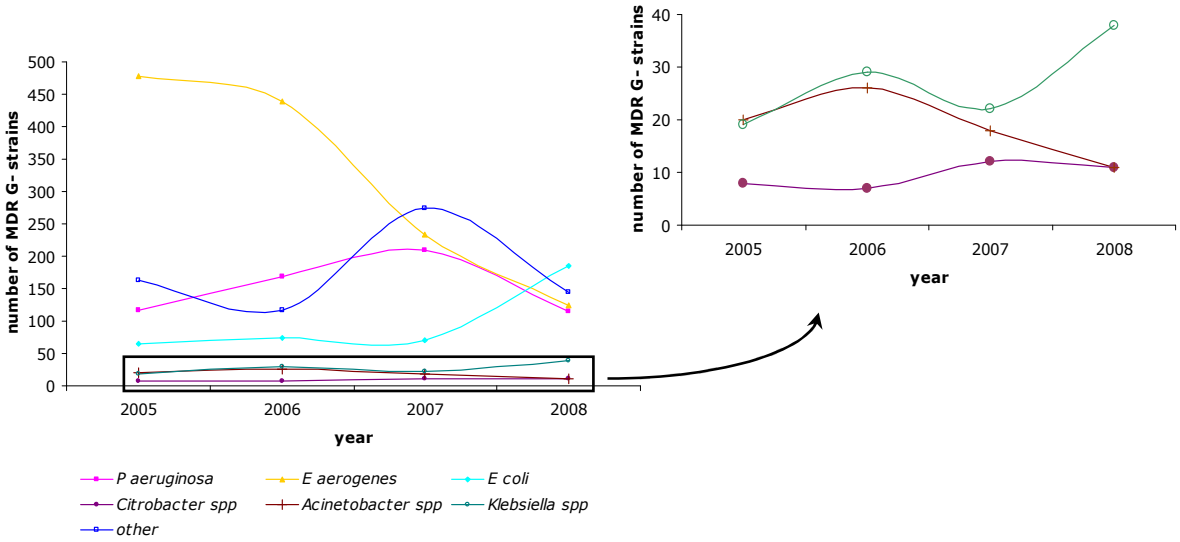
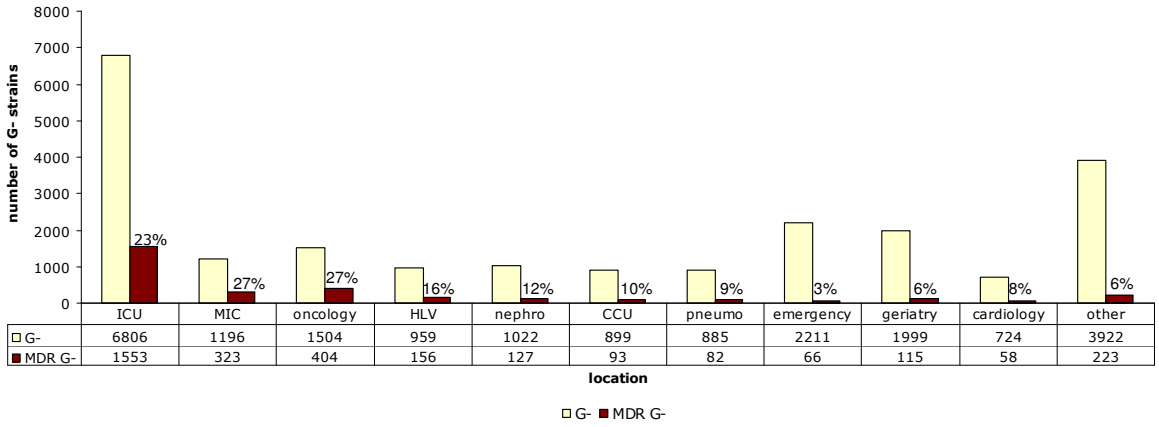


Figure 12: Evolution of MDR Gram-negative bacteria in the Virga Jesse hospital from 2005 till 2008. Infectio run 22/06/2009

In the period 2005-2008 MDR Gram-negative bacteria were mostly isolated from the departments of oncology (404/1504, 27%), medical intensive care (MIC, 323/1196, 27%) and surgical intensive care unit (ICU, 1553/6806, 23%) (Figure 13).



	ICU	MIC	oncology	HLV	nephro	CCU	pneumo	emergency	geriatry	cardiology	other
G-	6806	1196	1504	959	1022	899	885	2211	1999	724	3922
MDR G-	1553	323	404	156	127	93	82	66	115	58	223

Figure 13: Gram-negative (G-) and multi-drug resistant (MDR) G- bacteria per location in the Virga Jesse hospital (2005-2008). Percentage of MDR strains per species is shown. ICU: surgical intensive care. MIC: medical intensive care. HLV: heart lung vessel. Nephro: nephrology. CCU: coronary care unit. Infectio run 23/06/2009

Figure 14 shows the most prevalent species among MDR Gram-negative bacteria in the above mentioned wards in the period 2005-2008. The most frequently isolated species over the past four years have been *E. aerogenes* (52%) in ICU and *P. aeruginosa* in MIC and oncology (33% and 41% respectively). Both *E. aerogenes* and *P. aeruginosa* decreased in all wards, however the slope and the year in which the decline started, differed for each ward. The trend of *E. coli* is rising in each ward. Notice the different scale between the ICU and both MIC and oncology (Figure 14).

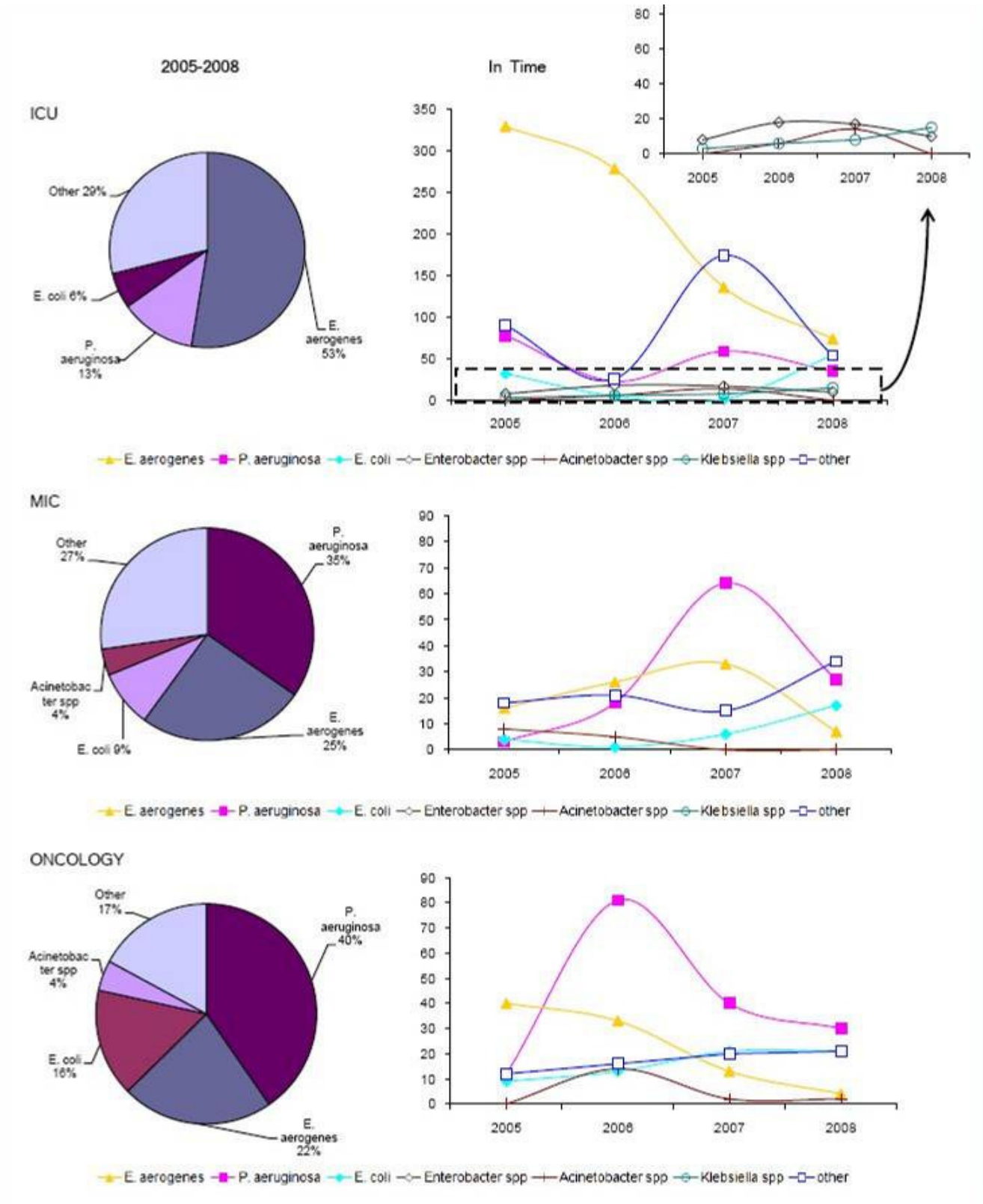


Figure 14: Composition and evolution of multi-drug resistant Gram-negative bacteria (2005-2008) in the surgical intensive care (ICU), medical intensive care (MIC) and oncology. Infectio run 23/06/2009

The majority of MDR Gram-negative bacteria were isolated from clinical samples, the remaining (ca. one third) from screening samples. Within screening samples, MDR Gram-negative bacteria were mostly derived from perineum (63%) and throat (32%). Concerning the clinical samples, respiratory (48%) and urinary tract (25%) were most often infected by these bacteria (Figure 15).

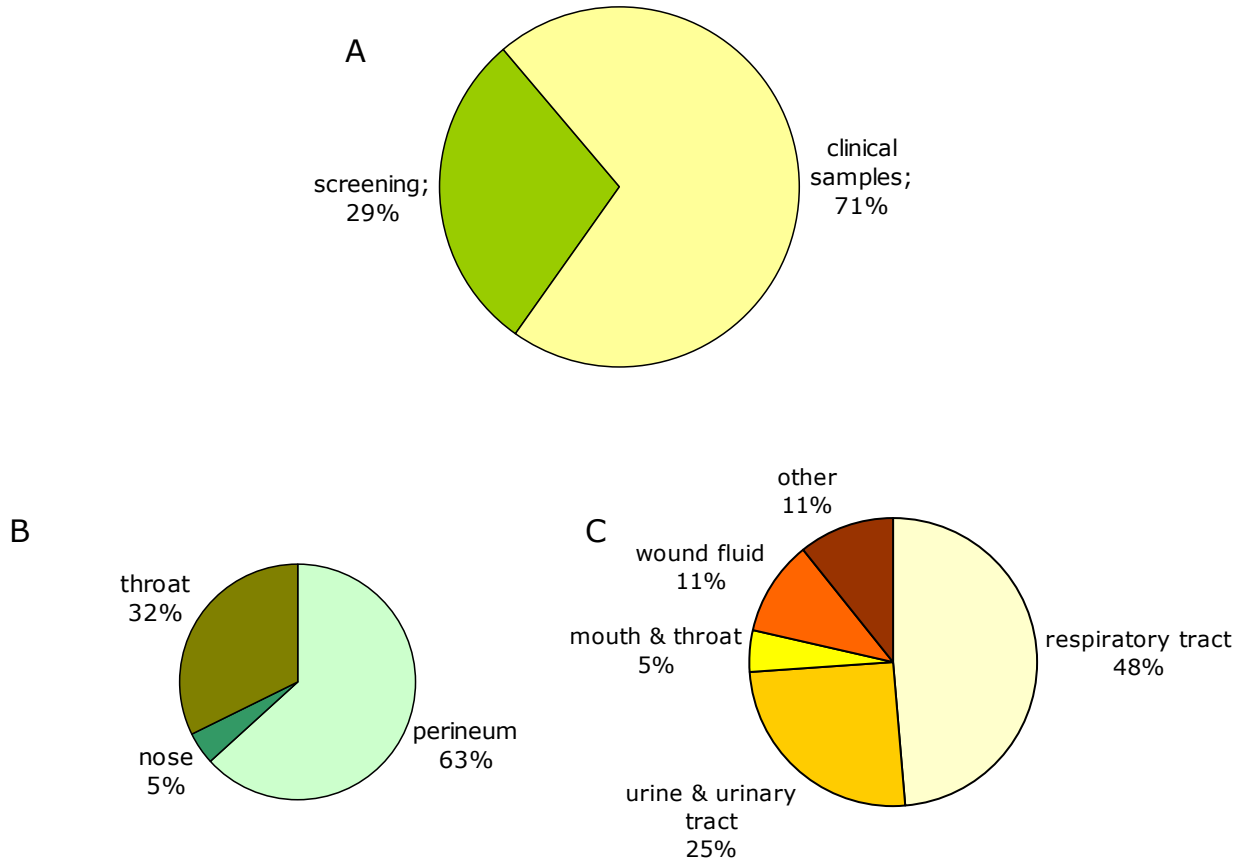


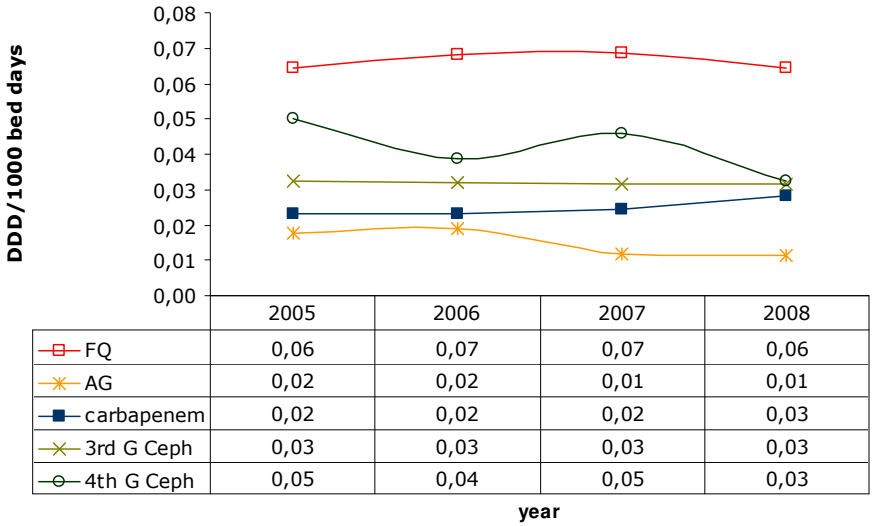
Figure 15: Multi-drug resistant Gram-negative bacteria, isolated in the Virga Jesse hospital in the period 2005-2008, sorted per specimen type (A). Both the screening (B) and clinical samples (C) are subdivided by source. Infectio run 23/06/2009.

3.2 Antibiotic use

Due to patients with a deficient immune system, the necessity of prophylaxis for certain diagnoses and therapies and the use of invasive devices, antibiotic use in hospitals is high[71]. In order to study a possible link between antibiotic use and resistance in hospitals, data on antibiotic consumption was collected for the VJH, Belgian and European hospitals.

3.2.1 Virga Jesse hospital

The pharmacy of the VJH collects only data on use of third line and reserve antibiotics (Figure 16). Hence no information on the hospital use of penicillins combined with a β-lactamase inhibitor, first and second generation cephalosporins is available. The group FQ consists of moxifloxacin and ciprofloxacin (of which the latter contributed most to total consumption of the FQ), amikacine represents the AG, meropenem the carbapenems, both CAZ and CTX the third generation cephalosporins (of which CTX contributed most) and FEP the fourth generation cephalosporins. Consumption is expressed in defined daily dose (DDD) per 1000 bed days, as recommended by the World Health Organisation[71]. DDD is the assumed average maintenance dose per day for a drug used for its main indications in adults[72].

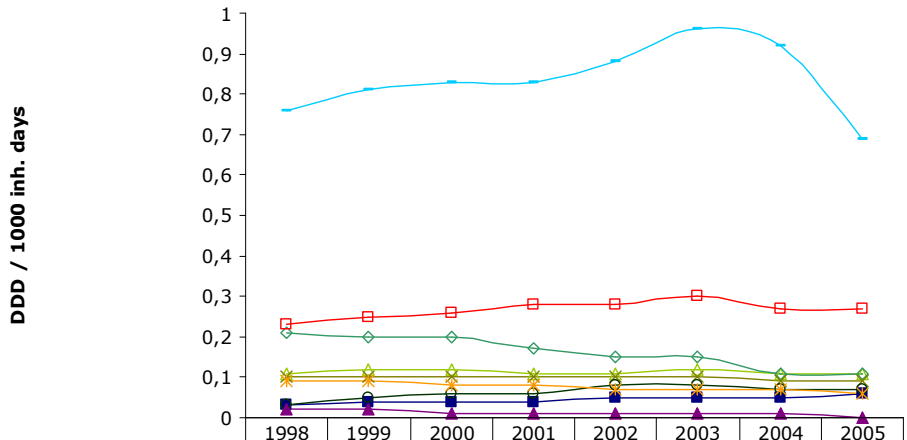


	2005	2006	2007	2008
FQ	0,06	0,07	0,07	0,06
AG	0,02	0,02	0,01	0,01
carbapenem	0,02	0,02	0,02	0,03
3rd G Ceph	0,03	0,03	0,03	0,03
4th G Ceph	0,05	0,04	0,05	0,03

Figure 16: Antibiotic use in the Virga Jesse hospital (2005-2008). DDD: defined daily dose. FQ: fluoroquinolones. AG: aminoglycosides. G. Ceph: generation cephalosporins.

3.2.2 Belgian hospitals

In Belgian hospitals (Figure 17), penicillin antibiotics combined with a β-lactamase inhibitor, were mainly used in 1998-2005. Of the other antimicrobial classes, FQ were most prescribed. Although the use of antibiotics appeared to be quite stable in the studied period, some shifts were seen in the lower range of the graph. For instance, whereas the use of carbapenems and fourth generation cephalosporins doubled from 1998 to 2005, the consumption of second generation cephalosporin and monobactams halved in the same period (coloured frames in Figure 17)[72].

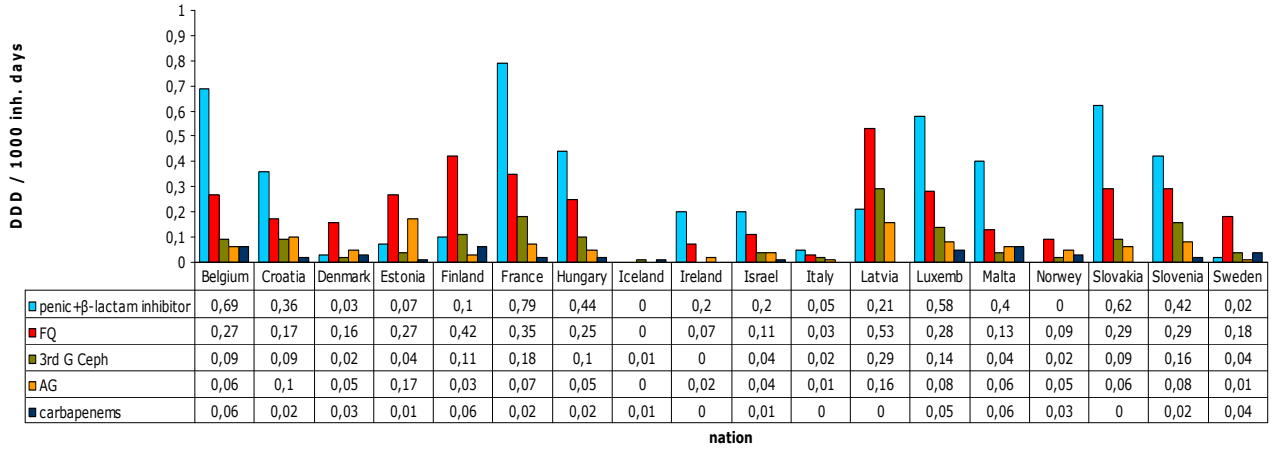


	1998	1999	2000	2001	2002	2003	2004	2005
Carbapenems	0,03	0,04	0,04	0,04	0,05	0,05	0,05	0,06
1st G Ceph	0,11	0,12	0,12	0,11	0,11	0,12	0,11	0,11
2nd G Ceph	0,21	0,2	0,2	0,17	0,15	0,15	0,11	0,11
3rd G Ceph	0,1	0,1	0,1	0,1	0,1	0,1	0,09	0,09
4th G Ceph	0,03	0,05	0,06	0,06	0,08	0,08	0,07	0,07
Monobactams	0,02	0,02	0,01	0,01	0,01	0,01	0,01	0
FQ	0,23	0,25	0,26	0,28	0,28	0,3	0,27	0,27
AG	0,09	0,09	0,08	0,08	0,07	0,07	0,07	0,06
penic.+β-lactam inh	0,76	0,81	0,83	0,83	0,88	0,96	0,92	0,69

Figure 17: Antibiotic consumption in Belgian hospitals (1998-2005). DDD: defined daily dose. Inh. Days: inhabitants per day. G Ceph: generation cephalosporins. FQ: fluoroquinolones. AG: aminoglycosides. Penic.+β-lactam inh: penicillin combined wirh β-lactamase inhibitor. [http://www.esac.ua.ac.be/]

3.2.3 *European hospitals*

The consumption of penicillins combined with a β-lactamase inhibitor, third generation cephalosporins, FQ, AG and carbapenems in European hospitals in 2005 is shown in figure 18. Generally, penicillins combined with a β-lactamase inhibitor were mainly used (> 0.5 DDD/1000 inh. days) in Belgium, France, Hungary, Luxembourg and Slovakia. FQ were frequently (> 0.4 DDD/1000 inh. days) used in Latvia, Finland and France. Iceland (although only data of carbapenems and third generation cephalosporins) and Italy, but also Norway (no penicillin data), Sweden, Denmark, Ireland (no information on carbapenems and third generation cephalosporins) and Israel are examples of nations using less antibiotics in hospitals. AG were mostly used in Latvian and Estonian hospitals (> 0.1 DDD/1000 inh. days)[72].



	Belgium	Croatia	Denmark	Estonia	Finland	France	Hungary	Iceland	Ireland	Israel	Italy	Latvia	Luxemb	Malta	Norwey	Slovakia	Slovenia	Sweden
penic+β-lactam inhibitor	0,69	0,36	0,03	0,07	0,1	0,79	0,44	0	0,2	0,2	0,05	0,21	0,58	0,4	0	0,62	0,42	0,02
FQ	0,27	0,17	0,16	0,27	0,42	0,35	0,25	0	0,07	0,11	0,03	0,53	0,28	0,13	0,09	0,29	0,29	0,18
3rd G Ceph	0,09	0,09	0,02	0,04	0,11	0,18	0,1	0,01	0	0,04	0,02	0,29	0,14	0,04	0,02	0,09	0,16	0,04
AG	0,06	0,1	0,05	0,17	0,03	0,07	0,05	0	0,02	0,04	0,01	0,16	0,08	0,06	0,05	0,06	0,08	0,01
carbapenems	0,06	0,02	0,03	0,01	0,06	0,02	0,02	0,01	0	0,01	0	0	0,05	0,06	0,03	0	0,02	0,04

Figure 18: Use of five antimicrobial classes in European hospitals in 2005. DDD: defined daily dose. inh days: inhabitants per day. Penic+β-lactam.: penicillin combined with β-lactamase inhibitor. FQ: fluoroquinolones. G Ceph: generation cephalosporins. AG: aminoglycosides. Luxemb: Luxembourg.

3.3 Resistance enzymes

3.3.1 *Virga Jesse Hospital*

Of the 2765 clinical relevant Gram-negative strains isolated in the study period, 111 were MDR. Of these 72 [56 *Enterobacteriaceae* (25 *E. coli*, 4 *Citrobacter spp*, 18 *Enterobacter spp* and 9 *Klebsiella spp*), 4 *Acinetobacter spp*, and 12 *P. aeruginosa*] were collected.

Enterobacteriaceae

Based on the screening algorithm (Figure 3), 54/56 (96%) MDR *Enterobacteriaceae* were tested for AmpC, 56/56 (100%) for ESBL, 15/56 (27%) for carbapenemases and 50/56 (89%) for MBL. AmpC was detected in 18/54 (33% of the tested strains) and ESBL in 40/56 MDR *Enterobacteriaceae* (71% of the tested strains), both when detection method was based on AmpC status and on species. One carbapenemase (7%) was detected in the 15 MDR strains which were tested for this enzyme. MBL were not detected (Figure 19).

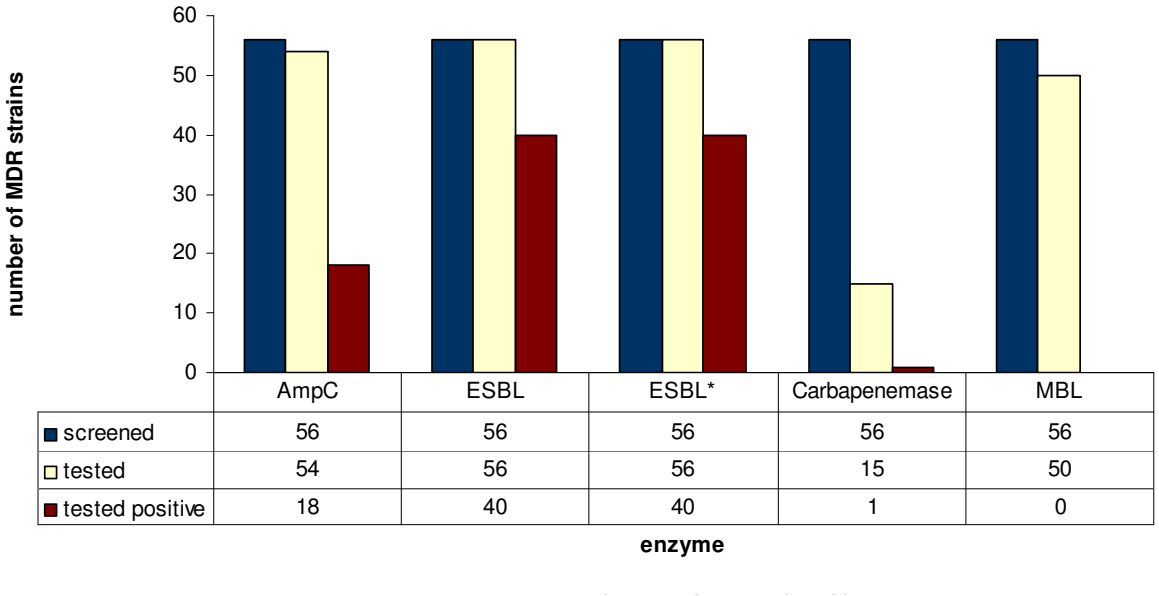


Figure 19: Screening and testing results of the multi-drug resistant (MDR) *Enterobacteriaceae*. Blue bars indicate the screened strains. White bars indicate the strains which fell within the selection criteria after screening and thus were tested for the corresponding enzyme(s). Red bars indicate strains in which a resistance enzyme is detected. AmpC: cephalosporinases. ESBL: extended-spectrum β-lactamases, detection method based on AmpC status. ESBL*: extended-spectrum β-lactamases, detection method based on species. MBL: metallo β-lactamases.

Only a small number of strains included in this study (n=4) belong to the *Citrobacter spp.* AmpC was present in 3/4 (75%) MDR strains. When detected based on AmpC status, ESBL was present in 3/4 (75%) MDR *Citrobacte*r strains. However, when the detection method based on species was used, 2 (50%) of these 4 MDR strains were positive for ESBL. Carbapenemases and MBL were not detected in *Citrobacter species*. The screening and testing results of *Enterobacter spp*, *E. coli* and *Klebsiella spp* are shown in figure 20. AmpC and ESBL are the enzymes most frequently detected. The one carbapenemase observed in this study, is present in a MDR *K. pneumoniae* strain. MBL were not detected at all in MDR *Enterobacteriaceae* (Figure 20).

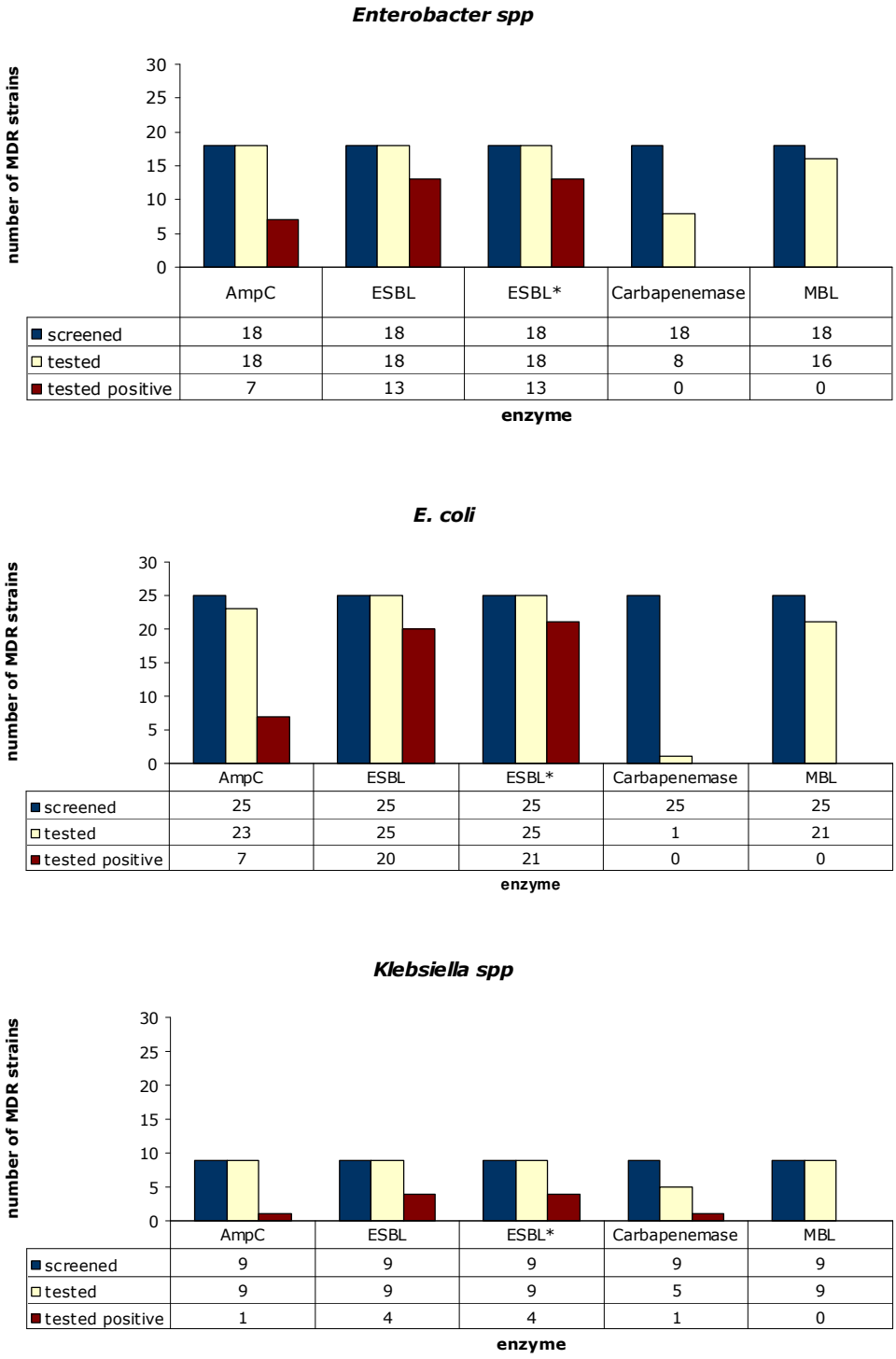


Figure 20: Screening and testing results of the multi-drug resistant (MDR) *Enterobacteriaceae* per species. Blue bars indicate screened strains. White bares indicate the strains which fell within the selection criteria after screening and thus were tested for the presence of the corresponding enzyme(s). Red bars indicate the strains in which a resistance enzyme is detected. AmpC: cephalosporinases. ESBL: extended-spectrum β-lactamases, detection method based on AmpC status. ESBL*: extended-spectrum β-lactamases, detection method based on species. MBL: metallo β-lactamases.

P. aeruginosa and *Acinetobacter spp*

As *P. aeruginosa* and *Acinetobacter spp* strains were not screened, each strain was tested for ESBL, MBL and, in case of *P. aeruginosa*, AmpC. None of the 4 collected *Acinetobacter* strains showed the presence of MBL or ESBL. AmpC was detected in 1/12 (8%), ESBL in 2/12 (17%) and MBL in 4/12 (33%) *P. aeruginosa* strains.

PCR and sequencing ESBL results

All MDR Gram-negative strains included in the study were tested for the presence of ESBL genes. PCR results were undecided for 8/12 (66%) *P. aeruginosa* strains. With regard to the other species, a positive result for the $bla_{TEM,\ CTX\text{-}M\ or\ SHV}$ PCRs was obtained for 44/60 (73%) strains, of which 17 (28%) showed a positive result for two genes. All genes that were detected were sequenced, except for the bla_{TEM} and $bla_{TEM'}$ genes in the strains positive for $bla_{TEM\text{-}24}$ (Table 9). Of the 35 bla_{TEM} positive strains, 33 (94%) were $bla_{TEM\text{-}24}$ and 2 (6%) $bla_{TEM\text{-}121,\ 114\ or\ 131}$. $bla_{CTX\text{-}M}$ was detected in 21/60 (35%) strains, 13 (22%) of which were $bla_{CTX\text{-}M\ 15}$. All bla_{SHV} genes observed in the VJH belonged to the isoform $bla_{SHV\text{-}12}$ (Table 9).

Table 9: Number of detected $bla_{TEM,\ CTX\text{-}M\ and\ SHV}$ genes per species.

	$bla_{TEM\text{-}24}$	$bla_{TEM\text{-}121,\ 114,\ 130}$	$bla_{SHV\text{-}12}$	$bla_{CTX\text{-}M\ 15}$	$bla_{CTX\text{-}M\ other}$
Acinetobacter spp	2				
Citrobacter spp	2		1		1
Enterobacter spp	12	1	3	1	
E. coli	13		1	12	6
Klebsiella spp	4	1			1

Discordant results were observed between molecular and phenotypic tests of *Enterobacteriaceae* and *Acinetobacter spp.* In case the ESBL detection method was based on AmpC presence, 23/60 (38%) MDR strains were discordant, mainly *Enterobacter spp* (11/18, 61%) and *E. coli* (9/25, 36%). When ESBL was detected without considering AmpC status (ESBL detection method based on species), the number of discordances was reduced to 6/60 (10%) MDR strains (*Enterobacteriaceae* and *Acinetobacter spp*) or 4/56 (7%) strains (if only *Enterobacteriaceae* were considered).

3.3.2 Belgium

AmpC has been detected in Belgian *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* strains [73-76]. Recently the first extended-spectrum AmpC expressing *E. coli* have been described[14].

In the late 1990's, the enzymes SHV-4 and TEM-24 (respectively in *K. pneumoniae* and *E. aerogenes*) were epidemic in Belgian and French hospitals[77]. Resistance mechanisms of multi-resistant *E. aerogenes* (MREA) include overproduction of chromosomal β-lactamases, ESBL production, modified porin expression and active efflux[78]. Since 2006, MREA has decreased remarkably[79]. Of the CTX-M family, CTX-M 15, CTX-M 1 and CTX-M 2 have been described in Belgium[77]. Other ESBL such as PER-1, GES-1, BEL-1 and VEB-1 have been detected as well, mainly in *Pseudomonas* and *Acinetobacter spp*[80, 81].

Carbapenemases detected in Belgium are OXA-48 (*Enterobacteriaceae*), VIM-2, OXA-9,10, BEL-1 and IMP-7 (*Pseudomonas spp)* and OXA-20, 26, 58 and 69 (*Acinetobacter spp)*[73, 82, 83].

In Belgium, MBL were initially detected in 2004. Three years later, an epidemic spread of VIM-2 producing *P. aeruginosa* caused infections in several Belgian hospitals[84] .

3.3.3 Europe

AmpC, both chromosomal and plasmid-encoded, has been described in European countries such as Belgium, United Kingdom, Ireland and Germany[20, 27, 43, 73, 74].

CTX-M-15 is an ESBL generally present in Europe. Other CTX-M isoforms can be grouped regionally: variants 9, 10 and 14 in southern nations (Spain and Portugal), CTX-M3 in Eastern Europe. SHV-12 is most prevalent in Italy, Spain and Poland, whereas SHV-5 is linked to Croatia, Greece, Hungary and Poland. TEM-3 and TEM-4 are mainly present in *K. pneumoniae* derived from Spain and France. TEM-24 on the other hand, mainly observed in Belgium, France, Portugal and Spain, prefers *E. aerogenes*. Co-production of different ESBL (for instance SHV or TEM in combination with CTX-M or carbapenemase) is increasingly reported in Europe. ESBL producing strains are not only resistant to β-lactam antibiotics, but also to FQ and AG [85]. ESBL other than TEM, SHV and CTX-M remain rare in Europe and when detected, these enzymes are mainly restricted to *Acinetobacter spp* (e.g. PER-1 and VEB-1 in France) and *P. aeruginosa* (e.g. PER-1 in Turkey)[86-88].

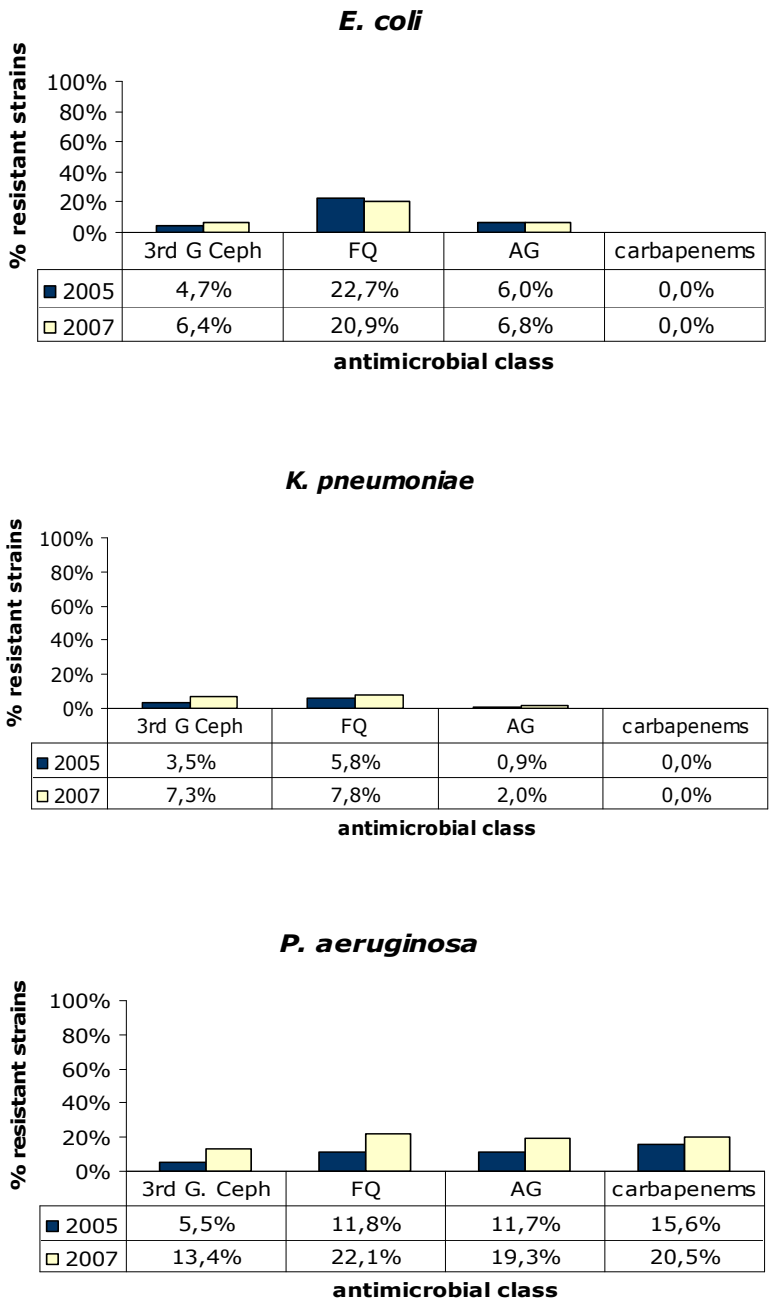
Carbapenemases in *Acinetobacter spp* were reported in at least 12 European countries. Although some of these carbapenemases belonged to the MBL class, most of them were class D enzymes such as OXA-2,10, 23-27 and OXA-40, 48, 51, 58[20, 87, 88]. KPC-producers have been found in Europe on a few occasions[87].

MBL mainly detected in Europe belong to the IMP- and VIM-families. These resistance enzymes were reported in France, Greece, Italy and Poland[89]. In Germany, the very rare GIM-type was described the first time in 2002[25].

3.4 Antibiotic resistance

3.4.1 Virga Jesse Hospital

In figure 21, resistance of *E. coli, K. pneumoniae* and *P. aeruginosa* to third generation cephalosporins, FQ, AG and carbapenems in 2005 and 2007 is shown. Of all *E. coli* isolated in 2007, one strain out of five was resistant to FQ. Approximately 20% of *P. aeruginosa* isolated in the VJH in 2007 was resistant for FQ, AG and carbapenems.



Figuur 21: Resistance of *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* to third generation cephalosporins (3rd G. Ceph), fluoroquinolones (FQ), aminoglycosides (AG) and carbapenems in the Virga Jesse hospital in 2005 and 2007. In case of *P. aeruginosa*, the only third generation cephalosporin considered is ceftazidime. Infectio run: 22/06/2009

3.4.2 Belgium

Although most European countries provided data to the EARSS database in 2005 and 2007, Belgium was not one of them. *E. coli* was the only species of which resistance data were known in 2002, 2005 and 2007. Resistance of *E. coli* in Belgium did not increase between 2002 and 2007[2].

3.4.3 Europe

In order to visualise the evolution of antimicrobial resistance, maps showing the amount of resistance to third generation cephalosporins, FQ, AG and -where possible- carbapenems were created for *E. coli*, *K. pneumoniae* and *P. aeruginosa* (Figures 22-24)[2].

Given antimicrobial resistance, the southern and eastern part of the continent generally scores worse, the Scandinavian countries best. As shown in figure 22, antimicrobial resistance of *E. coli* increased in virtually all European countries between 2002 and 2007. In contrast to 2005 and 2007, resistance of *K. pneumoniae* (Figure 23) and *P. aeruginosa* (Figure 24) was not well documented in 2002: only Norway provided data. *K. pneumoniae* (Figure 23) is especially resistant in (South-)East Europe. Greece is the only nation in Europe where more than 5% of *K. pneumoniae* was resistant to carbapenems. In a number of South-Eastern European countries, *P. aeruginosa* showed in 2007 a carbapenem resistance of more than 25% (Figure 24)[2].

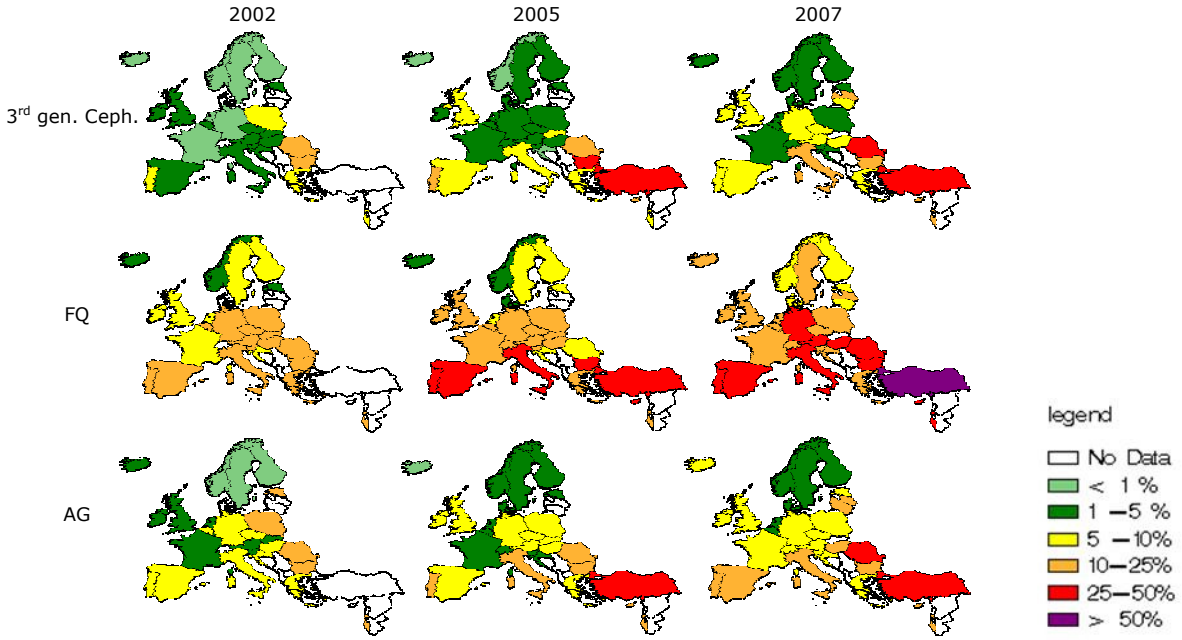


Figure 22: Evolution of antimicrobial resistance of *Escherichia coli* in European hospitals (2002, 2005, 2007). 3rd gen. ceph.: third generation cephalosporins. FQ: fluoroquinolones. AG: aminoglycosides [EARSS]

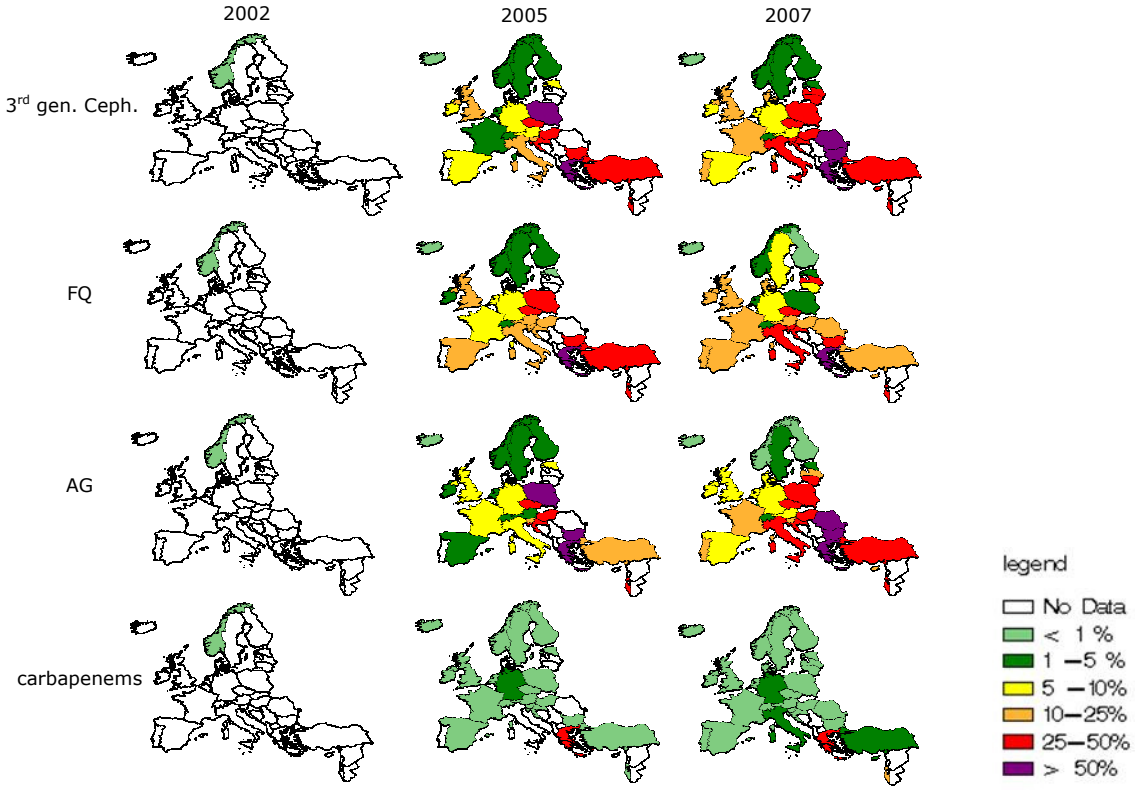


Figure 23: Evolution of antimicrobial resistance of *Klebsiella pneumoniae* in European hospitals (2002, 2005, 2007). 3rd gen. ceph.: third generation cephalosporins. FQ: fluoroquinolones. AG: aminoglycosides [EARSS]

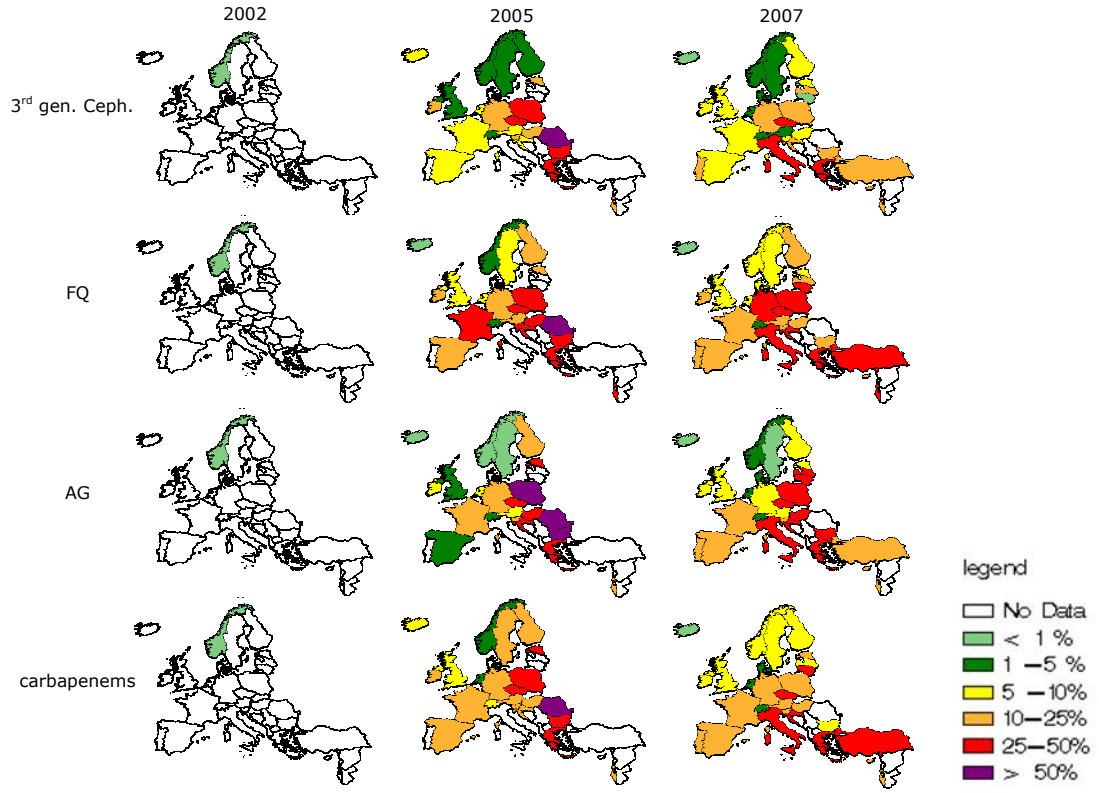


Figure 24: Evolution of antimicrobial resistance of *Pseudomonas aeruginosa* in European hospitals (2002, 2005, 2007). 3rd gen. ceph.: third generation cephalosporins. FQ: fluoroquinolones. AG: aminoglycosides [EARSS]

4 DISCUSSION

This research project focuses on MDR Gram-negative bacteria in the VJH and aims to develop a screening algorithm for the laboratory detection of the most common enzymes causing resistance in these strains. The study may contribute to the management of β-lactamases in the hospital and hence decrease the risk of compromised patient care and allow infection control measures (e.g. patient isolation). These measures are necessary to prevent resistance organisms to become endemic in the hospital[49].

Multi-drug resistance is defined as resistance to at least two different antimicrobial classes. In the period 2005-2008, *E. coli* was the species most frequently isolated, but *E. aerogenes* turned out to be the main MDR Gram-negative bacterium in the VJH. This could be related to the MREA strains, epidemic in Belgium since 1996-1997[78]. Evolution graphs showed in 2007 a noticeable decrease of MDR Gram-negative species isolated in the VJH, except for *E. coli* and *Klebsiella spp.* When ESBL in Europe shifted from TEM and SHV to CTX-M, the target species changed as well. Instead of *Enterobacter spp, E. coli* was joining *K. pneumoniae* as important host for ESBL[77]. This trend is also seen in the VJH and in Belgium. In the VJH, MDR *E. aerogenes* were decreasing and MDR *E. coli* and *Klebsiella spp* were increasing in the period 2005-2008. In Belgium, a reduced incidence of MREA and a rise of ESBL-containing *E. coli* is described in the same period. Incidence of ESBL containing *K. pneumoniae* is relatively stable in Belgium[79]. There is no real explanation for the marked decreases of the other isolated MDR Gram-negative species since 2007. It could be possible that the epidemic spread of MBL producing *P. aeruginosa* in several Belgian hospitals strengthened the normal surveillance and infection control measures[84].

Gram-negative bacteria were in 2005-2008 mainly present in the oncology, MIC and ICU of the VJH, wards in which patients were often immunocompromised and where antibiotic prescription was consequently high. The 'intensive' character of nursing in these wards facilitates the horizontal spread of MDR bacteria via health care workers. Although variable over the years, MDR germs prevalent in ICU, MIC and oncology were *E. aerogenes*, *P. aeruginosa* and *E. coli*. As a result, these strains originating from earlier mentioned wards could be the focus of a systematic resistance enzyme screening. Clinical samples, of the respiratory and urinary tract contained most MDR Gram-negative strains.

In the study period, MDR *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter spp* were collected. *Enterobacteriaceae* were screened to select strains for resistance enzyme detection, *P. aeruginosa* and *Acinetobacter spp* on the other hand, were tested without a screening step.

Enterobacteriaceae screened as CAZ $\leq$ 22 mm and CTX $\leq$ 27 mm or FOX < 18 mm were tested for the presence of **AmpC**. These criteria are derived (CAZ, CTX) or modified (FOX) from Jacoby *et al*[41]. Instead of selecting only FOX resistant strains, both intermediate and resistant strains were included in order to prevent possible unjustified exclusion. According to these screening criteria, the AmpC test had to be performed for 96% of the MDR *Enterobacteriaceae*. In a setting where presence of these enzymes is systematically investigated, it would be advantageous to add this test to the screening plate. This would be feasible to do because the APB-containing disks could be stored for a month at 4 °C and because the disks used for the AmpC test were placed centre-to-centre at 30 mm, just like in a disk dispenser[42, 45].

AmpC is detected in a simple, sensitive and specific way by means of boronic acids which are able to inhibit AmpC enzymes reversibly[90, 91]. According to Jacoby *et al*, specificity of this test is a concern

because strains producing a class A KPC β-lactamase may test false positive. The phenotypic test could not distinguish between the various families of plasmid-mediated AmpC enzymes and might overlook chromosomally determined AmpC β-lactamases with an extended spectrum[13]. Besides, distinction between overproduced AmpC and AmpC acquired by plasmids is impossible[92].

In this study, the same test was performed for all MDR *Enterobacteriaceae*, but the interpretation differed according to the species. In *E. coli* and *Klebsiella spp* the test is considered positive if APB enhanced the inhibition zone of CAZ at least 5 mm[42]. In case of *Enterobacter spp* and *Citrobacter spp,* the test is considered positive if APB restores –at least partially- the activity of CAZ (no minimum increase of the zone diameter was given, personal communication Glupczynski). To be able to interpret the test objectively, the test for these species is considered positive when APB increases the inhibition zone with at least 2 mm. However, this decision could lead to the inclusion of some false positive results. When AmpC detection will be performed in routine, it is important to define a cut off value for *Enterobacter* and *Citrobacter spp*, based on the comparison of phenotypic results with molecular characterization of AmpC β-lactamase genes as described by Perez-Perez and analytical isoelectric focusing results[93]. By doing so, it should be kept in mind that the diversity of AmpC β-lactamase genes expand continuously. Hence, the six families of genes covered by the multiplex PCR as described by Perez-Perez might be insufficient[42].

According to Tenover *et al*, organisms containing AmpC often test susceptible to extended-spectrum cephalosporinases, despite the fact that they do not respond clinically to these drugs. So results of susceptibility testing for cephalosporinases should be interpreted differently in AmpC positive strains[42]. Consequently, detection of AmpC in routine is recommended, especially for species as *E. coli*, *Klebsiella spp* and *Proteus spp.* Whether the interpretation of advanced spectrum cephalosporins (ASC) like cefepime should be modified as well, is not certain yet. Organisms containing AmpC but no ESBL often remained susceptible to ASC. This observation advocates the for strategy of not modifying susceptibility test results for ASC. However, false negative ESBL test results, acquisition of new ESBL genes in AmpC harbouring organisms and the inoculum effect (clinical relevance of the amount of β-lactamase producing organisms at the site of the infection) should be considered as well[42].

AmpC was detected in 33% of the MDR *Enterobacteriaceae*. This finding was in line with Sundin's statement that AmpC enzymes are ubiquitous enough for all clinical microbiology laboratories to be alert for their presence[49]. It should be noticed that AmpC was detected in only 7/18 (39%) MDR *Enterobacter spp* and in 3/4 (75%) MDR *Citrobacter spp,* while in these species, AmpC is chromosomally present. Possibly, AmpC is not always expressed.

P. aeruginosa strains were not screened, but all tested for AmpC as described by Henrichfreise *et al*. For some strains this test could not be used because nor in the presence nor in the absence of the inhibitor cloxa, a CAZ inhibition zone was detected[43]. For the positive control strain M5112, no interpretable result was obtained. Since a clearly positive result was obtained for at least one strain, test results were considered as valid.

According to Manchande *et al*, AmpC detection in *Acinetobacter spp* is possible by the modified three dimensional test[94]. However, as this test is not feasible in a routine clinical laboratory, AmpC enzymes were not detected in *Acinetobacter* strains.

ESBL was tested when screening revealed inhibition zones of CAZ ≤ 22 mm or CTX ≤ 27 mm or CFT ≤ 25 mm for *Enterobacteriaceae*. The criteria are derived from the CLSI guidelines 2009[18].

Initially, the method for ESBL detection in *Enterobacteriaceae* was based on the result of AmpC testing. In case of *Klebsiella spp*, *E. coli* and *P. mirabilis* without AmpC expression, ESBL was phenotypic detected by means of the combination disk method as described by the CLSI[18]. The test is easy to perform and to interpret[92]. Using more than one third generation cephalosporin enhances the sensitivity of the test[49]. According to Drieux *et al*, the CLSI test is not suited for species stably overproducing AmpC. Derbyshire *et al* stated too that synergy between CLA and an indicator cephalosporin is not ideal to detect ESBL in the presence of AmpC (both chromosomal and plasmid-encoded) because CLA may induce this enzyme, which then could attack the indicator cephalosporin and by doing so, mask the inhibition of any co-present ESBL[92]. Hence, the adapted DDST, described by Drieux *et al,* was performed for strains in which AmpC was detected and/or expressed AmpC chromosomally[45]. However, molecular confirmation of this ESBL detection method revealed 21/56 (38%) discordant results. Retrospectively it appeared that 19/21 (90%) of the discordant results were based on the ESBL detection test for strains with AmpC. Whether the bad performance of the test is due to the test itself or a bad referral by the AmpC detection test is not certain. All *Enterobacteriaceae* on which the ESBL test for strains with AmpC was performed, were retested using an alternative ESBL detection method. This approach decreased the number of discordances to 4/56 (7%). For the alternative ESBL detection method, strains were separated based on species, independent of the AmpC status. For *Klebsiella spp, E. coli* and *Proteus spp*, double disk tests CAZ $\pm$ CLA and CTX $\pm$ CLA were performed. For *Enterobacter* and *Citrobacter spp*, CTX was replaced by FEP. After improving the ESBL detection tests, 40/56 MDR *Enterobacteriaceae* (71%) tested positive. This finding was according to Sundin *et al* who stated that ESBL are ubiquitous enzymes[49]. In this study, ESBL was detected in 13/18 (72%) MDR *Enterobacter spp*, in 21/25 (84%) MDR *E. coli* and in 4/9 (44%) MDR *Klebsiella spp*. A MREA surveillance study performed in 2008 showed ESBL presence in 24.2% of *E. aerogenes*, in 4.8% of *E. coli* and in 8% of *Klebsiella spp*. Differences in ESBL prevalence can be due to different inclusion criteria: in this study, only the most resistant (MDR) strains were included whereas the surveillance study considered all strains. Notice too that in this study the *Enterobacters* were not restricted to *E. aerogenes*[79].

P. aeruginosa were tested for ESBL according to Aubert *et al*[46]. FEP, a cephalosporin which is known to be only a weak AmpC-inducer, was placed on MH containing the AmpC inhibitor cloxa[13, 45]. In 2/12 (17%) MDR *P. aeruginosa* strains, ESBL was phenotypical detected. Molecular confirmation was not possible as 8/12 (67%) MDR *P. aeruginosa* strains revealed no PCR results, probably due to insufficient DNA extraction. In the future, DNA extraction of *P. aeruginosa* strains should be optimized before repeating molecular ESBL detection. Of the 4 strains which were successfully amplified, 1 contained the ESBL SHV-2a.

Acinetobacter spp were tested for the presence of ESBL similarly as *P. aeruginosa*: synergy between CAZ or FEP and T/C on a cloxa containing MH-agar indicates ESBL presence. Again it is expected that the cephalosporinase inhibitor cloxa would enhance the ability to detect ESBL producing *Acinetobacter spp*[47]. However, in contrast to the molecular results which showed TEM-24 presence in 2/4 (50%) MDR strains, no ESBL was phenotypical detected. Considering both MDR *Enterobacteriaceae* and MDR *Acinetobacter spp*., the discordance rate of the screening algorithm was 6/60 (10%).

Spread of ESBL containing *A. baumannii* might be facilitated by an insufficient detection[47]. PCR and sequencing results show that ESBL are no longer exceptional in *Acinetobacter spp*. The ESBL PER and VEB have been described in Belgian and French *A. baumannii* strains[80]. According to Turner, *Acinetobacter spp* could also contain TEM and SHV[95].

When the inhibition zone diameters of *Enterobacteriaceae* for CAZ, CTX and CFT were respectively ≤ 22 mm, ≤ 27 mm or ≤ 21 mm, a second screening was performed with ETP and MEM disks. Presence of **carbapenemases** was tested when the diameter of ETP was between 19 and 21 mm or when MEM's inhibition zone was between 16 and 21 mm[18]. Concerning ETP, it is important to consider individual colonies within the inhibition zone as they can suggest the presence of a carbapenemase[49]. Screening criteria for carbapenemases are modified from the CLSI guidelines to prevent possible unjustified exclusion. This modification seems right as the one MDR strain tested positive for carbapenemase in this study, would have not been selected based on the CLSI screening guidelines. Despite the fact that CLSI guidelines must not be questioned based on one discordant result that was not genetically confirmed, it seems worthwhile to keep the selection criteria broad. According to the CLSI guidelines of 2009, the modified Hodge test must be used to detect carbapenemases in *Enterobacteriaceae*. The test is described as simple and feasible. Besides it detects very weak carbapenemases such as OXA-23 and GES-5/6[11, 48]. According to Anderson *et al*, sensitivity and specificity of the modified Hodge test is 100%[96]. Sundin however mentioned possible specificity problems due to AmpC enzymes and porin mutations decreasing the entry of the carbapenems in the cell[49]. Other disadvantages of the test are the difficult interpretation and the impossibility to identify the class of the present carbapenemase[48, 97]. Although ETP is not the best hydrolysed substrate, it was demonstrated to be the best molecule for KPC-detection, at least in *Enterobacter spp*[98]. In this study period, a carbapenemase was found in one MDR *K. pneumoniae* strain. This low prevalence is comparable to findings of a Belgian multicentre study performed by Glupczynski *et al* in 2008 (personal communication).

Carbapenem resistance in *Pseudomonas spp* can be caused by impermeability, loss of OprD porins, increased activity of efflux pumps, MBL production and derepressed AmpC[99, 100]. In this study, only MBL and AmpC were detected. Carbapenem resistance in MBL and AmpC-negative *Pseudomonas spp* strains could be due to decreased permeability or increased efflux.

Acinetobacter spp can become resistant to carbapenems because of intrinsic AmpC and OXA 59-61 enzymes and/or because of acquired MBL and carbapenem hydrolyzing oxacillinases (CHDL)[82]. In this project, only MBL enzymes were detected. In carbapenem resistant, MBL negative *Acinetobacter spp,* the presence of AmpC, OXA or CHDL is suspected.

The **MBL** detection test was carried out if *Enterobacteriaceae* screened CAZ ≤ 22 mm and T/C < 20 mm. These criteria are modified from Franklin *et al*[40]. However, it appeared retrospectively that the originally described selection criteria (CAZ ≤ 14 and T/C ≤ 14) are more efficient (40% less testing without the loss of a positive result)[40].

Although important both for therapy and infection control, MBL detection by clinical laboratories is hindered due to the absence of international guidelines and to the fact that MBL vary in their ability to cause resistance to CAZ or IPM, two substrates commonly used for MBL screening. The variability in β-lactam resistance phenotypes could be the result of outer membrane protein alterations and rearrangements in the MBL carrying plasmid[25, 26]. As all MBL need a zinc ion in their active site, the metal chelator EDTA can be used as inhibitor of the enzyme[40]. In this study, the DDST with IPM and EDTA discs 10 mm apart was used. According to Queenan and Galani, the test is convenient to screen potential MBL producers. Sensitivity of the test in earlier studies was 100% for *Enterobacteriaceae* and *Pseudomonas spp* and 95.7% for *Acinetobacter spp*[11, 51]. Furthermore the test is easy to use and relatively easy to interpret. Because of its low cost, the test is feasible for routine use. Disadvantages of the test are the non-standardized disks (as blank disks were loaded with EDTA manually, deviation of load is posiible) and the possibility of testing MBL

producing *Enterobacteriaceae* false negative when using IPM because of their carbapenem susceptibility[25]. Besides, the result (synergy or no synergy) is qualitative rather than quantitative, which makes the interpretation somewhat subjective. However according to Österblad *et al* this disadvantage could be ruled out by judging the test positive if the zone diameters of IMP+EDTA and IMP differ at least 5 mm[101]. Doing the MBL detection test, it appeared that placing the disks edge-to-edge at 10 mm, the result was not always easy to interpret. As suggested by Sundin *et al*, disks were placed also at an edge-to-edge distance of 5 mm[49]. In the end however, both distances were always judged the same. During the study, no MBL were detected in MDR *Enterobacteriaceae*.

All *P. aeruginosa* and *Acinetobacter spp* strains were tested as described for the *Enterobacteriaceae*. Of the 12 collected MDR *P. aeruginosa* strains, 4 (33%) were MBL positive. Possibly, these strains are a train of the epidemic spread of VIM-2 positive *P. aeruginosa* in 2007 in Belgian hospitals[84]. In the MDR *Acinetobacter* spp strains, no ESBL were found.

Prevalence of **resistance enzymes** detected in the VJH could not be compared with Belgian and European prevalences because no prevalence data of resistance enzymes are currently available for Belgium and Europe. However, it is possible to compare the different types of enzymes, detected in this research project with enzyme types described in Belgium and Europe. AmpC have been found in the VJH (MDR *Enterobacteriaceae*), Belgium (*K. pneumoniae*, *P. aeruginosa*, *Acinetobacter spp*) and Europe[20, 43, 73-75]. In the VJH, only ESBL TEM, SHV and CTX-M were traced, of which mainly TEM-24, CTX-M 15 and SHV-12 were detected. The finding of neither TEM-24 nor CTX-M 15 is surprising as these enzymes have been described both in Belgium and Europe. In this study, all SHV found were SHV-12, one of the most prevalent enzymes within the SHV family[86]. A similar result was found in another Belgian study performed by Rodriguez-Villalobos *et al* in 2006[102].SHV-4, the enzyme described to be prevalent in Belgium, was not found[77]. CTX-M 2, which represented 14% of all CTX-M types according to one Belgian study, was not found in the VJH[77]. Although carbapenemases have been described in Belgium (OXA, BEL, IMP) and Europe (mainly in *Acinetobacter spp*), it is not unexpected to find only one carbapenemase as a surveillance study performed by Glypczynski et al in 2008 revealed similar results[20, 73, 81-83, 87, 88](personal communication). In the VJH, MBL were restricted to MDR *P. aeruginosa* strains. This could be a train of the epidemic spread of VIM-2 producing *P. aeruginosa* in 2007[84]. To confirm this, molecular identification and pulsed field gel electrophoresis would have to be performed. In Europe, MBL mainly described are IMP, VIM and –in Germany- GIM[25, 89].

The surveillance and information system EARSS provides comparable data on the prevalence and spread of major disease-causing bacteria with **antimicrobial resistance** in Europe. Data are derived from public-health laboratories serving hospitals in 31 European countries[2]. Hence, by using data considering single regions with numerous collection sites, EARSS decreases sample bias seen in several large international programmes[103]. As indicated earlier, not all nations started to collect resistance data as early and for as many species. Countries which started later show often higher resistances than others. This may indicate a delayed awareness of the resistance problem, and thus a delayed policy to deal with it.

Generally, Southern Europe is suffering more from antimicrobial resistances than the north. This discrepancy could be explained by different infection control policies in European hospitals. According to Struelens *et al*, in Northern and Western Europe hand hygiene products are more frequently available at the

bedside, more infection control protocols and reports are present and there are sufficient numbers of isolation rooms and skilled staff, in contrast to Eastern and Southern Europe[104].

Generally resistance increased from 2002 to 2007: *E. coli, K. pneumoniae* and *P. aeruginosa* became more resistant to third generation cephalosporins, FQ, AG and carbapenems in almost all European countries. In Belgium, resistance of *E. coli* to third generation cephalosporins, FQ and AG did not increase in this five year period. A possible explanation could be the decreased/stabilized antibiotic use since 2000, both in ambulatory and hospital care [105]. In 2005 and 2007, resistance of *E. coli* to third generation cephalosporins, FQ and AG was similar in the VJH, in Belgium and in Western Europe. Regarding *K. pneumoniae*, resistance to the same antibiotics observed in the VJH in 2005 and 2007 was in the lower European range. In 2005 and 2007, Carbapenem resistance of *P. aeruginosa,* was comparable between the VJH and Central European countries. Resistance of the same strains to third generation cephalosporins, FQ and AG was somewhat higher than in other West-European countries but comparable to France.

It was stated that **antibiotic use** evokes resistance[106]. In the VJH, the third generation cephalosporin, CTX was clearly more prescribed than CAZ as the latter was reserved for *P. aeruginosa* strains. In Belgian and most European hospitals, penicillin combined with a β-lactamase inhibitor was the most used antibiotic. Of the other antimicrobial classes, mainly FQ were prescribed. Of the antibiotics for which data were available in the VJH, FQ were mostly used.

Antibiotic use in the VJH could not be compared with Belgium and Europe due to the use of different units. The hospital collects data on use of third line and reserve antibiotics expressed in DDD/1000 bed days, as advised by the World Health Organisation. The ESAC database in contrast, expresses consumption as DDD/1000 inhabitants per day because reliable data on bed days are not available for all European countries and because it would facilitate comparison with ambulant antibiotic use, expressed in DDD/1000 inhabitants[71]. Still it is possible to try to find a link between antibiotic use and the observed resistances in Europe. In general, an incubation period is present between the use of drugs and the appearance of resistance. Hence, consumption data of 2005 were compared with resistance data of 2007. FQ accumulated highest resistance (no European resistance data on penicillins combined with β-lactamase inhibitors). This is not unexpected as these drugs were highly used in European hospitals. France and Latvia, which contrasted sharp with Iceland, Sweden, Norway and Denmark, showed high antibiotic use in hospitals. The same distinction could be observed in the European maps showing antimicrobial resistances. However, there are also unexpected observations which did not follow the link between consumption and resistance. Finland was amongst the countries with the highest antibiotic use in hospitals, while being part of Scandinavia where antibiotic resistance is less a problem. Next, Italy, Malta and Croatia turned out to use little antibiotics in hospitals, despite their location in Southern Europe. These discrepancies might be explained by the fact that ambulant antibiotic consumption is left aside.

5 CONCLUSION AND SYNTHESIS

The goal of this research project is to study the epidemiology of MDR Gram-negative bacteria and to develop a screening algorithm for the laboratory detection of the most common enzymes causing resistance in these strains.

During 2005-2008 the main MDR Gram-negative bacterium isolated in the VJH was *E. aerogenes*. However, its number was decreasing remarkably since 2005. The number of MDR *P. aeruginosa* and *Acinetobacter spp* was decreasing as well. In contrast, the number of MDR *E. coli* and *Klebsiella spp* showed a marked increase since 2007 (respectively 2.7 fold and 1.7 fold). MDR Gram-negative strains were mainly present in the ICU, MIC and oncology units, although in these units, a similar decrease of MDR Gram-negative strains was seen. MDR Gram-negative micro-organisms were primarily isolated from clinical samples derived from the respiratory and urinary tract.

In the study period, mainly ESBL (71%) and AmpC (33%) were detected in MDR *Enterobacteriaceae*. Most ESBL were TEM-24, CTX-M 15 or SHV-12. One MDR *P. aeruginosa* strain out of three (33%) contained MBL. Carbapenemase was detected in 1/15 (7%) MDR *K. pneumoniae* strain.

Based on the results of this research project, the hypothesis is rejected. In stead of an increase, epidemiology graphs showed a decrease of most MDR species in the VJH since 2007. Although increasing, absolute numbers of MDR *E. coli* and *K. pneumoniae* remained low in the study period. However, conclusions should be drawn carefully as data of only one year past 2007 were available.

Limitations of the study are the incomplete molecular confirmation and the restriction of the molecular ESBL detection to the most important genes. Besides, the study is monocentric and the collection time restricted (6 months) Because of weekends and holidays, 39/111 (35%) of the MDR Gram-negative strains could not be collected.

In the future, phenotypic tests for carbapenemases, MBL and AmpC will be confirmed genetically. In addition, the number of ESBL genes detected will be extended. It would also be interesting to determine the relation between the collected strains by genotyping (e.g. pulsed field gel electrophoresis). Finally, in a future study, strains deriving from different hospitals in Limburg and Flanders should be included in order to allow comparison of the detected resistance mechanisms in the different regions. After all, it is important to know which resistance mechanisms are present in other regional hospitals as they could be transferred rapidly by patient transfer or by the spread of resistance mechanisms, for instance via plasmids[49].

The developed screening algorithm seems to be a useful tool for the routinely detection of resistance enzymes in the clinical laboratory, especially in *Enterobacteriaceae*. However, before it can be implemented, phenotypic detection tests of AmpC, carbapenemases and MBL need to be confirmed molecularly. By identifying resistance enzymes, the lab could act as an early warning system to alert the medical community for (new) resistance mechanisms present in clinically important bacteria. Early identification of resistant bacteria is necessary to minimise their spread and to help to select appropriate antibiotics[10]. In the VJH, the majority of the detected resistance enzymes belong to the ESBL and AmpC β-lactamases. MBL were found in MDR *P. aeruginosa* and carbapenemase was detected in one MDR *K. pneumoniae* strain.

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