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Molecular Characterization of VIM Carbapenemases in the Arabian Peninsula

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MOLECULAR CHARACTERIZATION OF VIM CARBAPENEMASES IN THE ARABIAN PENINSULA

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This Thesis is submitted in partial fulfilment of the requirements for the Master of Medicine and Health Sciences in Microbiology and Immunology degree

Under the direction of Professor Tibor Pal

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Declaration of Original Work Page

I, Nour Yahfoufi, the undersigned, a graduate student at the United Arab Emirates University (UAEU) and the author of the thesis/dissertation titled "Molecular Characterization of VIM Carbapenemases in the Arabian Peninsula" hereby solemnly declare that this thesis/dissertation is an original work done and prepared by me under the guidance of Prof. Tibor Pal in the College of Medicine and Health Sciences, Department of Microbiology and Immunology At UAEU. This Work has not previously formed the basis for the award of any degree, diploma or similar title at this or any other university. The Materials borrowed from other sources and included in my thesis/dissertation have been properly acknowledged.

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ABSTRACT

The emergence and global spread of carbapenem resistant *Enterobacteriaceae* is an alarming world-wide phenomenon that also affects the Middle East due to limited treatment options for such infections and their common association with high level of fatality. The most important mechanism of such resistance is caused by the production of various carbapenemase enzymes. In the Arabian Peninsula, so far, NDM and OXA48-like carbapenemases have been reported, while elsewhere other enzymes, e.g. VIM, IMP and KPC are also commonly found. Our aim was to systematically look for the VIM-type carbapenemases among local isolates and to characterize their genetic background.

Initially, screening isolates from Abu Dhabi hospitals, we identified a single *Enterobacter cloaceae* strain carrying the VIM-4 allele. This was the first such isolate ever reported from the Peninsula. Subsequently, investigating isolates from Kuwait, Saudi Arabia, Oman and the UAE, we identified a further 11 isolates, one *E. cloaceae* from Saudi Arabia, two from Oman, one from Kuwait and also one *Escherichia coli* from this country. Besides these, the latter country also provided six *Klebsiella pneumoniae* isolates. All strains produced the VIM-4 variant of the enzyme as determined by the sequencing of their genes. In all cases, the gene was located on plasmids of varying sizes, either non-typable or belonging to the IncA/C group; most of them were conjugative and they commonly harbored other ß-lactamase genes, such as CTX-M or CMY-4. In all strains the VIM-4 gene was located within a class I integron - with some variations between the gene casettes present - similar to strains previously identified in North Africa and Italy,

suggesting the possibility of spread. Clonal typing revealed that the relatively high incidence of VIM-producer *Enterobacteriaceae* encountered in Kuwait was not due to the spread of a particular clone, but most probably was the result of the transfer of an IncA/C plasmid, co-harboring bla_{VIM-4} and bla_{CMY-4}, into *Klebsiella pneumoniae* and *E. coli*.

Our data show that, beyond NDM and OXA-48-like, VIM type carbapenemases are the third most common isolates in the Arabian Peninsula. Further investigation is needed to monitor the spread of clones and genes in the region.

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Nour Yahfoufi

DEDICATION

I would like to dedicate this thesis to my distinguished professors, Prof. Tibor Pal and Dr. Agnes Sonnevend; to all researchers at the UAEU especially those in the Microbiology and Immunology Department; to the UAE with the hope that this work will be of benefit to the country; to my mother who has been a source of constant love and support to me; to my father, who has been my role-model for hard work, persistence and personal sacrifices and to my dear husband, who has been a source of encouragement and inspiration to me.

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ABBREVIATIONS

A. baumanni	*Acinetobacter baumanii*
Approx.	Approximately
Arg	Argnine
AM	Amikacin
AZT	Aztreonam
B. fragilis	*Bacillus fragilis*
CC	Clonal complex
CAZ	Ceftazidim
CDC	Centers for Disease Control and Prevention
C. freundii	*Citrobacter freundii*
CFU	Colony forming unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
COL	Colistin
cont.	Continued
CRE	Carbapenem Resistant *Enterobacteriaceae*
CTX	Cefotaxim
CTX-M	Cefotaximase
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleoside triphosphates
E. coli	*Escherichia coli*
E. cloacae	*Enterobacter cloacae*
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EPEC	Enteropathogenic *E. coli*
ERIC	*Enterobacteriaceae* Repetitive Intergenic Consensus
ERT	Ertapenem
ESBL	Extended spectrum beta-lactamase
EtBr	Ethidium Bromide
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
GM	Gentamicin
IDSA	Infectious Diseases Society of America
IMI	Imipenem
K. pneumoniae	*Klebsiella pneumonia*

Kb	Kilobase pairs
KPC	*Klebsiella pneumoniae* carbapenmase
LPS	Lipopolysaccharide
MDR	Multi-drug resistance
MEM	Meropenem
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
mRNA	Messenger Ribonucleic acid
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistance *Staphylococcus aureus*
NAG	*N-acetylglucosamine*
NAM	*N-acetylmuramic acid*
NDM	New Delhi metallo-beta-lactamase
OM	Outer membrane
OMP	Outer membrane protein
OS	Oligosaccharide
OXA	Oxacillinase
PBA	Phenylboronic acid
PBPs	Penicillin binding Proteins
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PMQR	Plasmid-mediated quinolone resistance
P. aeruginosa	*Pseudomonas aeruginosa*
P. otitidis	*Pseudomonas otitidis*
P. putida	*Pseudomonas putida*
qep	Quinolone efflux pump
QRDR	Quinolone resistance determinig regions
R	Recipient
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
RT	Room Tempreture
Sat	Secreted outotransporter toxin
SSC	Saline Sodium citrate
SDS	Sodium dodecyl sulfate
S. marcescens	*Serratia marcescens*
Se	Serine
SHV	Sulfhydryl reagent variable enzyme

spp.	Species
ß-lactamase	Beta-lactamase
ß-lactamase	Beta-lactam
ST	Sequence type
S. maltophilia	*Stenotrophomonas maltophilia*
SXT	Sulfamethoxazole
TC	Transconjugants
TE	Tris/EDTA buffer
TBE	Tris/Borate/EDTA
TF	Transformants
TGC	Tigecycline
TET	Tetracyclin
TOB	Tobramycin
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTI	Urinary tract infection
UV	Ultraviolet
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World Health Organization

I. INTRODUCTION

I.1. *Enterobacteriaceae*

The *Enterobacteriaceae* is the largest family of medically important Gram-negative bacteria. Currently, it includes 44 genera and 176 named species (Baumler et al. 2013). *Enterobacteriaceae* are ubiquitous organisms, found not only in animals and humans but also in water, soil and plants. These organisms are inhabitants of the intestinal flora of most animals including humans (Murray et al. 2005, Pham et al. 2007).

I.1.1. Physiology and Cell Structure

The average size of an *Enterobacteriaceae* cell is between 0.3 to 1.0 x 1.0 to 6.0 μm. They are Gram-negative, non-spore-forming bacilli. They are facultative anaerobes and under optimal conditions most representatives have a generation time of 20-30 min (Wilks et al. 2003, Murray et al. 2005). As typical Gram negative bacteria, the envelop of *Enterobacteriaceae* consists of an inner and an outer membrane with a periplasmic space in between (**FIGURE 1.**). The outer leaflet of the outer membrane is the major cell wall (O) antigen of *Enterobacteriaceae,* i.e. the heat-stable lipopolysaccharide (LPS) (Azari et al. 2013) (Murray et al. 2005). The outer membrane also contains proteins. Some are important ligands, some have sensor/signaling functions, while others (i.e. porins)

I.1.2. Pathogenic Potential

With considerable variation between species and even between strains regarding their actual virulence, some members of the family are considered genuine enteric pathogens (e.g. *Salmonella*, *Shigella*, the diarrhea-causing *E. coli* pathotypes). Some others may colonize the gut as part of the microbiota, but when getting to other, mostly extraintestinal body sites, they may cause serious, often life-threatening infections (e.g. *E. coli*, *Klebsiella*). Obviously, these infections are even more serious in hosts with compromised defense capacities. Finally, there are members of the family which are clearly opportunistic pathogens, i.e. usually not affecting the otherwise healthy hosts (e.g. *Serratia, Enterobacter*) (Holst 2007, Nordmann et al. 2012).

Depending on the site of infection, the diseases caused vary from cystitis to pyelonephritis, enteritis, septicemia, pneumonia, peritonitis, meningitis, and device-associated infections. Thus, *Enterobacteriaceae* can affect practically any body sites. (Nordmann et al. 2012). They are responsible for more than 70% of urinary tract infections (UTI), 30% to 35% of all bacteremia, and several intestinal infections (Murray et al. 2005). As some members also infect animals, or are carried by them, they cause zoonotic infections (most *Salmonella* serovars and *Yersinia species*). Others are strict human pathogens (*Shigella* species and *S.* Typhi). Among *E. coli* infections, examples of both zoonotic transmission (e.g. enterohaemorrhagic *E. coli*) and strictly human sources (e.g. enterotoxigenic or enteroinvasive *E. coli*) can be found (Nataro and Kaper 1998, Murray et al. 2005). *Enterobacteriaceae* spread easily between individuals either by direct contact transmission, with the aid of contaminated fomites, or by contaminated food and water (Baron 1996).

Importantly, some even some potentially pathogenic members of the *Enterobacteriaceae* family (such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*), are part of the commensal flora and co-habit the gut with hundreds of other members of the microbiota. This fact, combined with the ease with which these strains are capable of acquiring genetic material through horizontal gene transfer, explains the extreme flexibility of some family members leading to their highly versatile pathogenic and resistant features (Partridge 2011, Stokes and Gillings 2011, Nordmann et al. 2012).

I.2. The Global Problem of Antibiotic Resistance

Antibiotic resistance is considered as one of the most pressing problems of contemporary medicine. It has emerged as one of the foremost public health concerns of the 21st century as organisms are able to develop resistance rapidly to any antibiotics introduced (van Duin et al. 2013). Antibiotic resistance threatens the control of infectious diseases, increases morbidity and mortality and imposes enormous costs on societies. In the European Union, about 25000 patients die each year from infections caused by selected multidrug resistant (MDR) bacteria and 1.5 billion euros are the annual estimated associated costs (Leung et al. 2011). Likewise the Infectious Diseases Society of America (IDSA) considers antimicrobial resistance as "one of the greatest threats to human health worldwide" and the annual additional costs of infections in the USA caused by form channels through which various compounds, among others some antibiotics, reach the inner structures of the cells (Azari et al. 2013).

Beyond the somatic O polysaccharide antigen, other surface antigens may or may not be present on the strains. Certain *Enterobacteriaceae* have noticeable capsules (K antigen) such as *Klebsiella*, *Enterobacter* and *Escherichia* strains, while others are surrounded by a loose slime layer, or nothing at all. Depending on the genus, species and strain they can be motile, with peritrichous flagellae representing the H antigen. While the O antigen determines the serogroup, this, combined with the H (and sometimes with the K) antigen describes the serotype of the isolate.

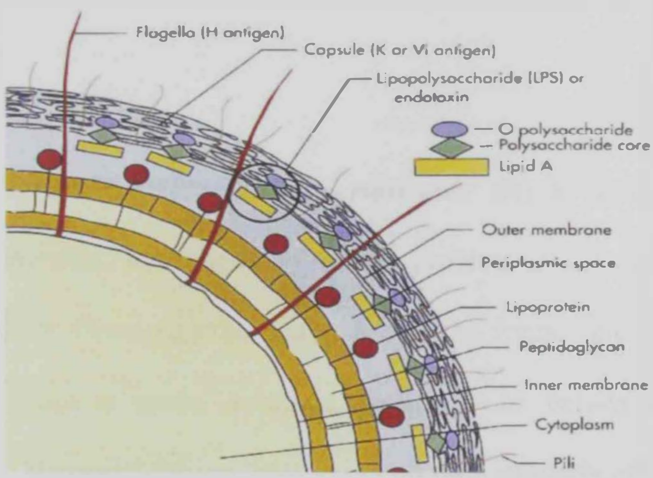

FIGURE 1. Cell wall structure of *Enterobacteriaceae* (Murray et al. 2005)

Several *Enterobacteriaceae* have fimbriae or pili associated with the capacity of the strain to adhere to various surfaces (Whitfield 1995, Weissman et al. 2003, Holst 2007).

resistant organisms, as compared to susceptible organisms, are estimated between $21 billion and $34 billion respectively (Spellberg et al. 2011).

Therefore, antimicrobial resistance has been the focus of the World Health Organization for several years: in 1998, the World Health Assembly implemented a resolution urging member states to take action against it. In 2001, the WHO outlined a global strategy on containment of antimicrobial resistance, besides a number of recommendations. In 2005, a new World Health Assembly resolution on antimicrobial resistance was implemented, a warning about the slow progress was issued and the rational use of antimicrobial agents by providers and consumers was called for. Finally in 2011, antimicrobial resistance was the focus of the 2011 World Health Day of the World Health Organization (WHO) (Leung et al. 2011).

While the problem of drug resistance affects almost all genera of pathogenic bacteria, the magnitude of the problem is not the same for Gram-positive and for Gram-negative bacteria. For the former group there are potent alternatives once a strain develops resistance to beta-lactams or even to vancomycin. However, for the latter group no such similarly effective, second line treatment is available. Actually, new anti-Gram-negative antibiotics, representing completely new classes are not even in the development/production pipelines and are unlikely to be available in the foreseeable future. Furthermore, resistance to the less-efficient or more toxic alternatives are emerging, resulting in pan-resistant strains (Stokes and Gillings 2011, Toleman and Walsh 2011, Zhanel et al. 2011, Walsh and Toleman 2012, van Duin et al. 2013).

I.2.1. Extended Spectrum Beta-Lactam Resistance Among Gram-Negative Rods in the Arabian Peninsula

The Middle East, specifically the Arabian Peninsula, has not been spared the problem of drug resistance, either. It is not surprising as actually some of these countries can be considered extremely vulnerable to the problem. They are surrounded by regions (the Indian subcontinent, North Africa etc.) often facing an extremely high rate of resistance. In some of the countries the rate of expatriate residents may reach 80%, mostly coming from these areas. Some of these countries, like the UAE, are tourist and commercial hubs with very high incoming and outgoing traffic. Characteristically, most of the Gulf countries have a highly advanced healthcare system with highly sophisticated technical interventions being routinely exercised. This, of course, means that the number of patients with compromised defense systems is high and it also means the extensive use of high-power antibiotics. Regretfully, however, this advanced level of curative medicine is often not matched with equally well-organized and well-maintained antibiotic regulation.

In the last two decades *E. coli,* followed by *K. pneumoniae*, *P. aeruginosa* and MRSA have been the most common microorganisms in the Gulf with emerging resistance (Aly and Balkhy 2012).

In Saudi Arabia, resistance to extended-spectrum cephalosporins due to ESBL production varies from 6% up to 38.5%. In addition, considerable levels of resistance are now encountered in the community. A study has revealed that

12.3% of healthy individuals were asymptomatic fecal carriers of ESBL-producing *E. coli* and *K. pneumoniae* (Zowawi et al. 2013). Regarding ESBLs genotypes, bla_{SHV} -like, as well as bla_{CTX-M} -like, and bla_{TEM} were common among ESBLs isolated from Al-Dhahran city in 2006 and in two hospitals in Riyadh in 2007, with bla_{SHV} dominating among *K. pneumoniae*. Regarding non-fermenting pathogens, in *P. aeruginosa* the bla_{GES}, bla_{VEB} and bla_{OXA} genes, while in *Acinetobacter* bla_{PER} and bla_{GES} were encountered (Zowawi et al. 2013).

The emergence of carbapenem-resistant *Enterobacteriaceae* in Saudi Arabia has been noticed since 2000. In a study done between 2002 and 2003 in the Eastern province of Saudi Arabia, 14% of ESBL-producing *E. coli* and *K. pneumoniae* isolates showed increased MICs to imipenem and meropenem (Zowawi et al. 2013). An outbreak of carbapenem-resistant *K. pneumoniae* in Saudi Arabia was reported from Riyadh from December 2009 to August 2010 where all isolates had altered outer membrane OMP36K and carried the carbapenemase gene bla_{OXA-48} (Zowawi et al. 2013). The rate of carbapenem-susceptible *P. aeruginosa* isolated from the ICU of a tertiary hospital in Riyadh was 66% in 2004 but declined to 26% by 2009 (Al Johani et al. 2010). Metallo-β-lactamases (MBL) have emerged as a common mechanism of carbapenem resistance in *P. aeruginosa* in Saudi Arabia with VIM being the most prevalent MBL type. *P. aeruginosa* isolates harboring bla_{IMP} and bla_{OXA-10} have been also identified (Zowawi et al. 2013). OXA-23 and OXA-40 were found in *Acinetobacter* (Zowawi et al. 2013).

In Kuwait, the prevalence of ESBLs has been also on the rise. A study on urine isolates between 2005 to 2007 showed that 26% and 12% of *K. pneumoniae* from hospital, and community-acquired urinary tract infections were ESBL producers. The respective figures for *E. coli* were 28% and 17% (Al Benwan et al. 2010). Another study in 2006 reported even higher figures, i.e. 62% for *E. coli* and 82% for *K. pneumoniae* (Jamal et al. 2009). bla_{CTX-M} genes have been the most commonly found ones in *E. coli* with CTX-M-15 being the dominant type. Other ESBL genes such as bla_{TEM}-like genes have been detected in *Salmonella* sp. isolates , $bla_{SHV-112}$ in *K. pneumoniae* and bla_{VEB} in *P. aeruginosa* isolates (Zowawi et al. 2013). Carbapenemase-producing strains of *E. coli* and *K. pneumoniae* have also been encountered in Kuwait (Zowawi et al. 2013). *Acinetobacter* resistance to carbapenems is now a major problem in the country with 64.3% resistance to imipenem and 66.1% resistance to meropenem. Several of the strains express OXA-58 type carbapenemase (Afzal-Shah et al. 2001, Coelho et al. 2006, Jamal et al. 2009).

In Omani hospitals ESBL producing *E. coli* and *Klebsiella* isolates are also common. A study between 2004 and 2005 showed that 60% of ESBL producers were *E. coli* and 40% were *K. pneumoniae*, although it was not revealed of what proportions these species were ESBL producers (Rafay et al. 2007). The most commonly identified genes were bla_{SHV} and bla_{CTX-M} (Zowawi et al. 2013). Several carbapenemases were also detected in the Sultanate of Oman: NDM-1, OXA-48 and OXA-181 have been encountered in *K. pneumoniae* and in *E.coli* (Poirel et al. 2011b, Potron et al. 2011b, Pfeifer et al. 2012).

In Qatar, ESBL production was demonstrated in 27.8% of *E. coli* and 18% of *K. pneumoniae* isolates during a study between 2007 and 2008 of 425 of blood culture isolates collected from the same hospital (Khan et al. 2010). From this country data on carbapenem resistance in *Enterobacteriaceae* have not been available, but in 2007-2008 it was found to be 41.5% among *Acinetobacter* and 14.3% *P. aeruginosa* of blood culture isolates, respectively (Khan et al. 2010).

In the Kingdom of Bahrain, a recent study showed that 22.6% of 11,886 *Enterobacteriaceae* isolated from 2005 to 2006 were ESBL producers, mostly from inpatient specimens with *E. coli* being the major ESBL producer (52.5%), followed by *K. pneumoniae* (24.3%) and *Proteus* spp. (17.6%) (Bindayna et al. 2009). Carbapenemases (OXA-23, OXA-58 and OXA-72) were identified in *Acinetobacter* isolates (Zowawi et al. 2013).

The presence of ESBL producing organisms is well-documented in the UAE, with CTX-M-15, SHV, TEM and PER being the most common types (Rotimi et al. 2008, Al-Zarouni et al. 2012, Opazo et al. 2012). Interestingly, CTX-M-15 have been also identified in isolates of Enteroaggregative *E. coli* (EAEC) with a gene associated with IS*Ecp1* on a plasmid, i.e. the first of its kind in this group of pathogens (Sonnevend et al. 2006). By now the spread of carbapenemase-producing strains is also described both in *Acinetobacter* (Ghazawi et al., 2012), as well as in *Enterobacteriaceae* (Sonnevend et al. 2012; Sonnevend et al. 2013).

Until the time when this project was initiated, only NDM and OXA type carbapenemases, but not VIM, IMP or KPC type enzymes, had been found in the Peninsula.

I.3. The Carbapenems

Carbapenems are derived from thienamycin, a naturally occurring antibiotic produced by the soil microorganism *Streptomyces cattleya (Papp-Wallace et al. 2011)*. Developed in the 1980s, imipenem and meropenem, the first members of the carbapenem class, had a broad spectrum of antimicrobial activity that included coverage of *P. aeruginosa*, making them important components of the armory of drugs used to treat nosocomial infections. Until that time, almost all *Enterobacteriaceae* were susceptible to carbapenems (Papp-Wallace et al. 2011).

Among carbapenems, imipenem, panipenem, and doripenem are potent against Gram-positive bacteria while meropenem, ertapenem, and doripenem have slightly more activity against Gram-negative organisms. However, both imipenem and panipenem are deactivated by dehydropeptidase I of the human renal brush. Therefore, they are co-administered with an enzyme inhibitor, i.e. cilastatin (Papp-Wallace et al. 2011).

As with all β-lactams, carbapenems are structurally related to penicillins "penams". The dissimilarity is that in them a carbon ("carba-") replaces the sulfur atom at position 1, a double bond exists between C-2 and C-3 ("-penem") and a hydroxyethyl side chain is present in place of the acylamino group shared by penicillins and cephalosporins, resulting in resistance to most β-lactamases (**FIGURE 2.**) (Bradley 1997, Mouton et al. 2000, Papp-Wallace et al. 2011).

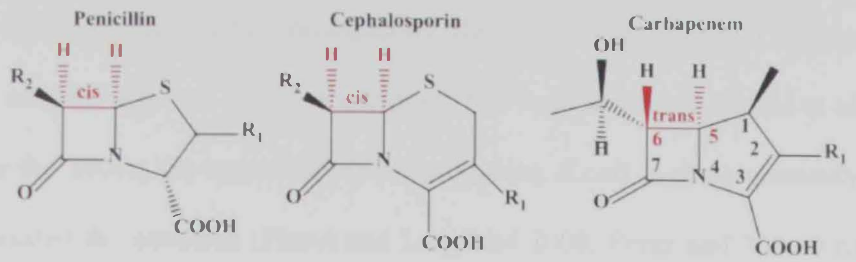

FIGURE 2. Structural comparison of the β-lactam antibiotics (Papp-Wallace et al. 2011).

Carbapenems are bactericidal antibiotics. Their mode of action is similar to that of other β-lactam antibiotics, i.e. they inhibit bacterial transpeptidases involved in peptidoglycan synthesis of the cell wall by binding with high affinity to penicillin binding proteins (PBPs). Unlike other β-lactam antibiotics, and similar to aminoglycosides and fluoroquinolones, they display a significant post-antibiotic effect against Gram-negative bacterial pathogens (Hashizume et al. 1984, Majcherczyk and Livermore 1990, Papp-Wallace et al. 2011).

Currently, carbapenems are frequently prescribed for severe sepsis, particularly for patients with recent health care-associated exposures (Martin et al. 2013). Their efficacy has been emphasized by several studies (Endimiani et al. 2004, Paterson et al. 2004). The use of carbapenems, measured in a sample from 35 university hospitals in the United States, rose by 59% between 2002 and 2006 (Pakyz et al. 2008).

I.4. Carbapenem Resistance in *Enterobacteriaceae*

In the 80s and early 90s *Enterobacteriaceae* started becoming resistant to advanced, 3rd and 4th generation cephalosporins by acquiring extended-spectrum beta-lactamases (ESBLs). Consequently, the use of carbapenems, i.e. drugs not, or only weakly, affected by these enzymes, has been increased (Rahal et al. 1998). Since the 2000s, the spread of ESBL-producing *E.coli* in the community further aggravated the situation (Pitout and Laupland 2008, Perez and Van Duin 2013). Therefore, the prevalence of carbapenem resistant *Enterobacteriaceae* (CRE) isolated from medical samples continues to rise worldwide (van Duin et al. 2013).

Alarmingly, by 2009–2010, the National Healthcare Safety Network from the Centers for Disease Control and Prevention (CDC) had shown that 12.8% of *K. pneumoniae* isolates related to bloodstream infections were not susceptible to carbapenems (Sievert et al. 2013). In 2012, 3.9% of short-stay acute-care hospitals and 17.8% of long-term acute-care hospitals described the minimum of one CRE health care-associated infection. Moreover, the rate of *Enterobacteriaceae* that are CRE have increased fourfold over the last 10 years (Perez and Van Duin 2013).

Resistance to antibiotics other than the β-lactam class is regularly present in these strains making them really multi-drug resistant (MDR). Frequently, the genes of resistance to aminoglycosides, tetracycline, sulphonamides are co-localized on the same plasmid as the genes related to β-lactam resistance (Martinez-Martinez 2008). Importantly, different carbapenems differ in their activity on non-fermenting Gram-negative bacteria. Pathogens, such as *A.*

baumanni or *P. aeruginosa* exhibit natural resistance to the so called Group 1 carbapenems (exemplified by ertapenem), while Group 2 agents are effective against them, unless there are some acquired resistance mechanism present (Sousa et al. 2013).

The treatment choices for CRE are limited to colistin (polymyxins), some aminoglycosides, tigecycline, and fosfomycin, i.e. agents considered as "drugs of last resort" (Ouderkirk et al. 2003, Falagas and Kasiakou 2005, Endimiani et al. 2009, Perez and Van Duin 2013). Polymyxins and tigecycline generally have *in vitro* activity against CRE. The use of these drugs is not without controversies, however. Polymyxins are considerably toxic, and because of its pharmacokinetic features, tigecycline is not ideal to treat blood-stream infections (Perez and Van Duin 2013). Furthermore, its bacteriostatic nature makes it less optimal in patients with decreased immunological fitness. To aggravate the situation even further, resistance to both drugs is also emerging (Ouderkirk et al. 2003, Nix and Matthias 2010, Tarchini 2010, Prasad et al. 2012, Perez and Van Duin 2013).

The use of high-dose prolonged-infusion carbapenem therapy is a possible approach in a combination regimen in CRE infections when carbapenem MICs are ≤4 mg/L (Daikos and Markogiannakis 2011). Moreover, certain human studies and ones using murine models suggest that double-carbapenem therapy may be effective in some instances (Bulik and Nicolau 2011). Nevertheless, the effectiveness of carbapenem therapy, whether in combination regimens, in a high-dose prolonged infusion, or the "double carbapenem therapy" need to be further studied (Nicolle et al. 2005, Bulik and Nicolau 2011, Tumbarello et al. 2012).

In *Enterobacteriaceae*, two major mechanisms are responsible for carbapenem resistance. One is in combination with overexpression of β-lactamases having, on their own, limited activity against carbapenems, and the decrease of the intracellular concentration of the drug. This may take place either by limiting their uptake or, seldom, by increasing their efflux antibiotics. The second, more important, mechanism is the production of various carbapenemase **(FIGURE 3.)** (Nordmann et al. 2012).

I.4.1. Non-Carbapenemase-Mediated Resistance

Porins of *Enterobacteriaceae* are specific proteins that form hydrophilic channels, or pores, that permit a selective uptake of different types of molecules such as essential nutrients and other compounds, including antibiotics, through the outer membrane **(FIGURE 3.)** (Koebnik et al. 2000, Pages et al. 2008). Hence, these channels can serve as a bottle-neck for compounds passing the outer membrane as their actual number impacts the amount of drugs reaching the periplasmic space. OmpF and OmpC porin families are the first porins in *Enterobacteriaceae* that contribute to the uptake of antibiotics (Pages et al. 2008).

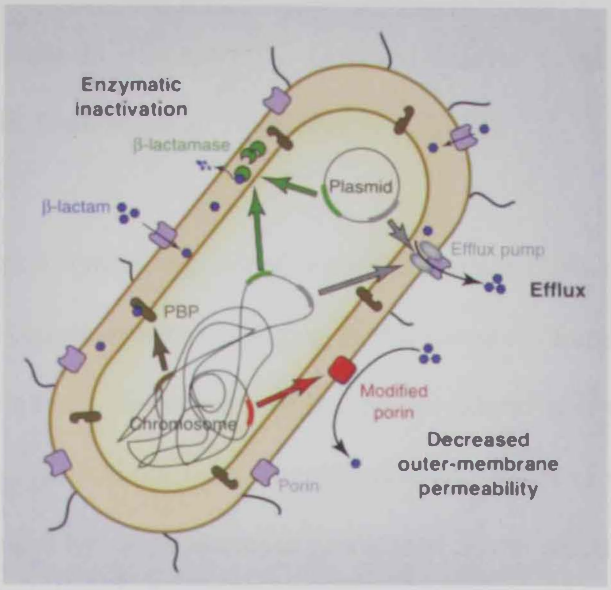

FIGURE 3. Mechanisms of β-lactam resistance in *Enterobacteriaceae* (Nordmann et al. 2012).

A decrease in porin expression, or any change in the activity of outer membrane porins, can affect antibiotic resistance. This decrease in the number of porins in the outer membrane might be observed as a response to antimicrobial products so antimicrobial exposure itself may regulate porin synthesis. (Armand-Lefevre et al. 2003, Nikaido 2003, Chia et al. 2009, Doumith et al. 2009, Shin et al. 2012). Limitation of the drug uptake alone, however, is unlikely to cause clinically relevant carbapenem resistance. On the other hand, while enzymes with limited carbapenemase activity may not be efficient enough alone against high drug concentrations, once the uptake is limited by porin loss, the decreased amount reaching the periplasmic space might be hydrolyzed sufficiently enough. This is particularly the case if less effective enzymes are getting overexpressed, to cause a significant, clinically-relevant decrease in susceptibility (Armand-Lefevre et al. 2003, Martinez-Martinez 2008, Doumith et al. 2009, Shin et al. 2012).

Different enzymes, e.g. AmpC enzymes, certain ESBLs, some other class A (see later) cephalosporinases belong to this category. Importantly, resistance due to this combined mechanism, while it can be extensive, is usually considered less important, particularly from an epidemiological point of view, compared to resistance caused by carbapenemase production. Porin mutants often also lose some of their fitness as they became inefficient in the uptake of not only antibiotics, but also of other, physiologically-important compounds. Furthermore, this resistance trait is unlikely to spread as it is related to mutations in the chromosome, instead of localized on mobile elements. (Lartigue et al. 2007, Pages et al. 2008, Doumith et al. 2009, Garcia-Fernandez et al. 2010).

An alternative cause of developing carbapenem resistance without producing carbapenemases is the overexpression of efflux pumps, such as the overexpression of the AcrA efflux pump component in *Enterobacter aerogenes* that resulted in the development of imipenem resistance (Bornet et al. 2003). Nevertheless, this mechanism is considered second in importance compared to porin-enzyme-related resistance.

I.4.2. Carbapenemases

The most effective defense of the bacterial cell against carbapenems is due to the production of special groups of β-lactamases with high affinity to members of this group of β-lactams, i.e. to carbapenemases. SME-1 and IMI-1 were the first carbapenemases identified in *Enterobacteriaceae* in the United Kingdom in 1982 and IMI-1 in the USA in 1984, respectively (Yang et al. 1990, Rasmussen et al. 1996). The first serine carbapenemase reported was NmcA, a chromosomally-encoded enzyme, discovered in an *E. cloacae* clinical isolate (Naas and Nordmann 1994). Since then, carbapenem-resistant *Enterobacteriaceae* have been reported globally, mainly as a consequence of the extensive acquisition of carbapenemase genes (Queenan and Bush 2007). Enzymes with considerable carbapenemase acitivity (i.e. higher than those of ESBLs and AmpC enzymes) can be found in three of the four Ambler classes of β-lactamases **(TABLE 1.)** (Nordmann et al. 2012). (ESBLs and class C AmpC enzymes with minimal carbapenemase activity were discussed earlier).

Class A Carbapenemases

Regarding the Ambler class A carbapenemases, three main types are known: the NmcA/IMI, SME, and KPC enzymes (Walther-Rasmussen and Hoiby 2007). All these enzymes hydrolyze a broad variety of β-lactams including penicillins, cephalosporins, carbapenems, and aztreonam **(TABLE 1.)**. They have a serine-containing active site at position 70 (Ambler numbering of class A β-lactamases). They are inhibited *in vitro* by clavulanic acid and tazobactam

(TABLE 1). A fourth type of Ambler A group enzymes related to the GES type β-lactamases was also identified. Originally this group was considered to be a 'classical' ESBL because GES-1 did not possess carbapenemase activity. However, lately, its weak but significant carbapenemase activity has been recognized (Nordmann et al. 2012).

At present, KPC enzymes are the clinically most significant enzymes among class A carbapenemases. The first strain of *K. pneumoniae* producing KPC was isolated in 1996 in the Eastern part of the USA (Yigit et al. 2001). Up until now, multiple KPC variants have been recognized from KPC-2 to KPC-12 (11 variants). KPC-producing strains spread widely and have been identified in different countries such as the entire US territory, Puerto Rico, Columbia, Italy, Greece, Israel, and China (Navon-Venezia et al. 2009, Nordmann et al. 2009). Outbreaks of KPC-producing strains have also been reported in many European countries and in South America (Navon-Venezia et al. 2009, Nordmann et al. 2009).

KPC enzymes have been described mainly from nosocomial *K. pneumoniae* isolates and to a lesser degree from other enterobacterial species. Fatality to infections due to KPC-producing strains is high, around 50% or more, due to multi-drug resistance among KPC-producing strains that may result in failure in first line therapy (Borer et al. 2009, Nordmann et al. 2009).

Class D carbapenemases

The class D carbapenemases called OXAs for 'oxacillinases', includes over 200 enzymes. Several, but not all variants exhibit carbapenemase activity, although at very different levels. Actually, most have weak carbapenem hydrolyzing ability and do not inactivate expanded-spectrum cephalosporins **(TABLE 1.)**. They are inhibited by NaCl but not by clavulanic acid or EDTA **(TABLE 1.)** (Nordmann et al. 2012).

Most class D carbapenem hydrolyzing enzymes have been found in *Acinetobacter spp*., where $bla_{OXA\text{-}51}$ is considered a species-defining gene (Turton et al. 2006). OXA-48 has been discovered in *Enterobacteriaceae*. The first identified strain producing OXA-48 was a *K. pneumoniae* isolate recovered in Turkey in 2003 (Poirel et al. 2004). Today, OXA-48-like enzymes represent a group increasingly encountered in many countries of the world (Poirel et al. 2012). Nosocomial outbreaks due to OXA-48-like enzyme producing strains have been described in Turkey and in several other countries (Carrer et al. 2008, Nordmann et al. 2011a). A single plasmid of approximately 62 kb is the main source of the $bla_{OXA\text{-}48}$ gene disseminated in a variety of enterobacterial species (Potron et al. 2011a, Nordmann et al. 2012) .

TABLE 1. The most important carbapenemases in *Enterobacteriaceae* (Nordmann et al. 2012).

Ambler class	Enzyme	Location	Hydrolysis spectrum						Inhibitor
				Cephalosporins (generations)					
			Penicillins	1st	2nd	3rd	Aztreonam	Carbapenems	
A	SME-1 to -3	Chromosome	++	++	-	+	+	+	Clavulanate, tazobactam, sulbactam, NXL-104
	NMC-A	Chromosome	++	++	-	+	-	++	
	IMI-2	Plasmid	++	++	-	+	-	++	
	GES-4, -5, -6	Plasmid	++	++	+	+	-	+	
	KPC-2 to -12	Plasmid	++	++	-	++	+	++	Clavulanate, tazobactam, boronic acid, sulbactam
B	IMP-1 to -33	Plasmid	++	++	++	++	-	++	EDTA
	VIM-1 to -33	Plasmid	++	++	++	++	-	++	
	NDM-1 to -6	Plasmid	++	++	++	++	-	+	
	KHM-1	Plasmid	++	++	++	++	-	++	
D	OXA-48	Plasmid	++	++	+/-	+/-	-	+	NaCl
	OXA-181	Plasmid	++	++	+/-	+/-	-	+	

Class B carbapenemases

Class B enzymes are called metallo-β-lactamases (MBL) as they require Zn^{2+} ion(s) in their active site. Consequently, they can be inhibited by chelators of divalent cations such as ethylenediaminetetraacetic acid (EDTA) **(TABLE 2.)** (Zhao and Hu 2010). These enzymes have a broad spectrum of hydrolytic activity, they hydrolyze all penicillins, cephalosporins, and carbapenems, with the exception of monobactams **(TABLE 2.)** and they are not inactivated by commercially available β-lactamase inhibitors (clavulanic acid, tazobactam, or sulbactam) (Miriagou et al. 2010, Cornaglia et al. 2011).

The first MBLs identified were chromosomally encoded enzymes and the bacteria were mainly opportunistic and soil-inhabitants (*Bacillus cereus*, *Aeromonas spp*., and *Stenotrophomonas maltophilia*) (Zhao and Hu 2010). The first MBL-related nosocomial outbreak was reported from Europe (Lauretti et al. 1999; Cornaglia et al. 2011). Since the 1990s, a remarkable number of transferable MBL genes has been described in *Enterobacteriaceae* and *Pseudomonas spp.*. The most common types of acquired MBLs in *Enterobacteriaceae* are members of the IMP and VIM groups and the various alleles of the recently described NDM group (Walsh et al. 2005). The VIM and IMP MBLs have disseminated mostly in *Pseudomonas aeruginosa* and, at least in some regions, in *Acinetobacter baumannii* and *Enterobacteriaceae*, particularly *K. pneumoniae*. Moreover, they have been identified in different species as *Salmonella enterica, E. coli, Enterobacter spp., and Pseudomonas spp.* (Miriagou et al. 2010).

Other acquired MBLs include SPM-1, GIM-1, SIM-1, DIM-1, AIM-1, KHM-1; (Gupta 2008, Sekiguchi et al. 2008). It was the emergence and fast global spread of the NDM type enzymes which propelled MBLs into the center of attention. NDM-1-producing bacteria were initially found mainly in the UK, India, and Pakistan, but by now it has spread globally not sparing any of the continents (Kumarasamy et al. 2010, Nordmann et al. 2011b).

Resistance caused by acquired MBLs among major Gram-negative pathogens such as *Enterobacteriaceae*, has serious clinical and epidemiological implications and is a matter of considerable concern all over the world (Cornaglia et al. 2007). Fatality associated with MBL production varies from 18% to 67% (Daikos et al. 2009). The lack of clinically available inhibitors eliminates even the theoretical possibility to interfere with its action *in vivo*. Furthermore, their frequently plasmid-coded nature allows an efficient inter-strain and inter-species transfer further emphasizing the clinical and epidemiological importance of MBLs (Walsh et al. 2005).

I.5. Metallo-β-Lactamases

In 1989, within the Bush-Jacoby-Medeiros functional classification, based on their sequences, MBLs were grouped into three subclasses, B1, B2 and B3 (Fast and Sutton 2013). Members of the three subclasses vary in the structure of their active sites. The active site in enzymes of subclasses B1 and B3 has two zinc ions, while in enzymes of subclass B2 there is only one zinc ion and these are characterized by narrower substrate specificities (Garau et al. 2004). Every subclass has several different types of MBLs, and several types have multiple allelic variants. In order for a MBL enzyme to be classified as a new subclass, minimum 30% of amino acid diversity is required (Cornaglia et al. 2007).

Subclass B1 enzymes share more than 23% identity, while subclass B2 enzymes present only 11% of identity with subclass B1 members. Subclass B3 MBLs have only nine conserved residues when compared with the other MBLs (Bebrone 2007). All of the acquired types of MBLs belong to subclass B1. They are the ones most often captured, and further spread by mobile genetic elements, a fact explaining their wide-spread nature compared to the other two subclasses (Bebrone 2007).

I.5.1. Inhibitors of Metallo-β-Lactamases

One of the most effective antibacterial strategies in the treatment of bacterial infections is the combination of β-lactams and β-lactamase inhibitors. The success of these agents is clearly highlighted by the efficiency of amoxicillin and clavulanate (Miller et al. 2001).

MBLs are different from the serine β–lactamases as they have a wide active-site groove making them able to accommodate most β -lactam substrates and hence to have a broad spectrum of activity. They are resistant to the inactivation effects of serine inhibitors, such as clavulanic acid and sulbactam, as these compounds are poor substrates. Clavulanic acid interacts directly with class

A enzymes and forms a stable covalent intermediate, while MBLs do not form such intermediate (Walsh et al. 2005).

Aztreonam is not hydrolyzed by any of the MBLs, so it has been investigated whether it might be used as a therapeutic MBL inhibitor. When combined with high doses of aztreonam, a significant decrease in lung bacterial counts was observed when imipenem was given at the highest doses recommended for humans. Nevertheless, the treatment was unable to eradicate pneumonia in animals caused by *P. aeruginosa* producing VIM-2 enzyme (Bellais et al. 2002).

To further complicate the matter, subclasses of MBLs exhibit considerable variations in their active site structure, so a single inhibitor effective against all MBLs is highly unlikely to be found (Daiyasu et al. 2001). Furthermore, good affinity of the inhibitor to the enzyme *per se* does not guarantee a considerably lower MICs against β-lactams, as was shown experimentally in *P. aeruginosa* containing MBL genes (Daiyasu et al. 2001, Schilling et al. 2003).

To find clinically useful, specific inhibitors for MBLs is particularly difficult since such inhibitors must not have activity towards human metallo-enzymes (e.g. angiotensin converting enzyme). Several inhibitors were studied, such as thioesters derivatives, trifluoromethyl alcohols and ketones, sulfonyl hydrazones, tricyclic natural products, succinic acid derivatives, biphenyl tetrazoles, cysteinyl peptides, carbapenem and penicillin derivatives, degradation products of cephalosporins, simple thiol compounds such as mercaptoacetic acid and thioesters, thiomandelic acid, captopril, derivatives of benzohydroxamic acid, and others. Regretfully, they had moderate activity against one or two metallo-β-lactamases, only (Bebrone 2007, Fast and Sutton 2013).

Consequently, effective inhibition of MBLs can be achieved only by chelation, i.e. removal of the divalent cations required for the function of the enzyme. Inhibitors effective *in vitro* are metal chelators EDTA, O-1, 10-phenanthroline and dipicolinic acid. Understandably however, using them clinically, *in vivo*, is not a feasible option (Bebrone 2007).

I.5.2. Genetic Organization of MBL Genes

Genes of MBLs can either be located on the chromosome (often referred to as resident MBLs) or can be mapped on mobile elements. As these latter ones are often transferred horizontally, these enzymes represent the majority of acquired MBLs (Bebrone 2007). Resident MBLs are found in only a few species of clinical significance, e.g. *S. maltophilia*, *Bacillus* spp., *B. fragilis* and *P. otitidis* (Yano et al. 2001). Regarding acquired MBLs, several types have been detected and described. IMP-type, VIM-type, SPM-type, and NDM-type enzymes have been detected in multiple strains of *Enterobacteriaceae*, *P. aeruginosa*, *A. baumannii*, and other Gram-negative non-fermenters (Bebrone 2007).

The vast majority of acquired MBLs belong to the subclass B1, while enzymes belonging to sucbclass B2 are coded on less mobile genes located on the chromosome. Concerning B3 enzymes, they are generally on large plasmids, as well as on the chromosome (Avison et al. 2001, Bebrone 2007).

Genes coding for transferable MBLs are generally carried on gene cassettes of type 1 or type 3 integrons. Integrons are assembly platforms that capture exogenous open reading frames (ORFs) by site-specific recombination allowing their expression, thus transforming them into functional genes. All integrons are made up of three regions of importance for the incorporation of exogenous genes: a gene (*intI*) encoding an integrase of the tyrosine-recombinase family; a primary recombination site (*attI*); and a promoter (Pc) directed outwards to realize the transcription of the captured genes (Hall and Collis 1995). Integron-encoded integrases can capture different units of circularized DNA or gene cassettes. Integration takes place at the *attI* site, downstream of the resident promoter, which permits the expression of the genes in the cassette. Gene cassettes that are integron-inserted contain a single gene and an imperfect inverted repeat at the 3′ end of the gene called an *attC* site (or 59-base element). The *attC* sites are a varied family of nucleotide sequences that are important for the recognition by the integrase.

Related to the variety of genes encoding integrase, eight classes for integrons have been recognised and characterized (Zhao and Hu 2011). The dissemination of MBLs among Gram-negative bacilli is mediated by class 1 integrons. A typical class 1 integron contains a 5′conserved segment (5′-CS), a variable region and a 3′ conserved segment (3′-CS) (Zhao and Hu 2011). Class 1 integron has two promoters, P1 and P2. Regarding the 3′-CS, it often consists of a partly deleted *qac* gene (*qacED1*) fused to a *sul1* gene, and is responsible of resistance to antiseptics and sulfonamide. The antibiotic resistance gene cassettes are normally introduced between the 5′-CS and 3′-CS (**FIGURE 4.**) (Fluit and Schmitz 1999, Zhao and Hu 2011).

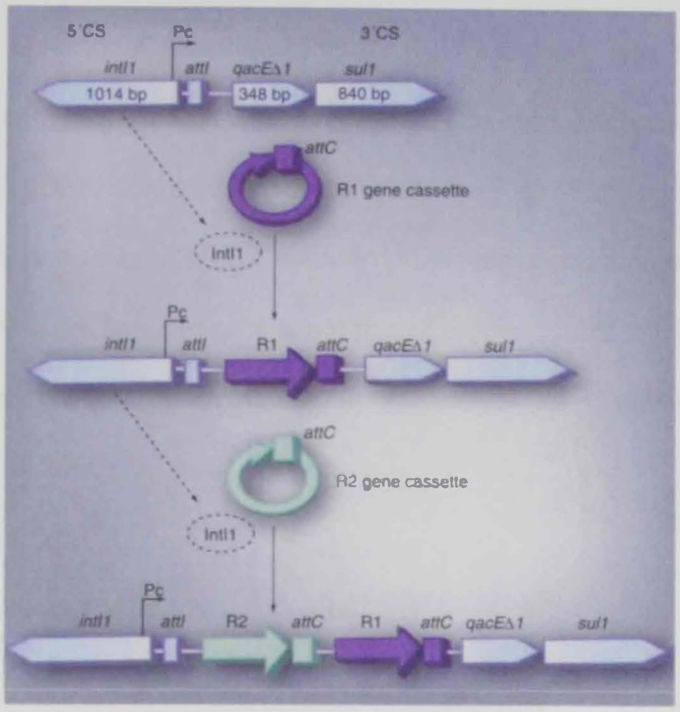

FIGURE 4. The class 1 integron and the process to capture gene cassettes (Zhao and Hu 2011).

Gene cassettes are circular DNA molecules of around 1 kb. They are inserted by a specific site of recombination between the 5'- and 3'- conserved segments of an integron. The number of gene cassettes can vary between 0 for In0 to at least five (Fluit and Schmitz 1999). Several copies of gene cassettes can be presented in the same integrin, for example the two copies of *oxa2* that exist in In1 (Stokes and Hall 1992, Fluit and Schmitz 1999).

Most integrons containing gene cassettes for MBLs also carry other gene cassettes encoding for other antibiotic classes, disinfectants, or other β-lactamase genes. So, integron transfer might result in a single-step transfer of a complex multidrug-resistant phenotype (Fluit and Schmitz 1999, Cornaglia et al. 2011).

The majority of genes encoding for IMP- and VIM- type MBLs are found in class 1 integrons (see later in details for VIM-type enzymes), while certain IMP- type enzymes might be found in class 3 integrons (Walsh et al. 2005). MBL genes may also be carried by ISCR (insertion sequence common regions) as in the case of bla_{SPM-1}, where the gene is not part of a gene cassette, neither found on a class 1 integron, but beside the ISCR variant ISCR4 (Toleman et al. 2002, Toleman et al. 2006).

I.5.3. Detection of MBLs

Currently, no standardized phenotypic methods exist to detect MBLs. The actual MIC to carbapenems exhibits great variations according to the enzymes expressed and to the genus and species. With some *Enterobacteriaceae* and some *Acinetobacter spp.* isolates, MIC values, particularly with imipenem, can be as low as 1 and 2 mg/L (Yan et al. 2001a, Scoulica et al. 2004). *Pseudomonas* carrying MBLs strains usually exhibit higher carbapenem MICs than *Enterobacteriaceae*.

Methods to detect carbapenemases, e.g. the modified Hodge test (MHT), often fail to give positive reaction with some of the MBLs, particularly NDM type enzymes, as the presence of divalent cations may not be enough to support their action. Supplementing the media with them increases the sensitivity of this test **(FIGURE 5.)** (Girlich et al. 2012).

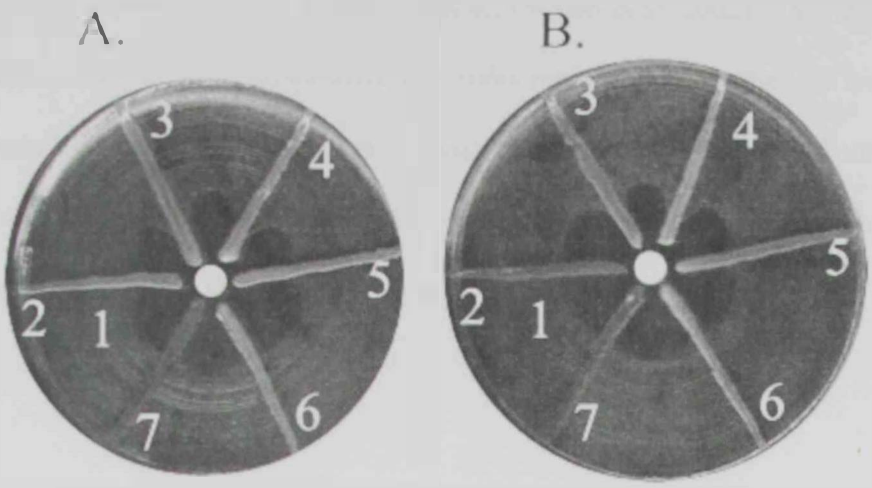

FIGURE 5. Modified Hodge Test (A) and the effect of extra zinc sulphate added (B)

Organisms tested: 1, E. coli JM109; 2, K. pneumoniae COO (CTX-M-15 + porin loss); 3, K. pneumoniae BIC (OXA-48); 4, K. pneumoniae POZ (KPC-2); 5, E. coli GEN (NDM-1); 6, E. coli RIC (NDM-1); 7, E. coli ALL (NDM-1). Zinc sulfate improved the MHT for E. coli RIC and not for E. coli ALL. (Girlich et al. 2012).

Specific detection of MBLs is usually based on the fact that chelators can block their action and is done by the use of a variety of inhibitor plus β-lactam combinations **(TABLE 2. -** ***see page 32*****)** (Yan et al. 2004). The degree of inhibition of MBLs differs with different compounds and the MBLs enzymes have different resistance to different substrates. Ceftazidime and imipenem are two substrates commonly used in screening MBLs.

For most microbiology laboratories, the E-test MBL strip is preferred as a screening system. The strip is partly permeated with an imipenem gradient across several dilutions and the other half with another imipenem gradient soaked with a constant concentration of EDTA (**FIGURE 6.**) (Walsh et al. 2002). This test may not be able to detect all MBL-positive *Enterobacteriaceae* due to the low level of "resistance". The disk approximation test may work better, particularly if multiple substrates (imipenem, ceftazidime, and meropenem) are used in combination with multiple inhibitors (EDTA and mercaptopropionic acid) (Arakawa et al. 2000, Yong et al. 2002).

FIGURE 6. E-test MBL strip of an *Acinetobacter sp.* expressing a VIM-2 MBL (Walsh et al. 2005)

In research laboratories, bacterial cell extracts are inspected for their carbapenem hydrolyzing ability and whether this hydrolysis is EDTA sensitive. This method is considered the non-molecular "gold standard". Results show the production of the enzyme irrespective of the strains' genotype. However the problem of using EDTA in combination with imipenem is that a number of MBL-negative *P. aeruginosa* showed reduced imipenem MICs in the presence of

EDTA. That might be caused by the effect of zinc on OprD (outer membrane porin that allows the entry of carbapenem) and the newly described CzcR-CzcS system (Conejo et al. 2003, Perron et al. 2004).

In case of positive screening results, verification is often achieved by molecular techniques in reference laboratories. The techniques used to detect MBLs are PCR or DNA hybridization.

Taken together MBLs represent a major, emerging problem, as (Bebrone 2007; Cornaglia et al. 2011; Rossolini et al. 2001; Saavedra et al. 2003):

- they are efficient carbapenemases with broad spectrum activity
- they have a potential to be transferred horizontally
- their genes often co-locate and cluster with other resistance genes
- we do not have clinically useful inhibitors
- their genes are present in several environmental species serving as reservoirs

My thesis will focus on a special group of MBLs, the so called VIM-type enzymes.

TABLE 2. MBL detection techniques (Walsh et al. 2005)

Test	Substrate-inhibitor combination	Advantages	Disadvantages
Disc approximation	Ceftazidime + 2-mercap-toproprionic acid	Easy to use	Not standardized and not always easy to interpret
Disc diffusion	Imipenem + EDTA	Easy to use and relatively easy to interpret	Not standardized. Bacteria can be imipenem sensitive
Microdilution test	Imipenem + EDTA and 1,10-phenanthroline	Based on reduction in MICs, easy to interpret	Specialized and labor intensive
E-test	Imipenem + EDTA	Easy to use and relatively easy to interpret	Bacteria can be imipenem sensitive, borderline cases may be missed
Carbapenem hydrolysis	Meropenem + EDTA	Very sensitive and deemed to be the gold standard	Highly specialized, labor intensive, and interpretation not straightforward
PCR		Easy to perform, specific for gene family	Requires tailor-made DNA primers, cannot differentiate between variants, may not detect new variants
DNA probes		Specialized, labor intensive	Probe required for each gene family, cannot differentiate between variants
Cloning and sequencing		Molecular gold standard	Labor intensive, interpretation of data requires experience

I.6. VIM-Type MBLs

The VIM-type enzymes form one of the most common groups of acquired MBLs. In some regions, like in Italy, their frequency actually exceeds that of IMP type MBLs by a ratio of 4:1 (Rossolini et al. 2008).

I.6.1. The Variety of VIM Enzymes

The VIM-1 (Verona integron-related metallo ß-lactamase-1) was discovered in Verona, Italy in 1997 in a *P. aeruginosa* strain. bla_{VIM-1}, the gene encoding for the VIM-1, was integrated into a class 1 integron located on the bacterial chromosome (Lauretti et al. 1999, Zhao and Hu 2011). The isolate was resistant to a set of β-lactams, including piperacillin, ceftazidime, imipenem, and aztreonam. The MIC of imipenem was >128mg/L. Biochemical analysis of this strain indicated a carbapenemase activity that was inhibited by EDTA and restored after addition of Zn^{2+}. The cloning of the encoding gene revealed its relation to BCII from *B. cereus*, sharing only 39% amino acid identity. The hydrolytic pattern of the cloned bla_{VIM-1} was typical of class B enzymes, i.e. hydrolyzing most β-lactams except aztreonam; obviously, resistance of the *P. aeruginosa* strain to monobactams was not due to MBL-related mechanisms (Lauretti et al. 1999). The class 1 integron, bearing the bla_{VIM-1} gene, carried an integrase gene typical of class 1 integrons, as well as an *aacA4* gene cassette encoding resistance to aminoglycosides (Walsh et al. 2005).

After the initial detection of VIM-type enzymes in *P. aeruginosa* and other Gram-negative non-fermenting strains, these enzymes also appeared in *Enterobacteriaceae* (Zhao and Hu 2011). As of today, more than twenty VIM allotypes were identified (VIM-1–27). VIM variants have a characteristic geographical distribution with VIM-1 and VIM-4 found in Europe, VIM-3 in Taiwan, VIM-6 in Asia and VIM-7 in the USA, while VIM-2 is distributed worldwide (Zhao and Hu 2011). The VIM-type MBLs have broader substrate specificities than the IMP-types, and are capable of hydrolyzing 6-α-methoxy-penicillins.

Additionally, the VIM-type enzymes have a substantially high affinity for carbapenems which is distinctive in the metallo-β-lactamases. In this respect a considerable functional heterogeneity was found among different VIM allotypes. Nevertheless no clinical consequence or significance was associated with these differences (Docquier et al. 2003, Cornaglia et al. 2011).

In most cases the mature VIM enzymes consist of 266 amino acid residues, while VIM-7 has an amino acid deletion in position 7 with 265 amino acids and VIM-18 has 262 residues with a deletion of four amino acids from position 145 compared with other VIM variants. The amino acid similarities of the most common VIM variants range from 72.9 to 99.6% (Zhao and Hu 2011) (**TABLE 3.** - *see page 37*).

Below, we provide a brief account on the most important alleles of these enzymes.

bla$_{VIM-2}$ was isolated from a strain of *P. aeruginosa* that showed resistance to most β-lactams (ceftazidime, cefepime, and imipenem) but not to aztreonam. The strain was recovered from the blood culture of a neutropenic patient in 1996 in southern France (Poirel et al. 2000). *bla*$_{VIM-2}$ was found to be on a non-conjugative plasmid of ca. 45 kb and located within a gene cassette carried by a class 1 integron. β-lactamases VIM-1 and VIM-2 have 90% amino acid identity (**TABLE 3.**) (Poirel et al. 2000, Docquier et al. 2003).

Sequence heterogeneity is mostly observed in the NH2- and carboxy-terminal regions of VIM-1 and VIM-2. Lately, other *P. aeruginosa* strains that carry the same *bla*$_{VIM-2}$ gene cassette with the same resistance profile have been identified in France. However, the *bla*$_{VIM-2}$ gene cassettes were integrated in different class 1 integrons, In58 and In59. This integron usually carries aminoglycoside resistance genes, as well as sulfonamide resistance gene in the 3' conserved element (Poirel et al. 2001).

Recently, VIM-2-producing *P. aeruginosa* strains have been also identified in Italy and Greece, and eventually have also been reported from other countries, such as Japan, South Korea, Portugal, Spain Poland, Croatia, Chile, Venezuela, Argentina, Belgium, and most recently in the United States (Zhao and Hu 2011). Moreover, VIM-2 has been detected in other species such as *C. freundii* in Taiwan, in *S. marcescens* in South Korea, and *E. cloacae* in South Korea (Walsh et al. 2005).

VIM-3, was found in *P. aeruginosa* isolates in Taiwan. It differs from VIM-2 by two amino acid changes and is located on the chromosome (Yan et al. 2001b).

VIM-4 was first discovered in a *P. aeruginosa* in a patient with post-surgical cerebrospinal infection in Greece (Pournaras et al. 2002). The strain was highly resistant to carbapenems. The bla_{VIM-4} was carried by a class 1 integron. Compared to VIM-1, VIM-4 has only one different nucleotide resulting in a Ser-to-Arg modification at amino acid position 175 (Ser175Arg) (Pournaras et al. 2002, Libisch et al. 2004). Subsequently, a VIM-4-producing *P. aeruginosa* was isolated in Sweden (Giske et al. 2003) and the same enzyme was also identified in other species such as in *K. pneumoniae* and *E. cloacae* strains in Italy (Luzzaro et al. 2004). Interestingly, while that bla_{VIM-4} gene in *K. pneumonia* and *E. cloacae* was located on the same plasmid, the MICs of imipenem and meropenem for these isolates were different (2 and 0.5 mg/L for *K. pneumoniae* and 0.25 and 0.12 mg/L for *E. cloacae*, respectively) (Luzzaro et al. 2004).

TABLE 3. Comparison of the numbers of different amino acids among VIMs (Zhao and Hu 2011)

	VIM-2	VIM-3	VIM-4	VIM-5	VIM-6	VIM-7	VIM-8	VIM-9	VIM-10	VIM-11	VIM-12	VIM-13	VIM-14
VIM-1	25	27	1	5	27	62	26	26	25	26	8	19	26
VIM-2		2	24	27	2	68	1	1	1	1	17	32	1
VIM-3			26	29	1	70	3	3	3	1	19	32	3
VIM-4				4	26	61	25	25	24	25	9	18	25
VIM-5					29	65	28	28	27	27	13	20	28
VIM-6						70	3	3	3	1	19	18	25
VIM-7							69	69	68	69	64	65	69
VIM-8								1	2	2	18	33	2
VIM-9									2	2	18	33	2
VIM-10										2	18	32	2
VIM-11											18	33	2
VIM-12												23	18
VIM-13													33

VIM-5 differs in five amino acids from VIM-1 **(TABLE 3.)**. This allele was encountered in Turkey in *K. pneumoniae* and in *P. aeruginosa* isolates showing resistance not only to all β-lactams but also to aztreonam (Bahar et al. 2004). The VIM-6 enzyme was also detected in a *P. putida* isolate in Singapore. It differs from VIM-2 by a glutamine/arginine change at position 59 and by an asparagine/serine at position 165 and by one amino acid from VIM-3. Besides exhibiting MICs of >32 mg/L for imipenem and >256 mg/L for ceftazidime, the strain was also resistant to aztreonam (128 mg/L) (Koh et al. 2004).

VIM-7, has been isolated from a *P. aeruginosa* strain in Houston, Texas that was resistant to all β-lactams, including aztreonam, and to all other available antibiotics except polymyxin B. It forms a third subgroup among the VIM-type β - lactamases. The $bla_{VIM\text{-}7}$ gene was located on an approximately 24-kb plasmid and is likely to be integron-borne (Toleman et al. 2004).

Based on the phylogenetic tree (Zhao and Hu 2011) and considering the number of different amino acid residues, VIMs may be divided into three subgroups **(FIGURE 7.)**:

- Subgroup 1 involves VIM-1, -4, -5, -12, -13, -19, -25 and -26; they have 91.4–99.6% identity and 1–23 different amino acid residues.

- Subgroup 2 involves 15 members that are VIM-2, -3, -6, -8–11, -14–18, -20,-23 and -24, which share 97.4–99.6% identity and have 1–7 different amino acid residues.
- Subgroup 3 has only one member, VIM-7, which presents 72.9–77% similarity and 61–72 different amino acid residues after comparison with the other VIM variants.

There are no systematic data currently available on the relationship between the VIM amino acid sequences and their substrate specificities or affinities (Zhao and Hu 2011).

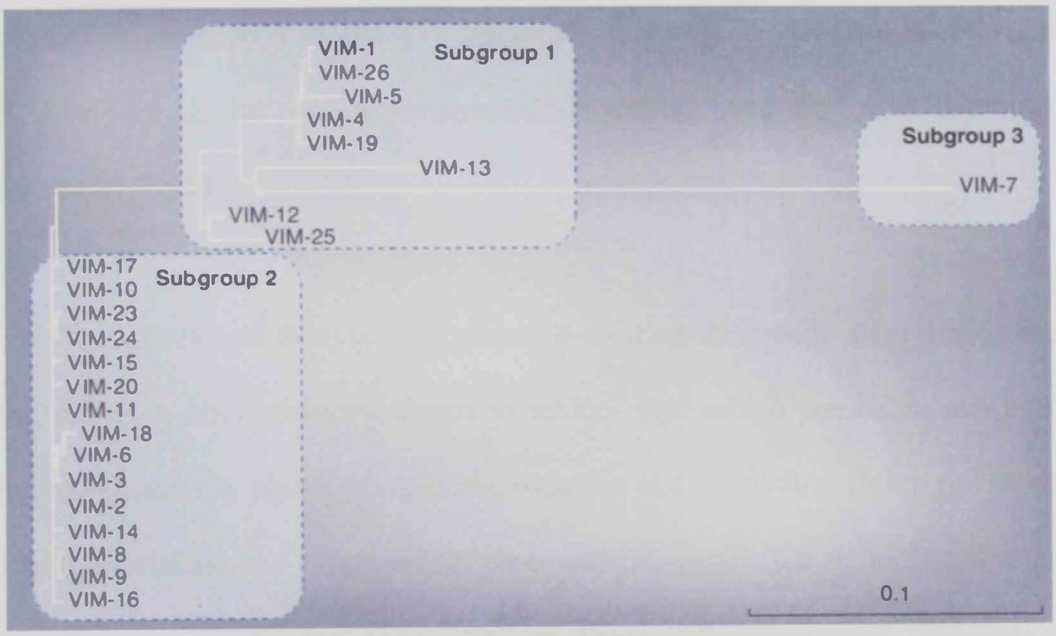

FIGURE 7. Phylogenetic tree of the VIM family based on the amino acid sequences (Zhao and Hu 2011)

I.6.2. Integrons Carrying bla_{VIM}

Most of the bla_{VIM} genes contained by class 1 integrons that are generally inserted into transposons localized on plasmids or in the chromosomes. However, there are exceptions, e.g. the *P. putida*-derived transposon, which carries bla_{VIM-2} (*tnpA*–*tnpR*–ISP*pu17*–*aacA4*–bla_{VIM-2}–aadA1–bla_{OXA-9}–tnpR–bla_{TEM-1}–ISP*pu18*) but this lacks the integron structure (Zhao and Hu 2011).

In the majority of cases, bla_{VIMs} are found on the integron with one or more aminoglycoside resistance genes, such as *aacA4, aacA7, aadA1, aadA2, aadB* and *aacC1*. Of these aminoglycosides resistance genes, *aacA4* is the most common, being present in some 50% of the 110 integron structures: it codes for acetyltransferase AAC(6′)-Ib C, i.e. an aminoglycoside-modifying enzyme gene (Ramirez and Tolmasky 2010).

Examples of β-lactamase genes co-existing frequently with bla_{VIM} includes the bla_{OXA-2}, conferring resistance to amino- and ureido-penicillin and important hydrolytic activity against cloxacillin, oxacillin and methicillin (Naas and Nordmann 1999). Obviously, the expression of all co-existing genes that are harbored usually by the same integron, including the resistance determinants to antiseptics and sulfonamide in the 3′-CS (*qacED1/sul1*), results in the emergence of multi-drug resistant bacteria.

I.6.3. The Spread of VIMs

To date, VIMs have been identified in at least 23 species of Gram-negative bacilli in more than 40 countries (Zhao and Hu 2011). Regarding VIM-4, it is found mainly in Europe and spreads fast in *Enterobacteriacae*, *P. aeruginosa* and other non-fermenters (Cornaglia et al. 2011). VIM-4 was reported from: *P. aeruginosa* isolates from Greece, Sweden, Poland, Australia, Hungary and Norway; *K. pneumonia* from Italy and Hungary; *Klebsiella oxytoca* from Hungary; *Enterobacter cloacae* from Greece and Italy; *Acinetobacter* spp. from Greece; *Pseudomonas putida* from Belgium and *Aeromonas hydrophila* from Hungary (Pournaras et al. 2002, Giske et al. 2003, Libisch et al. 2004, Patzer et al. 2004, Walsh et al. 2005, Peleg et al. 2006, Ikonomidis et al. 2007, Figueiredo et al. 2008, Kristof et al. 2010, Zhao and Hu 2011).

In the region of North Africa, VIM-4 producing *K. pneumoniae* have been discovered in Tunisia in strains also carrying CTX-M-15 ESBL, and CMY-4 AmpC enzyme. The $bla_{VIM\text{-}4}$ gene was a part of a class 1 integron (Ktari et al. 2006). Moreover, a study done at the University of Edinburgh has identified two isolates of *E. cloacae* (E1720 and E4293) from Egypt that carry the $bla_{VIM\text{-}4}$ and $bla_{CTX\text{-}M\text{-}14}$ genes on a same plasmid where $bla_{VIM\text{-}4}$ was found as a gene cassette in a class I integron (Dimude and Amyes 2013).

While NDM and OXA-48-like carbapenemases are increasingly common in the Arabian peninsula (Shibl et al. 2013, Sonnevend et al. 2013, Zowawi et al. 2013), at the time of initiating our research no VIM-producing *Enterobacteriaceae* have been reported with the exception of a conference abstract without any follow up data (Dashti A et al. 2011) . Therefore we initiated an active search for VIM-type enzymes among carbapenem resistant local isolates. The current thesis summarizes the results of these efforts.

II. AIMS AND OBJECTIVES

II.1. General Aim

The general aim of the study was to provide detailed insight into the molecular epidemiology of VIM-producing *Enterobacteriaceae* in the Arabian Peninsula.

II.2. Specific Objectives

- To establish whether VIM-producing *Enterobacteriaceae* are present in Abu Dhabi
- To characterize such isolate(s), to establish the allelic type of the enzyme produced and its genetic background
- To collect similar isolates from countries of the Arabian Peninsula
- To compare their
    - susceptibility patterns
    - type of VIM produced
    - its genetic background
    - plasmids carrying the bla_{VIM} genes
    - genes coding for resistance to drugs other than carbapenems
- To establish their clonal relationship

III. MATERIALS AND METHODS

III.1. Bacterial Strains

The subject of the first part of our study was an *Enterobacter cloacae* strain (ABC104) identified to carry bla_{VIM} from a collection of 34 carbapenem resistant *Enterobacteriaceae* submitted to our laboratory between 2009 and April 2012 from the United Arab Emirates to confirm the presence of any carbapenemase genes.

For the second part of the study, altogether 166 independent *Enterobacteriaceae* isolates isolated between 2009 and April, 2013 from Kuwait, (27 isolates: Prof. Vincent Rotimi and Dr. Wafa Jamal, Kuwait University, Kuwait), the Kingdom of Saudi Arabia (54 isolates: Prof. Atef Shibl, King Saud University, Riyad), from the Sultanate of Oman (63 isolates: Dr. Seif Al-Abri and Dr. Aminah Al-Jardani, Royal Hospital, Muscat), and further 22 isolates recovered between May 2012 and April, 2013 from the United Arab Emirates were examined. Isolates of the latter group were received mostly from different Abu Dhabi hospitals, while a few others were obtained from Sharjah and Dubai. All strains were chosen on the basis of showing decreased susceptibilities to any of the carbapenems tested by any of the methods used by the isolating laboratories according to the CLSI standards valid at the time of their isolation. Once received, their susceptibility was confirmed (*see later*) and only those 166 isolates, which exhibited decreased susceptibility to any carbapenems by the current CLSI standards (CLSI 2012) were included into the collection. Screening of these identified 11 strains as carrying bla_{VIM} (*see the description of the PCR reaction later*), which were the subject of our subsequent work. Strains were speciated by the providing laboratories. Once the susceptibilities were confirmed, strains were stored in 20% glycerol at -80 °C in duplicates, in two freezers connected to two independent electric circuits.

III.2. Antibiotic Susceptibility Testing

During the first study (i.e. the identification of the first VIM expressing strain in the Gulf region) the quantitative susceptibility of the isolates and their derivatives were established by E-test (BioMerieux) against imipenem, meropenem, ertapenem, ceftazidime, cefotaxim, cefepime, azrtreonam, cefoperazone, trimethoprim/ sulphamethoxazole, chloramphenicol, amikacin, gentamicin, tobramicin, netilmicin, ciprofloxacin, moxifloxacin, levofloxacin, tetracycline, minocycline and colistin. In all subsequent studies antibiotic sensitivity testing was routinely performed by the Kirby-Bauer disk diffusion method using ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, gentamicin, amikacin, tobramycin, chloramphenicol, tetracycline, and trimethoprim/sulphamethoxazole discs purchased from MAST Group Ltd. Quantitative susceptibilities to ceftazidim, cefotaxim, imipenem, ertapenem, meropenem and aztreonam were determined by the broth microdilution method.

All tests were carried out and evaluated by the CLSI standards (CLSI 2012). For all antibiotic sensitivity assays, *Escherichia coli* ATCC 25922 was used as a susceptible control. Since for tigecycline there are no current CLSI breakpoint values available, we used the ones recommended by EUCAST (www.eucast.org/clinical-breakpoints).

For the disc diffusion test and E-test, fresh cultures grown on Tryptic-soy agar (TSA) plates were suspended in sterile 1xPBS to 0.5 McFarland density and applied onto ready-made Mueller-Hinton agar (MHA) plates (Pangulf Lab Solution) with a swab. Antibiotic disks or E-tests were applied using a dispenser or sterile forceps.

For the microdilution assay, antibiotics were serially diluted in 100 μl volume in 96 well microplates (Nunc) and inoculated by a Multipoint inoculator (MAST) to give a final concentration of 5×10^5 colony forming units (CFU)/ml.

III.3. Phenotypic Assays to Assess the Presence of Various Beta-lactamases

The double disc synergy test was used to confirm ESBL production using a disc containing amoxicillin/clavulanic acid placed in the center of the inoculated plate and discs containing various cephalosporins and aztreonam placed around 20 mm apart from the central disc. The strains exhibiting the "keyhole" were considered as an ESBL producer (Sonnevend et al. 2013).

The general presence of carbapenemases were assessed by the modified Hodge test (CLSI 2012). Mueller Hinton agar plates (Pangulf Lab Solution) were inoculated with a 0.5 McFarland suspension of *E.coli* ATCC 25922. Meropenem, ertapenem or imipenem susceptibility disks were placed on the center of the plate. The test strain, a carbapenem susceptible negative control (E. coli K-12 J53) and KPC producing positive control (GR-KPC2) were streak-inoculated towards the disc. The plate was incubated overnight at 37^{o}C. After 16-24 hours the plate was examined for a cloverleaf-type indentation at the intersection of the test organism and the *E.coli* 25922.

The presence of specific carbapenemases was assessed based on inhibition by either phenylboronic acid (PBA) or by EDTA (Applichem). The former one inhibits Class A and C, while the latter one class B, i.e. MBL enzymes. The test was performed by inoculating Mueller–Hinton agar plates as described for the standard disc diffusion method and placing a disc of meropenem without any inhibitors, one with meropenem + 400 μg of PBA, and one with meropenem + 292 μg of EDTA. A minimum 5 mm increase in the diameter of the zone of inhibition in the presence of the inhibitor suggested the presence of the respective type of enzymes. In case PBA was found to be effective, the test was repeated in the presence of cloxacillin, as well. If it also showed efficacy, it suggested the involvement of AmpC type enzymes (Tsakris et al. 2010).

III.4. Extraction of Bacterial Genomic DNA for PCR

Three to 5 colonies of the bacterial strains grown overnight on TSA plates were picked by toothpick and suspended into two 200 μl of sterile distilled water in Eppendorf tubes. The tubes were incubated for 10 minutes at 99°C in a thermo-block (Eppendorf). They were centrifuged for 10 minutes at 14,800 rpm; the supernatant of both tubes was collected without touching the pellet, combined in a new autoclaved Eppendorf tube and kept at 4°C. This material was used for most experiments as the DNA target. Whenever doubts were raised on the quality of DNA material (e.g. fuzzy amplicons, unexpected results), DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manusfacturer's instruction.

III.5. Genotyping by PCR

Before submitting DNA samples to further testing, a 16S PCR reaction was carried out to establish the presence of bacterial DNA (Louie et al. 2002). dNTP's used were from Applied Biosystem, while other reagents: 10X buffer with loading dye, $MgCl_2$ buffer Q and Taq polymerase were purchased from Qiagen. The Ultra-pure distilled water was DNAse, RNAse free and was obtained from Gibco. The reactions were performed on Applied Biosystems 2700 and 2720 thermocyclers. In all reactions *E. coli* $J53_{RAZ}$ genomic DNA and ultra-pure distilled water were used as negative controls. All positive reactions first obtained were confirmed by direct sequencing (*see later*).

III.5.1. PCR Detection of Various Beta-Lactamase Genes

The targeted genes, the primers and parameters used in these PCR reactions with respective references are listed in (**TABLE 4.**). For AmpC genes detection a multiplex PCR (Perez-Perez and Hanson 2002) was used with six sets of ampC-specific primers given in (**TABLE 5.**). In case of obtaining a CIT-like bla_{AmpC} amplicon within the AmpC gene-targeting PCR reaction the entire gene was amplified using flanking forward primer5'-AACACACTGATTGCGTCTGAC-3' and reverse primers, 5'-CTGGGCCTCATCGTCAGTTA-3' (Perez-Perez and Hanson 2002). All amplicons of the above PCR reactions were purified with a gel purification Kit (Promega) as directed by the manufacturer and sequenced directly (*see later*).

III.5.2. Detection of Plasmid Mediated Quinolone Resistance and Ribosomal Methyltransferases Genes by PCR

Plasmid mediated target protection *qnr* genes were detected by a multiplex PCR (Cattoir et al. 2007). The two other plasmid mediated quinolone resistance genes *qepA* and *aac-6'-Ib-cr* were detected by simplex PCR methods published earlier (Poirel et al., 2011) with primers listed in (**TABLE 6.**). The primers and parameters to detect *armA, rmtA, rmtB, rmtC, and rmtD* and *npmA* are presented in (**TABLE 7.**) (Fritsche et al. 2008).

TABLE 4. Primers and conditions used to detect various beta-lactamase genes

Gene	Primer	Sequence (5'-3')	Initial denaturation	Cycle	Final extension	Amplicon size	Reference
bla$_{TEM}$	C	TCG GGG AAA TGT GCG CG	5'at 94°C	30 X (30" at 94°C- 30" at 60°C and 60" at 72°C)	10' at 72°C	971bp	(Cao et al. 2002)
	D	TGC TTA ATC AGT GAG GCA CC					
bla$_{SHV}$	OS-5	TTA TCT CCC TGT TAG CCA CC	5' at 94°C	30X(45" at 94°C, 55" at 60°C and 60" at 72°C)	5'at 72°C	798bp	(Cao et al. 2002)
	OS-6	GAT TTG CTG ATT TCG CTC GG					
bla$_{PER}$	PER-A	ATG AAT GTC ATT ATA AAA GC	5'at 94°C	35X (45s at 94°C, 60" at 52°C and 60" at 72°C)	10'at 72°C	925bp	(Cao et al. 2002)
	PER-B	AAT TTG GGC TTA GGG CAG AA					
bla$_{CTX-M}$	MA-1	SCS ATG TGC AGY ACC AGT AA	5' at 94°C	30X (30" at 94°C, 30" at 55°C and 60" at 72°C)	10' at 72°C	543bp	(Cao et al. 2002)
	MA-2	CCG CRA TAT GRT TGG TGG TG					
bla$_{OXA-48-like}$	OXA-F	GCGTGGTTAAGGATGAACAC	5' at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	438bp	(Poirel et al. 2011a)
	OXA-R	CATCAAGTTCAACCCAACCG					
bla$_{KPC}$	KPC-F	CGTCTAGTTCTGCTGTCTTG	5' at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	798bp	(Poirel et al. 2011a)
	KPC-R	CTTGTCATCCTTGTTAGGCG					
bla$_{VIM}$	VIM-F	GATGGTGTTTGGTCGCATA	5'at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	390bp	(Poirel et al. 2011a)
	VIM-R	CGAATGCGCAGCACCAG					
bla$_{IMP}$	IMP-F	GGAATAGAGTGGCTTAAYTCTC	5' at 94°C	40X (30"at 94°C, 40" at 52°C, 50" at 72°C)	10'at 72°C	232bp	(Poirel et al. 2011a)
	IMP-R	GGTTTAAYAAAACAACCACC					
bla$_{NDM-1}$	NDM1-Fo	TGCCGAGCGACTTGGCCTTG	5' at 94°C	30X (30"at 94°C, 30" at 60°C, 60" at 72°C)	7' at 72°C.	379bp	(Ghazawi et al. 2012)
	NDM1-Re	ACCGATGACCAGACCGCCCA					

TABLE 5. Primers and conditions used to detect AmpC β-lactamases genes (Perez-Perez and Hanson 2002)

Gene(s)	Primer	Sequence (5' – 3')	Initial denaturation	Cycle	Final extension	Amplicon size
MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	5' at 94°C	30 X (30" at 94°C, 30" at 64°C and 60" at 72°C)	10' at 72°C	520 bp
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C				
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA				462 bp
	CITMR	TTT CTC CTG AAC GTG GCT GGC				
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T				405bp
	DHAMR	CCG TAC GCA TAC TGG CTT TGC				
ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA				346bp
	ACCMR	TTC GCC GCA ATC ATC CCT AGC				
MIR-1, ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG				302bp
	EBCMR	CTT CCA CTG CGG CTG CCA GTT				
FOX-1 to FOX-5b	FOXMF	AAC ATG GGG TAT CAG GGA GAT G				190bp
	FOXMR	CAA AGC GCG TAA CCG GAT TGG				

TAB LE 6. Primers used to detect plasmid mediated quinolone resistance genes (Cattoir et al. 2007) (Fritsche et al. 2008)

Genes	Primer	Sequence (5'-3')	Product size	Initial Denaturation	Cycle	Extension
qnrA	QnrAm-F QnrAm-R	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	580bp	10' at 94°C	35 X (1' at 94°C, 1' at 54°C and 1' at 72°C)	10' at 72°C
qnrB	QnrBm-F QnrBm-R	GGMATHGAAATTCGCCACTG[c] TTTGCYGYYCGCCAGTCGAA[c]	264bp			
qnrS	QnrSm-F QnrSm-R	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	428bp			
qepA	QepA-F QepA-R	CTGCAGGTACTGCGTCATG CGTGTTGCTGGAGTTCTTC	403bp			
aac-6'-Ib-cr	AAC6'-Ib-cr1 AAC6'-Ib-cr2	TTGCAATGCTGAATGGAGAG CGTTTGGATCTTGGTGACCT	218bp			

[C] M= A or C;H = A or C or T; Y =C or T

TABLE 7. PCR primer sets utilized in the detection of aminoglycoside methyltransferase resistance genes (Fritsche et al. 2008).

Target	Primers	Sequences (5'-3')	Product size	Initial denaturation	Cycle	Extension
armA	armA-f armA-r	TATGGGGGTCTTACTATTCTGCCTAT TCTTCCATTCCCTTCTCCTTT	514 bp	5' at 94 °C	40 X (15" at 94°C, 30" at 58°C, and 1' at 72°C)	10' at 72°C
rmtA	rmtA-f rmtA-r	CTAGCGTCCATCCTTTCCTC TTTGCTTCCATGCCCTTGCC	635 bp			
rmtB	rmtB-f rmtB-r	TCAACGATGCCCTCACCTC GCAGGGCAAAGGTAAAATCC	459 bp			
rmtC	rmtC-f rmtC-r	GCCAAAGTACTCACAAGTGG CTCAGATCTGACCCAACAAG	752 bp			
rmtD	rmtD-f rmtD-r	CTGTTTGAAGCCAGCGGAACGC GCGCCTCCATCCATTCGGAATAG	376 bp			
npmA	npmA-f npmA-r	CTCAAAGGAACAAAGACGG GAAACATGGCCAGAAACTC	641bp			

III.6. Detection of Plasmids

The alkaline lysis method was used to determine the plasmid profile of the isolates using the method of Kado and Liu with slight modifications (Kado and Liu 1981). The strains were inoculated on half TSA plates to obtain confluent growth and incubated overnight at 37°C. Next day, cells were collected with toothpicks from the plate, suspended gently in 250 µl of lysing solution (3% SDS, 50 mM Tris, pH 12.56) to get a turbid suspension and mixed by gentle agitation until the preparation became homogenous and viscous. To complete cell lysis, the suspensions were incubated at 60°C in a thermo-block for 45 minutes while gently mixed manually every 15 minutes. 250 µl of phenol-chloroform (1:1) was added and by gentle shaking the mixture was liquefied. Centrifugation at 13,000 rpm for 15 minutes separated the layers. The top aqueous layer (approximately 60 µl) was transferred to clean tubes containing the loading dye without disrupting the precipitate at the interface (i.e. without touching the floating pellet). Samples were subjected to electrophoresis for 7 hours at 120 V in 0.8% agarose gel (Sigma) and 1X TBE. The gel was stained with ethidium bromide (EtBr) for 20 minutes, followed by de-staining for 10 minutes in 400 ml of MilliQ water with gentle shaking. Bands were detected in a Biometra UV transilluminator. Plasmids in 39R861 (154kb, 66.2kb, 37.6kb and 7.4kb) were used as plasmids molecular size controls, and *E. coli* $J53_{RAZ}$, i.e. a strain containing no plasmids, served to aid the identification of the chromosomal band.

III.7. Detection of Megaplasmids by S1 Nuclease Digestion

This method is used to detect very large plasmids (Barton et al. 1995, Basta et al. 2004). Strains were grown overnight in 15 ml TSB with or without 8µg/ ml ceftazidime at 37° C. Next day, approximately 4 x10^9 cells were collected by centrifugation. The cells were washed and re-suspended in 500 µl of EC buffer (10 mM Tris, 0.1 M EDTA, 1 M NaCl pH8.0). To 5 ml of melted plug agarose (Sigma), 10µl of RNase (10µg/ µl) (Qiagen) was added. 500µl of the melted agarose was mixed with 500µl of the bacterial suspension and immediately transferred to 1 ml syringes. After solidifying, the plugs were cut into 1 mm thick slices. The agarose plugs were incubated in 1 ml of EC buffer containing 20 µg/ ml RNase and 1 mg/ml lysozyme to lyse the cells within. Subsequently, the plugs were incubated at 50°C overnight in ES buffer (0.5M EDTA:1% N-laurylsarcosine) buffer supplemented with 1mg/ml proteinase K (Invitrogen). The ES buffer and the proteinase were inactivated by washing the plugs in 1ml of 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma) prepared in TE buffer. In order to linearize the circular megaplasmids, the plugs were washed twice in 1 mL 10mM Tris pH8.0 for 15 min at room temperature followed by digestion with 1 Unit of S1 nuclease in 200 µl of S1 buffer for 10 min at 37°C. S1 nuclease mixture was removed and replaced with cold ES buffer and the tubes were incubated on ice for 15 min. The plugs were loaded into wells of 1% horizontal agarose gels prepared in (0.5XTBE). Lambda Ladder PFG Marker (New England Biolabs) was used as size marker. The gel loaded was placed into the electrophoresis chamber of CHEF Mapper (Biorad). Electrophoretic conditions were the following:

6V/cm current with 18 hours run time and 120° angle with 5 sec initial to 25 sec final switch time with linear ramp.

III.8. Plasmid Replicon Typing

Plasmids are classified based on their incompatibility (Inc) groups (Datta and Hedges 1971, Novick 1987). Plasmids with the same replication control genes are incompatible, i.e. cannot co-exsist in the same cell (Datta and Hughes 1983, Couturier et al. 1988). Traditionally, incompatibility groups were established on basis of the incapacity of two plasmids (one of a known Inc group) to coexist within the same host cell (Anderson et al. 1977). Lately, it has been done by determining the sequence of the plasmids' replicons, initially by hybridization with a set of probes (Couturier et al. 1988) and today mostly by PCR (Gotz et al. 1996).

Template DNA was prepared from bacterial collections using a boiling lysis procedure, as previously described. We targeted 18 different replicons, i.e. FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P,W, T, A/C, K, B/O, X, Y, F, and FIIA, respectively, by PCR assigned into 5 multiplex, and 3 simplex reactions (Carattoli et al. 2005). The primers and the parameters used are shown in (**TABLE 8.** - *see page 58*).

In addition to the original typing set, the recently identified IncHI1B replicon type was sought by simplex PCR reaction (Dortet et al. 2012). Primers and conditions used are cited in the (**TABLE 8.**).

Four simplex PCR reactions were performed to screen for the IncX plasmid subgroups (Johnson et al. 2012). Primers and conditions used are cited in (**TABLE 8**). PCR was performed using the same conditions for all simplex PCRs.

III.9. Conjugation

For conjugation experiments, $J53_{RAZ}$ (Na-azid resistant in-house derivative of the rifampin resistant J53 *E. coli* K-12) were used as recipients. Conjugation was attempted by combining cultures of the donor and recipient in 1:5 ratios, for 4 hours at 37^0C without shaking, and then it was centrifuged at 3500 rpm for 15 minutes. The supernatant was removed and the pellet was re-suspended in 200 µl TSB and 100 µl of the suspension was added as a drop to the center of a TSA plate without any antibiotics (2 plates were prepared). Next day, the growth of both plates was collected in PBS. After centrifugation at 3,500 rpm for15 min, the pellet was washed and re-suspended in 3 ml of PBS. Serial dilutions of -1, -2, -3 were prepared and aliquots of 200µl were plated onto plates containing 100 µg/ml Na-azid and 8 mg/L ceftazidime. Next day, colonies were collected and subjected to repeated antibiotic susceptibility tests, plasmid electrophoresis, PCR and in case of necessity, to ERIC PCR and PFGE to ensure that transconjugants and not mutants of the donor or recipient were obtained. If the conjugation failed at 37°C, the experiment was repeated using 30°C incubation temperature.

TABLE 8. Primers used for Plasmids Replicon Typing

Primers	DNA sequence (5'-3')	PCR product size	Initial denaturation	Cycle	Final extension
Multiplex PCR to recognize IncHI1, IncHI2 and IncI1/Iγ (Carattoli et al. 2005)					
HI1 FW HI1 RV	GGAGCGATGGATTACTTCAGTAC TGCCGTTTCACCTCGTGAGTA	471 bp	5' at 94°C	30 X (60" at 94°C, 30" at 60°C and 60" at 72°C)	5' at 72°C
HI2 FW HI2 RV	TTTCTCCTGAGTCACCTGTTAACAC GGCTCACTACCGTTGTCATCCT	644 bp			
I1 FW I1 RV	CGAAAGCCGGACGGCAGAA TCGTCGTTCCGCCAAGTTCGT	139 bp			
Multiplex PCR to recognize X, L/M and N replicons (Carattoli et al. 2005)					
X FW X RV	AACCTTAGAGGCTATTTAAGTTGCTGAT TGAGAGTCAATTTTTATCTCATGTTTTAGC	376 bp	5' at 94°C	30 X (60" at 94°C, 30" at 60°C and 60" at 72°C)	5' at 72°C
L/M FW L/M RV	GGATGAAAACTATCAGCATCTGAAG CTGCAGGGGCGATTCTTTAGG	785 bp			
N FW NRV	GTCTAACGAGCTTACCGAAG GTTTCAACTCTGCCAAGTTC	559 bp			
Multiplex PCR to recognize FIA, FIB and W replicons (Carattoli et al. 2005)					
FIA FW FIA RV	CCATGCTGGTTCTAGAGAAGGTG GTATATCCTTACTGGCTTCCGCAG	462 bp	5' at 94°C	30 X (60" at 94°C, 30" at 60°C and 60" at 72°C)	5' at 72°C
FIB FW FIB RV	GGAGTTCTGACACACGATTTTCTG CTCCCGTCGCTTCAGGGCATT	702 bp			
W FW W RV	CCTAAGAACAACAAAGCCCCCG GGTGCGCGGCATAGAACCGT	242 bp			
Multiplex PCR to recognize Y, P and FIC replicons (Carattoli et al. 2005)					
YFW YRV	AATTCAAACAACACTGTGCAGCCTG GCGAGAATGGACGATTACAAAACTTT	765 bp	5' at 94°C	30 X (60" at 94°C, 30" at 60°C and 60" at 72°C)	5' at 72°C
PFW PRV	CTATGGCCCTGCAAACGCGCCAGAAA TCACGCGCCAGGGCGCAGCC	534 bp			
FIC FW FIC RV	GTGAACTGGCAGATGAGGAAGG TTCTCCTCGTCGCCAAACTAGAT	262 bp			

TABLE 8. - *cont.*

Primers	DNA sequence (5'-3')	PCR product size	Initial denaturation	Cycle	Final extension
Multiplex PCR to recognize A/C, T and FIIA replicons.(Carattoli et al. 2005)					
A/C FW A/C RV	GAGAACCAAAGACAAAGACCTGGA ACGACAAACCTGAATTGCCTCCTT	465 bp	5' at 94°C	30 X (60" at 94°C, 30" at 60°C and 60" at 72°C)	5' at 72°C
TFW TRV	TTGGCCTGTTTGTGCCTAAACCAT CGTTGATTACACTTAGCTTTGGAC	750 bp			
FIIS FW FIIS RV	CTGTCGTAAGCTGATGGC CTCTGCCACAAACTTCAGC	270 bp			
Three Simplex PCRs to recognize F, K and B replicons (Carattoli et al. 2005)					
F_{repB} FW F_{repB} RV	TGATCGTTTAAGGAATTTTG GAAGATCAGTCACACCATCC	270 bp	5' at 94°C	30 X (60" at 94°C, 30" at 52°C and 60" at 72°C)	5' at 72°C
K/B FW K RV	GCGGTCCGGAAAGCCAGAAAAC TCTTTCACGAGCCCGCCAAA	160 bp	5' at 94°C	30 X (60" at 94°C, 30" at 60°C and 60" at 72°C)	5' at 72°C
K/B FW B/O RV	GCGGTCCGGAAAGCCAGAAAAC TCTGCGTTCCGCCAAGTTCGA	159 bp	5' at 94°C	30 X (60" at 94°C, 30" at 60°C and 60" at 72°C)	5' at 72°C
Simplex PCR for IncHI1B plasmid (Dortet et al. 2012)					
IncHI1B-Fw IncHI1B-Rv	CAA AAC AGA GAG TAT TCA ACC C CTG ATT CTT TTC GAG ACA GGG	600 bp	5' at 94°C	30 X (60" at 94°C, 30" at 52°C and 60" at 72°C)	5' at 72°C
Four Simplex PCRs for IncX plasmids subgroups IncX1, IncX2, IncX3 and IncX4 (Johnson et al. 2012)					
X1 X1	GCTTAGACTTTGTTTTATCGTT TAATGATCCTCAGCATGTGAT	461 bp	5' at 95°C	30 X (60" at 95°C, 30" at 52°C and 60" at 72°C)	5' at 72°C
X2 X2	GCGAAGAAATCAAAGAAGCTA TGTTGAATGCCGTTCTTGTCCAG	678 bp			
X3 X3	GTTTTCTCCACGCCCTTGTTCA CTTTGTGCTTGGCTATCATAA	351 bp			
X4 X4	AGCAAACAGGGAAAGGAGAAGACT TACCCCAAATCGTAACCTG	569 bp			

III.10. Transformation

If we were unable to mobilize the bla_{VIM} -carrying plasmid into an *E. coli* K-12 recipient transformation of the plasmid was attempted. For these experiments *E. coli* DH5α was used as a recipient. Plasmid DNA was purified by the method of Kado and Liu (Kado and Liu 1981). The procedure described above for plasmid detection was continued by removal of traces of phenol from the aqueous preparations normally used for electrophoretic detection of the plasmid by mixing the solution with an equal volume of chloroform, and centrifuged at 15.000 rpm for 5 min. The aqueous layer was collected into a new tube, 1:10 volume of sodium acetate (pH 5.2) was added followed by 3 volumes of 99% ethanol. The preparation was incubated at -80°C for at least 30 min and centrifuged at 15000 rpm for 30min at 4°C. The pellet was washed with 1ml of 70% ethanol and centrifuged at 15000 rpm for 10 min. After air-drying, the pellet was resuspended in TE buffer (10mM Tris:1mM EDTA pH8 (Sigma)). For re-purification, the same steps were followed without adding chloroform. Five µg of DNA, as measured on a ND-1000 spectrophotometer (Nano Drop Technologies, USA) were used for every transformation.

Bullets of 150 µL competent *E. coli* DH5α cells were used for heat shock transformation. Cells were made competent by calcium chloride method. *E. coli* DH5α was grown at 37°C in shaken culture in Luria-Bertani broth till OD_{600} reached 0.5-0.7, centrifuged at 5000rpm for 10 min at 4°C, resuspended in cold 50mM $CaCl_2$ on ice, centrifuged at 5000rpm for 10 min at 4°C, resuspended again in cold 50mM

$CaCl_2$ on ice, left on ice for 20 min, centrifuged at 5000rpm for 10 min at 4°C, reusupended in 50mM $CaCl_2$ containing 20% glycerol, aliquoted in 150µl and snap freezed in liquid N. Aliquots were stored at -80°C. Heat shock transformation was accomplished by putting the mixture (purified DNA and competent cells) on ice for two minutes; then a thermo-block was used for heat shock at 42°C for 5 minutes and finally the preparation was left on ice for two minutes. The transformation mixture was incubated either for 1h or overnight at 37 °C with shaking; then it was plated onto plates containing 8 µg/mL ceftazidime. The type of plasmid content of transformants was confirmed by electrophoresis, susceptibility testing and PCR.

III.11. Hybridization

Plasmid gels were depurinated in 0.25 M HCl, followed by denaturation in 0.5 M NaOH, 1 M NaCl and finally neutralized in 1 M Tris, 0.6 M NaCl. All treatment steps were 15 minutes long at room temperature with gentle shaking and were repeated twice. Between each step gels were rinsed in sterile distilled water. The gel was capillary-transferred to Hybond N+ membranes (Roche) by soaking overnight in 20X SSC (Saline Sodium citrate). Next day the membranes were UV cross-linked at 70,000 micro-joules.

Hybridization probes were generated by PCR amplification of bla_{VIM-4}, IncA/C, IncN, bla_{TEM-1}, bla_{CMY-4}, $bla_{CTX-M-15}$ of genomic DNA of control strains, followed by DNA purification (Promega Kit), and quantitation using the ND-1000 spectrophotometer (Nano Drop Technologies, USA). The DNA fragments were labeled using the DIG DNA labeling kit (Roche). In brief, 200 ng of purified fragment was boiled for 10 minutes in a boiling water bath and quickly chilled on ice. The denatured DNA was conjugated with digoxigenin according to the manufacturer's instructions. Membranes were pre-hybridized at optimal hybridization temperature, which was calculated based on the size of the probe and GC ratio, using the formula recommended by the manufacturer.($T_{opt.}=T_m-20°C$ with $T_m=49.82+0.41(\%G+C)-600/L$ (L=length of the probe in base pairs)) in a pre-warmed hybridization buffer (5X SSC 1% blocking solution, 0.1 % N-lauryl sarcosine, 0.02% SDS) for 30 minutes followed by the addition of the hybridization buffer containing the probe. According to the previous calculations the membranes were hybridized overnight with gentle shaking at 69°C for VIM, 70°C for IncA/C, 68°C for IncN, 71°C for blaCMY-4, 72°C for CTX-M-15, and 69°C for TEM-1.

For post hybridization, the blots were washed twice with 2X SSC / 0.1% SDS (Sigma) at room temperature (RT) for 5 minutes with gentle shaking followed by two subsequent washings in 0.1X SSC / 0.1 % SDS at 68°C for 15 minutes also with constant agitation. The membrane was briefly rinsed in washing buffer at room temperature, incubated in blocking solution for 30 minutes, and again incubated in antibody solution provided with the kit for 30 minutes. The blots were washed twice in washing buffer, equilibrated in detection buffer and finally incubated without shaking in the color substrate solution for various lengths of time in the dark till the desired spot or band intensity was seen. The reaction was stopped using TE buffer

(10mM Tris:1mM EDTA pH8 (Sigma)). The blot was digitized using the Biometra gel documentation system. In order to re-probe the membrane, it was stripped with dimethyl formamide at 56°C followed by steps starting from the pre-hybridization.

III.12. Macrorestriction of the Chromosome Followed by Pulsed Field Gel Electrophoresis (PFGE)

In this technique a restriction endonuclease with rare cutting sites (*XbaI*) was applied to digest the entire bacterial chromosome embedded into agarose gel plugs to protect DNA from mechanical fragmentation. The very large DNA fragments generated by enzymatic digestion were electrophoretically separated in an electrical field with changing vector of the current (Gautom 1997). The pattern of the fragments provided the basis of comparison.

Bacterial strains grown on TSA plates were suspended in 2ml of cell suspension buffer (100mM Tris:100mM EDTA, pH8.0) up to a density of 3 McFarland unit. Suspensions were kept on ice. Simultaneously, 1% plug agarose (Sigma) was melted in 1% SDS in TE buffer (10mM Tris:1mM EDTA pH8 .0) and kept at 54°C. 500 µl bacterial suspensions, 25 µl of proteinase K (Invitrogen) (20 mg/ml) and 525 µl of 1% plug agarose were combined, mixed carefully, quickly transferred into 1 ml syringes and kept for 15-30 minutes at room temperature to allow the solidification of the agarose. Aliquots of 5 ml cell lysis buffer (50mM Tris:50mM EDTA pH8.0, 1% Sarkosyl) and 25 µl proteinase K 20 mg/mL were distributed into 50 ml tubes and

1mm thick slices of agarose plugs were directly cut into them. They were incubated for 2 hours at 50°C in a shaker water bath (200 rpm). Subsequently, the plugs were washed twice with 10 ml of preheated sterile MilliQ water for 20 minutes in a 50°C shaker water bath. Plugs were washed four times for 20 minutes with 10 ml of preheated TE buffer. Finally, plugs were stored in 5 ml of fresh TE buffer at 4°C.

Genomic DNA within the plugs were digested overnight at 37°C in a 100 µl restriction mixture made of 10 µl of NE buffer 4 (New England Biolabs), 1 µl of BSA (New England Biolabs), 30 U (1.5 µl) of *XbaI* enzyme (New England Biolabs) and 87.5 µl of sterile distilled water. Following digestion, the restriction mixtures were removed and the plugs were incubated in 250 µl of 0.5X TBE buffer for 30 minutes at room temperature. Subsequently, plugs were inserted into wells of 1.4% of agarose gel (Pulse Field Running Agarose A2929, Sigma) prepared in 0.5xTBE buffer. The two wells at the two sides of each gels contained a lambda-ladder PFGE marker (New England Biolabs) for standardization. Gels were run in CHEF Mapper (Biorad) electrophoresis chamber in 0.5X TBE buffer pre-chilled to 14°C. The running program consisted of 26 hours run at 6 V/cm with 120° angle and an initial switch time of 2.2 seconds and a final switch time of 54.2 seconds with linear ramp.

The gels were stained with ethidium bromide for 20 minutes, followed by de-staining in MilliQ water. Bands were detected and photographed under UV light in a Biometra gel documentation system. Gel pictures were stored as .tif files for further analysis. The GelCompare II software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze the banding patterns. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree graphically showing the level of relatedness between the isolates was created based on the Dice similarity coefficient (SD) (Dice, with a 1.5% position tolerance). Strains showing patterns with SD ≥ 80% were arbitrarily considered to represent a pulsotype.

III.13. Multilocus Sequence Typing of Selected Strains (MLST)

All strains were subject to a species-specific MLST protocol using their genomic DNA. The primers used are listed in (**(TABLE 9.)** - *see page 67*). PCR product was purified according to the manufacturer's instruction with PCR and gel purification kit (Promega) and both strands were directly sequenced using a 3130X genetic analyzer (Applied Biosystems). The sequence results were analyzed using MEGA5 program (Tamura et al. 2011).

For *E. coli* the MLST protocol of Wirth *et al.* was used (Wirth et al. 2006). The isolate was then assigned to sequence types using the tools on the *E. coli* MLST webpage (http://mlst.ucc.ie/mlst/dbs/Ecoli). *E. cloaceae* was subjected to the MLST protocol of (Miyoshi-Akiyama et al. 2013). Sequence types were determined according to the *E. cloaceae* MLST webpage (http://pubmlst.org/ecloacae/). For *K. peumoniae* the protocol of (Diancourt et al. 2005) was applied and *K. pneumoniae* MLST webpage (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) was used to establish the sequence types.

III.14. ERIC PCR

The Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) (Versalovic et al. 1991) method was used to rapidly confirm differences between donor, recipient for conjugation and or transformation. It was performed by using the ERIC2 primer (5'-AAG TAA GTG ACT GGG GTG AGC G-3'). A 7-minutes initial denaturation at 94°C was followed by 30 cycles of denaturation for 30seconds at 90°C, annealing for 60seconds at 50°C and extension for 8 minutes at 65°C. The final step was an extension for 16 minutes at 65°C. The patterns obtained with different strains (strictly amplified in the very same experiment only) were compared visually.

TABLE 9. Primers and conditions used for *E.coli*, *E.cloacae* and *K.pneumoniae* MLST

Gene targeted	Primers	Sequences (5'- 3')	PCR product size	Initial denaturation	Cycle	Final extension
***E.coli* MLST(Wirth et al. 2006)**						
adk	adk F	ATTCTGCTTGGCGCTCCGGG	583 bp	5 minutes at 94°C	35X (60" at 94°C, 60" at 56°C and 60" at 72°C)	10'at 72°C
	adk R	CCGTCAACTTTCGCGTATTT				
fumC	fumC F	TCACAGGTCGCCAGCGCTTC	806 bp			
	fumC R	GTACGCAGCGAAAAAGATTC				
gyrB	gyrB F	TCGGCGACACGGATGACGGC	911 bp			
	gyrB R	ATCAGGCCTTCACGCGCATC				
icd	icd F	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	878 bp			
	icd R	GGACGCAGCAGGATCTGTT				
mdh	mdh F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	932 bp			
	mdh R	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT				
purA	purA F	CGCGCTGATGAAAGAGATGA	816 bp			
	purA R	CATACGGTAAGCCACGCAGA				
recA	recA F	CGCATTCGCTTTACCCTGACC	780 bp			
	recA R	TCGTCGAAATCTACGGACCGGA				
***E.cloaceae* MLST (Miyoshi-Akiyama et al, 2013)**						
dnaA	dnaA-f2	AYAACCCGCTGTTCCTBTATGGCGGCAC	1151 bp	2' at 95°C	35X (15" at 95°C, 10" at 50°C and 60" at 72°C)	7' at 72°C
	dnaA-r	KGCCAGCGCCATCGCCATCTGACGCGG				
fusA	fusA-f2	TCGCGTTCGTTAACAAAATGGACCGTAT	906 bp			
	fusA-r2	TCGCCAGACGGCCCAGAGCCAGACCCAT				
gyrB	gyrB-F	TCGACGAAGCGCTCGCGGGTCACTGTAA	1153 bp			
	gyrB-R	GCAGAACCGCCCGCGGAGTCCCCTTCCA				
leuS	leuS-f2	GATCARCTSCCGGTKATCCTGCCGGAAG	845 bp			
	leuS-r	ATAGCCGCAATTGCGGTATTGAAGGTCT				
pyrG	pyrG-f	AYCCBGAYGTBATTGCRCAYMAGGCGAT	535 bp			
	pyrG-r	GCRCGRATYTCVCCCTSHTCGTCCCAGC				
rplB	rplB-f	GTAAACCGACATCTCCGGGTCGTCGCCA	746 bp			
	rplB-r	ACCTTTGGTCTGAACGCCCCACGGAGTT				
rpoB	rpoB-f	CCGAACCGTTCCGCGAACATCGCGCTGG	944 bp			
	rpoB-r2	CCAGCAGATCCAGGCTCAGCTCCATGTT				

TABLE 9. - *cont.*

Gene targeted	Primers	Sequences (5'- 3')	PCR product size	Initial denaturation	Cycle	Final extension
K. pneumoniae MLST (Diancourt et al. 2005)						
rpoB	VIC3	GGC GAA ATG GCW GAG AAC CA	501 bp	5' at 94°C	35X (60" at 94°C, 60" at 50°C and 60" at 72°C)	10' at 72°C
	VIC2	GAG TCT TCG AAG TTG TAA CC				
gapA	gapA173	TGA AAT ATG ACT CCA CTC ACG G	450 bp			
	gapA181	CTT CAG AAG CGG CTT TGA TGG CTT				
mdh	mdh130	CCC AAC TCG CTT CAG GTT CAG	477 bp			
	mdh867	CCG TTT TTC CCC AGC AGC AG				
pgi	pgi1F	GAG AAA AAC CTG CCT GTA CTG CTG GC	432 bp			
	pgi1R	CGC GCC ACG CTT TAT AGC GGT TAA T				
phoE	phoE604.1	ACC TAC CGC AAC ACC GAC TTC TTC GG	420 bp			
	phoE604.2	TGA TCA GAA CTG GTA GGT GAT				
infB	infB1F	CTC GCT GCT GGA CTA TAT TCG	318 bp			
	infB1R	CGC TTT CAG CTC AAG AAC TTC				
tonB	tonB1F	CTT TAT ACC TCG GTA CAT CAG GTT	414 bp			
	tonB2R	ATT CGC CGG CTG RGC RGA GAG				

III.15. Characterization of the Genetic Environment of bla_{VIM-4}

The genetic support of bla_{VIM-4} was determined by PCR mapping and sequencing using primers designed based on the genetic surrounding of bla_{VIM} published earlier (GenBank accession numbers AJ704863 and AY339625). (Miriagou et al. 2004, Colinon et al. 2007). All primers used for sequencing were designed with the help of Clone Manager v9.2 (Sci-Ed Software, Cary, NC, US). Primers amplified overlapping fragments of the molecular structure surrounding bla_{VIM}, which were sequenced with the amplification primers and with walking primers annealing approximately 500 bp apart. All PCR reactions were performed using 35 cycles of 30' 94 °C denaturation followed by 55 °C annealing for 1 min and extension at 72 °C for varying length of time (1'-3') depending on the size of the expected products. PCR and sequencing primers are listed in (**TABLE 10.**). Sequencing of amplicons was performed with the Big Dye Cycle Terminator V.3.1 (Applied Biosystems) using the 3130X Genetic Analyzer (Applied Biosystems). After checking the quality of the sequencing trace files with MEGA5.0, the sequence fragments were aligned and the complete genetic surrounding was constructed using the Clone Manager v9.2 (Sci-Ed Software, Cary, NC, US). Annotation and GenBank deposition of the sequences was done using the Sequin software available from the GenBank.

TABLE 10. Primers used in sequencing the molecular structures carrying the *bla*$_{VIM}$ gene

Primer name	5'-3' sequence	Annealing to AJ704863	Size of products * (bp)	Comment
AS_Classlint_L	TGT CGT TTT CAG AAG ACG GCT GC	5250	434	For amplification and sequencing the 5' end of Class I integron
AS_Classlint_R	CAA ACG TGC CGT AGA ACA AG	5683C		
AS_intI1_L	GGG AGG ACT TTC CGC AAC CG	5363	1084	For amplification and sequencing the blaVIM upstream region
AS_VIM_R	CGT TAC CAC CGC TGC GTT CG	6446C		
AS_VIM_GS_SI	GCC TTG ATG TTA CCC GAG AG	5937C	NA	
AS-VIM4GS-f	GAT GCG TGG AGA CCG AAA CC	6228	1295	For amplification and sequencing the blaVIM and immediate surrounding
AS-VIM4GS-r	TGC CTA ACG CCT GAG TTG AG	7522C		
AS_VIM_L	AAT CGC TCA GTC GCC GAG TA	7412	3750	For amplification and sequencing the blaVIM downstream region
AS_ISPa21_R	CTA TAA GAC ACG AGG TGT CTG	11161C		
AS_ISPa21_L	CAC CAC AAC CGC AAG AAA TA	10034	NA	
AS_ISPa21_seq	CGC GCA TCG ATT GTT CGT AG	10549	NA	
AS_smr_f	GCT GGA CTC TTT GAG ATT GG	9507	NA	
AS_dhfrI_R	ACC CTT TTG CCA GAT TTG GT	8597C	NA	
AS_aacA7_R	GAG CAA CCT CCG TGA ATC CA	7955C	NA	
AS_VIMdn_LS1	TTC GTT CAA GCC GAA CTT GC	8010	NA	
AS_VIMdn_LS2	AAT AGA CAT CGA GCC GGA AG	8477	NA	
AS_VIMdn_LS3	ACA TAG CGT TGC CTT GGT AG	9030	NA	
AS_orf5_R	TTA GAT TTC GAG TTC TAG GCG TTC TG	NA	3647 bp (if Class I integron classical 3' end is present)	Primer AS_orf5_R anneals to the 3' end of the class I integron, the two primers amplify the 3' region of class I integron if present
AS_smr_f	GCT GGA CTC TTT GAG ATT GG	9507		
AS_ISPa21_R	CTA TAA GAC ACG AGG TGT CTG	11161C	NA	Sequencing the amplicon produced by the PCR above
AS_ISPa21_L	CAC CAC AAC CGC AAG AAA TA	10034	NA	
AS_ISPa21_seq	CGC GCA TCG ATT GTT CGT AG	10549	NA	

TABLE 10. - *cont.*

Primer name	5'-3' sequence	Annealing to AY339625	Size of products * (bp)	Comment
AS_sul1_R1	TTG CCG ATC GCG TGA AGT TC	13000C	NA	Sequencing the amplicon produced by the PCR using primer AS_orf5_R and AS_smr_f
AS_sul1_R2	CACAACCTGGTCGATATCAC	13311C	NA	
AS_orf5_L	ATGGACAGCGAGGAGC	13249	NA	
AS_qacED1_L	GCG AAG TAA TCG CAA CAT CC	11978	NA	
AS_VIMdn_LS4	GAT CAG ATG CAC CGT GTT TC	12501	NA	

* NA: not applicable; these primers were used for sequencing only

IV. RESULTS

IV.1. VIM-Producing *Enterobacteriaceae* in Abu Dhabi

Screening a collection of 34 carbapenem non-susceptible clinical *Enterobacteriaceae* isolated in Abu Dhabi Emirate for various carbapenemase genes identified an *Enterobacter cloacae* strain positive for bla_{VIM} (ABC104).

ABC104 was resistant to imipenem (MIC=16 mg/L), meropenem (MIC=8mg/L), ertapenem (MIC=4mg/L), ceftazidime (MIC>128mg/L), cefotaxim (MIC>128mg/L), cefepime (MIC=64mg/L), aztreonam (MIC>128mg/L), cefoperazone (MIC=64mg/L), trimetoprime/sulphametoxazole (MIC=8/76mg/L), chloramphenicol (MIC=32mg/L), gentamicin (MIC=32mg/L), tobramycin (MIC=48mg/L), netilmicin (MIC=32mg/L), ciprofloxacin (MIC=32mg/L), moxifloxacin (MIC=12mg/L), levofloxacin (MIC=12mg/L), tetracycline (MIC=64mg/L), minocycline (MIC=32mg/L) and exhibited sensitivity to amikacin (MIC=6 mg/L) and colistin (MIC=0.125mg/L) only. It carried the bla_{VIM-4} gene on an IncA/C type plasmid of approximately 175 kb of size as detected by Southern blotting of the S1 digested genomic DNA (**FIGURE 8.**). ABC104 also harboured other β-lactamase genes: $bla_{CTX-M-15}$, bla_{TEM-1} and bla_{CMY-4}, as well, with bla_{CMY-4} being located on the same plasmid as bla_{VIM-4}. The $bla_{CTX-M-15}$ gene was carried on a plasmid of approximately 300 kb size, while a probe for bla_{TEM-1} hybridized with both plasmids (**FIGURE 8.**).

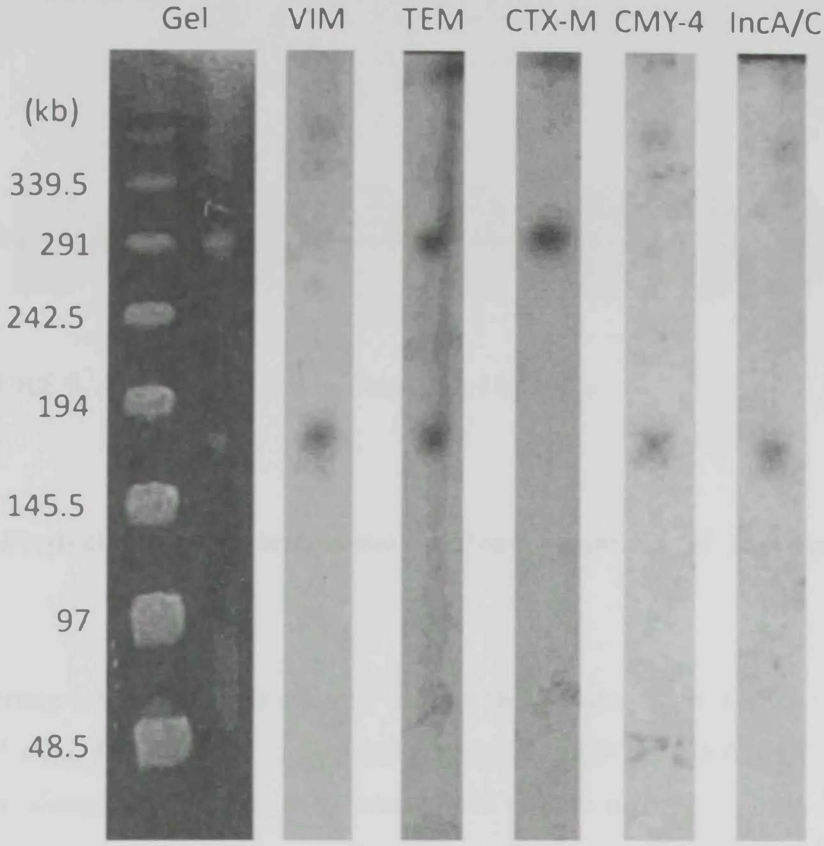

FIGURE 8. Southern blot of the S1 digested genomic DNA of *E. cloacae* ABC104. Gel, left lane: λ concatamer.

Specificity of the probes used are shown above the membrane strips

Attempts to conjugally transfer the bla_{VIM-4} carrying plasmid into an azid resistant derivative of *E. coli* J53 failed at 30°C, as well as at 37°C. Transformation of competent DH5α with plasmid preparation of wild type ABC104 was also attempted, but was unsuccessful.

The bla_{VIM-4} was identified as part of a class I integron by PCR mapping and sequencing (**FIGURE 9.**).

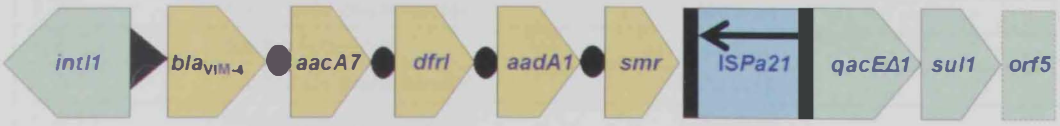

FIGURE 9. ABC104 bla_{VIM-4} bearing class I integron

IV.2. VIM-Producing *Enterobacteriaceae* in Four Countries of the Arabian Peninsula

Screening the second collection of further 166 isolates identified 11 strains positive for bla_{VIM}. Their names, species and country of origin are listed in (**TABLE 11.**). The prevalence of VIM producers was 29.6% in Kuwait (n=27), 3.1% in the Sultanate of Oman (n=63), 1.85% in the Kingdom of Saudi Arabia (n=54) and 1.79% in the UAE (n=56).

TABLE 11. VIM-producing *Enterobacteriaceae* of the Arabian Peninsula

Strain	Species	Country of isolation
KW1	*Klebsiella pneumoniae*	Kuwait
KW2	*Klebsiella pneumoniae*	Kuwait
KW3	*Enterobacter cloacae*	Kuwait
KW4	*Klebsiella pneumoniae*	Kuwait
KW6	*Klebsiella pneumoniae*	Kuwait
KW7	*Escherichia coli*	Kuwait
KW8	*Klebsiella pneumoniae*	Kuwait
KW11	*Klebsiella pneumoniae*	Kuwait
OM63	*Enterobacter cloacae*	Sultanate of Oman
OM69	*Enterobacter cloacae*	Sultanate of Oman
SA4/2	*Enterobacter cloacae*	Kingdom of Saudi Arabia
ABC104	*Enterobacter cloacae*	United Arab Emirates

IV.2.1. Antibiotic Susceptibility Testing

All strains exhibited resistance to all carbapenems and third generation cephalosporins tested. Modified Hodge test was positive with all isolates and EDTA, but not PABA, exhibited synergy with meropenem in all cases. All but *E. cloacae* SA4/2, were resistant to aztreonam, which suggested of co-harbored ESBL or AmpC cephalosporinase enzymes in the other 11 isolates. Strains were uniformly resistant to tobramycin, co-trimoxazole and tetracycline. Susceptibility to ciprofloxacin, chloramphenicol, gentamicin, amikacin and tigecycline (using the EUCAST breakpoints) were variable. Only one *K. pneumoniae* (KW11) exhibited resistance to colistin. Detailed susceptibility data are shown in **TABLE 15.**

IV.2.2. Detection of Non-Carbapenemase Coding Antibiotic Resistance Genes

The results of 21 different PCR reactions targeting common ESBL, AmpC cephalosporinase, plasmid mediated quinolone resistance and ribosome methylase genes, as well as their alleles identified by sequencing are listed in **TABLE 12.**

TABLE 12. Antibiotic resistance genes detected in the VIM-4 producer clinical isolates

Strain	ESBLs				AmpC β-lactamases	Plasmid Mediated Quinolone Resistance			Methyltransferases
	bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M}$	bla_{PER}		*qepA*	*qnrA, qnrB, qnrS*	*aac-6'-Ib-cr*	armA, RmtA, RmtB, RmtC, RmtD, NpmA
KW1	TEM-1	SHV-12	CTX-M-15	ND	CMY-4	ND	ND	POS	ND
KW2	TEM-1	SHV-12	CTX-M-15	ND	CMY-4	ND	ND	POS	ND
KW3	ND	ND	ND	ND	ND	ND	ND	ND	ND
KW4	ND	ND	ND	ND	CMY-4	ND	ND	POS	ND
KW6	TEM-1	ND	CTX-M-15	ND	CMY-4	ND	*qnrB*	POS	ND
KW7	ND	ND	ND	ND	CMY-4	ND	ND	ND	ND
KW8	ND	ND	ND	ND	CMY-4	ND	ND	ND	ND
KW11	TEM-1	SHV-1	CTX-M-15	ND	CMY-4	ND	ND	POS	ND
OM63	TEM-1	ND	CTX-M-15	ND	ND	ND	*qnrB*	POS	ND
OM69	TEM-1	ND	CTX-M-15	ND	ND	ND	*qnrB*	POS	ND
SA4/2	ND	ND	ND	ND	ND	ND	*qnrB*	ND	ND
ABC104	TEM-1	ND	CTX-M-15	ND	CMY-4	ND	ND	POS	ND

ND. Not detected

IV.2.3. Molecular Typing of VIM Producer Isolates

All strains carried the *bla*$_{VIM-4}$ allele as proven by sequencing of the *bla*$_{VIM}$ amplicons. PFGE typing of the strains showed that with the exception of two *K. pneumoniae* from Kuwait (KW1 and KW2) and the two *E. cloacae* from Oman, the strains were not related **(FIGURE 10. and 11.)** -below.

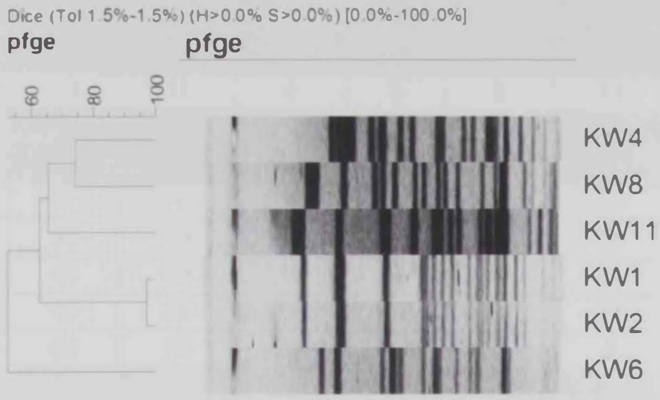

FIGURE 10. PFGE comparison of *K.pneumoniae* carrying VIM-4

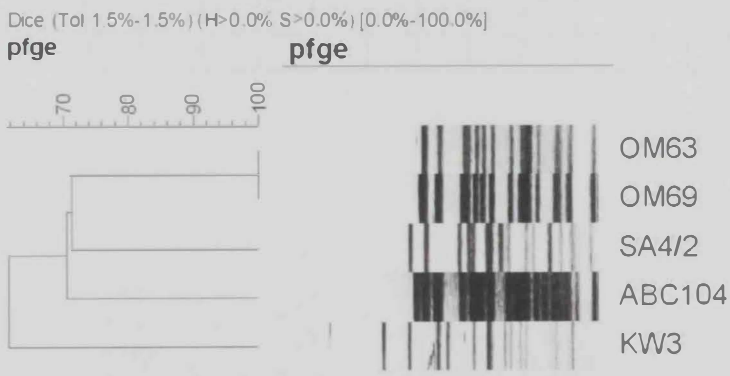

FIGURE 11. PFGE comparison of *E. cloacae* carrying VIM-4

The result of the multi-locus sequence typing of the 12 VIM-4 producer strains is shown in **(TABLE 13.)**. It is interesting to note that although OM63 and

OM69 were only 70% similar to ABC104 by PFGE, their sequence type was the same. Further clustering by MLST was only observed with strains KW1 and KW2, which were already grouped together by PFGE.

TABLE 13. Allelic profile and ST of VIM-4 producer isolates

Strain	Allelic profile							Sequence type (ST)
				E. coli				
	adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	
KW7	10	11	4	8	8	13	2	ST167
				E. cloacae				
	dnaA	*fusA*	*gyrB*	*leuS*	*pyrG*	*rplB*	*rpoB*	
KW3	4	4	4	6	92	30	6	ST184
ABC104	49	20	19	44	90	24	32	ST182
OM63	49	20	19	44	90	24	32	ST182
OM69	49	20	19	44	90	24	32	ST182
SA4/2	82	56	93	93	91	4	23	ST183
				K. pneumoniae				
	gapA	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	
KW1	4	1	124	1	7	4	91	ST1399
KW2	4	1	124	1	7	4	91	ST1399
KW4	18	22	26	23	31	13	49	ST138
KW6	17	19	39	112	122	18	148	ST1400
KW8	2	1	1	1	9	1	4	ST1401
KW11	3	4	6	1	7	4	38	ST147

IV.2.4. Localization of the *bla*$_{VIM-4}$ Gene

The southern blot of plasmid electrophoresis of the clinical isolates was hybridized with VIM-4 probe to prove its localization (**FIGURE 12.**). The size of plasmids that the probe hybridized with was approx. 175 kb in KW1, KW2, KW4, KW6, KW7, KW8 and ABC 104, >300 kb in KW11, approx. 80 kb in KW3 and approx. 50 kb in OM63, OM69 and SA4/2.

IV.2.5. Plasmid Replicon Typing of VIM-4 Producer Clinical Isolates

The results of the PCR based replicon typing (PBRT) are shown below in (**TABLE 14.**). Since the majority of strains harbored IncA/C plasmids known to frequently carry VIM genes, as well as *bla*$_{CMY-4}$, the membrane used to localize *bla*$_{VIM-4}$ was re-probed with both IncA/C and *bla*$_{CMY-4}$ probes. These hybridizations proved that, beyond *E. cloacae* ABC104, in *K. pneumoniae* KW1, KW2, KW4, KW6, KW8, KW11 and in *E. coli* KW7 *bla*$_{VIM-4}$ was co-localized with *bla*$_{CMY-4}$ on IncA/C plasmids (**FIGURE 12.**).

TABLE 14. The incompatibility types of plasmids detected in VIM-4 producer clinical isolates

Strain	Incompatibility Group		
KW1	IncA/C	IncN	IncFIIA
KW2	IncA/C	IncN	IncFIIA
KW3	Non-typable		
KW4	IncA/C	IncFIIA	
KW6	IncA/C	IncHI1B	
KW7	IncA/C	IncF	IncFIB
KW8	IncA/C		
KW11	IncA/C	IncHI1B	
OM63	IncX3		
OM69	IncX3		
SA4/2	Non-typable		
ABC104	IncA/C		

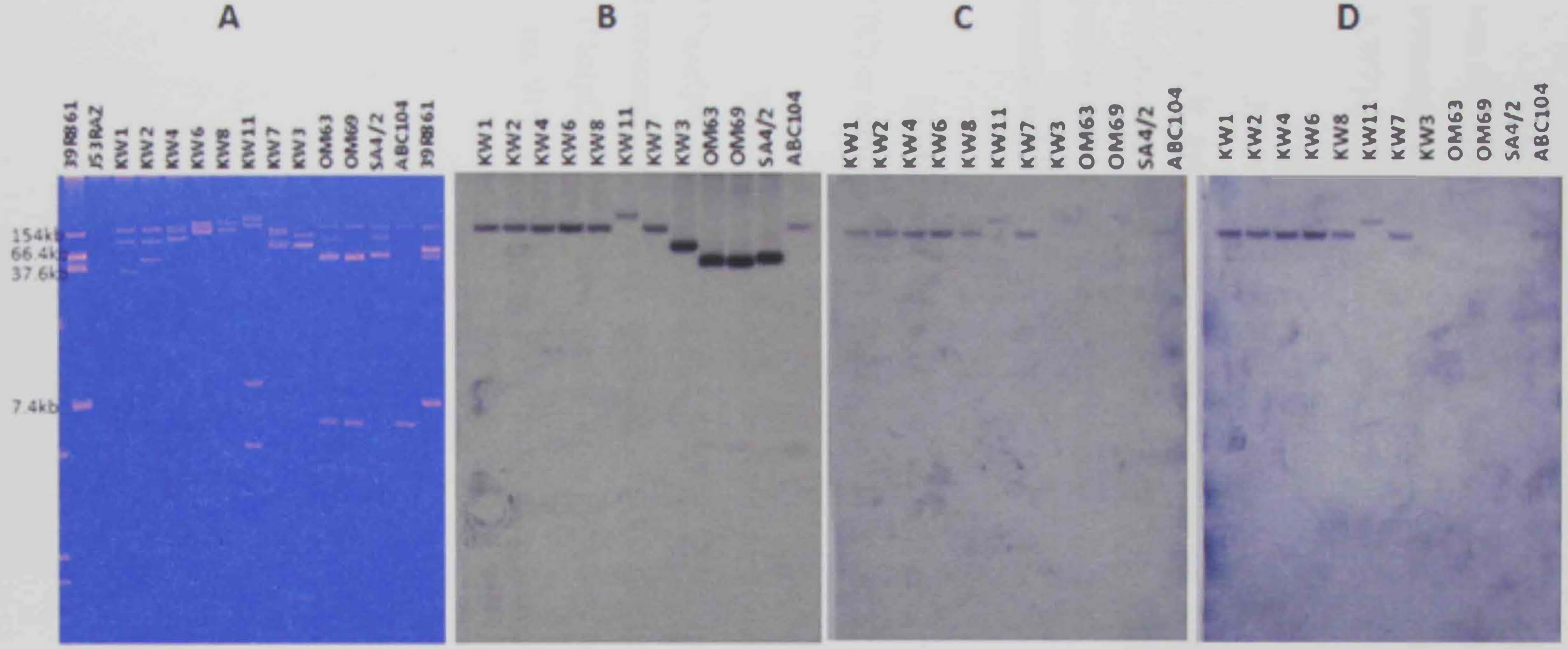

FIGURE 12. Plasmid profile of clinical isolates (A) and hybridization with VIM-4 (B), with IncA/C (C) and with CMY-4 probes (D)

IV.2.6. Conjugation and Transformation of the bla_{VIM-4} Carrier Plasmids

Conjugative transfer of plasmid carrying bla_{VIM-4} into the *E. coli* $J53_{RAZ}$ strain was attempted with the 12 clinical isolates at 37°C and 30°C, which resulted in seven tranconjugants: from 5 *K. pneumoniae* (KW1, KW2, KW4, KW6, KW8), one *E. coli* (KW7) and one *E. cloacae* (SA4/2), only.

From the five clinical isolates from which the conjugation had failed, plasmids purified were used to transform DH5α. VIM-4 carrier plasmids were transformed from *E. cloacae* OM63 and OM69 into DH5α, while from *K. pneumoniae* KW11 and *E. cloacae* ABC104 and KW3 the transformation was unsuccessful.

Antibiotic susceptibility of all wild-type (WT) and respective transconjugants (TC) or transformants (TF) are listed in (**TABLE 15.**).

PBRT and hybridization confirmed the presence of IncA/C plasmid in $J35_{RAZ}$(pKW4/1), $J53_{RAZ}$(pKW6/2), $J53_{RAZ}$(pKW7/9) and $J53_{RAZ}$(pKW8/7). Transconjugant $J53_{RAZ}$(pSA4/2/13) and the two transformants DH5α(pOM63/T1) and DH5α(pOM69/T5) were non-typable by PBRT. All these plasmid had the same size as the VIM-4 plasmids in the corresponding clinical isolates (**FIGURE 13.**).

TABLE 15. Antibiotic susceptibility of clinical isolates and their derivatives

		Microdilution mg/L						E-test		Disc diffusion						
		ERT	MEM	IMI	CAZ	CTX	AZT	TGC	COL	CIP	AM	GM	TOB	TET	SXT	CHL
$J53_{RAZ}$	R	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	NT	NT	S	S	S	S	S	S	S
DH5α	R	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	NT	NT	S	S	S	S	S	S	S
KW1	W	32	>128	32	>128	>128	>128	2	0.125	R	R	R	R	R	R	R
$J53_{RAZ}$ (pKW1/3)	TC	0.25	1	2	64	32	64	NT	NT	S	R	R	R	R	R	R
KW2	W	64	>128	32	>128	>128	>128	1.5	0.125	R	S	R	R	R	R	R
$J53_{RAZ}$ (pKW2/9)	TC	<0.125	2	2	64	32	64	NT	NT	S	S	I	R	R	R	R
KW3	W	32	32	8	>128	>128	>128	1	0.125	R	S	R	R	R	R	I
KW4	W	4	4	8	64	32	16	8	0.25	I	S	R	R	R	R	R
$J53_{RAZ}$ (pKW4/1)	TC	0.25	1	1	32	16	16	NT	NT	S	S	I	R	R	R	I
KW6	W	4	8	8	64	64	>128	4	0.125	R	S	R	R	R	R	R
$J53_{RAZ}$ (pKW6/2)	TC	0.25	1	0.5	32	16	16	NT	NT	S	S	R	R	R	R	R
KW7	W	4	4	2	>128	128	32	0.75	0.125	R	S	R	R	R	R	R
$J53_{RAZ}$ (pKW7/9)	TC	2	2	1	64	32	32	NT	NT	S	S	S	R	R	R	R
KW8	W	1	8	4	16	16	8	1	0.125	S	S	R	R	R	R	R
$J53_{RAZ}$ (pKW8/7)	TC	0.25	1	1	32	16	16	NT	NT	S	S	R	R	R	R	R
KW11	W	>64	>128	>128	>128	>128	>128	3	6	R	I	R	R	R	R	R
OM63	W	4	16	16	64	>128	128	1.5	0.125	R	S	S	R	R	R	S
DH5α (pOM63/T1)	TF	0.25	0.5	<0.25	8	8	<0.25	NT	NT	S	S	S	S	S	S	S
OM69	W	4	16	8	64	>128	128	1	0.125	R	S	S	R	R	R	S
DH5α (pOM69/T5)	TF	1	1	<0.25	8	16	<0.25	NT	NT	S	S	S	S	S	S	S
SA4/2	W	8	16	8	32	64	0.5	1.5	0.19	R	S	S	R	R	R	S
$J53_{RAZ}$ (pSA4/2/13)	TC	4	8	2	16	16	0.5	NT	NT	S	S	S	I	S	S	S
ABC104	W	4	8	16	>128	>128	>128	5	0.125	R	S	R	R	R	R	R

R : Recipient W: Wild Type TC: Transconjugant TF: Transformant
NT: not tested I : Intermediate R : Resistant S : Sensitive

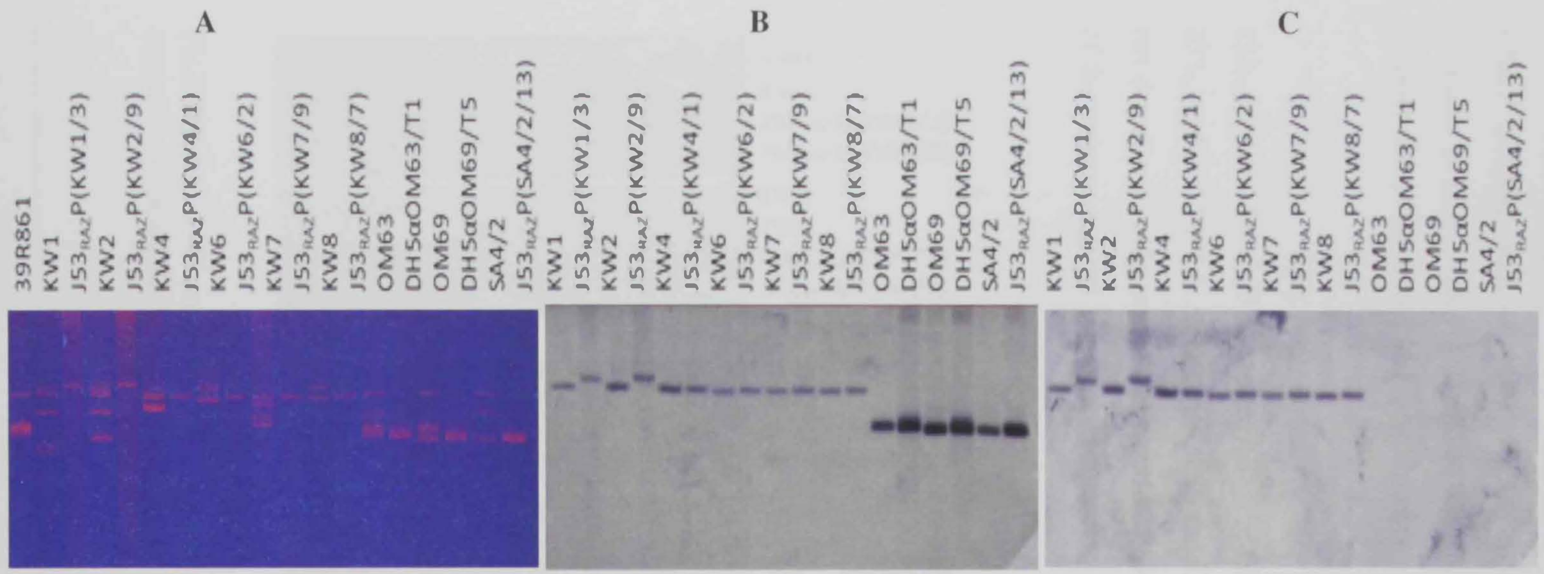

FIGURE 13. Wild Type and Transconjugants/Transformants Plasmid gel (A), hybridization with VIM-4 probe (B) and IncA/C probe (C).

Transconjugants $J53_{RAZ}$ (pKW1/3) and $J53_{RAZ}$ (pKW2/9) harboured plasmids increased in size compared to the VIM-4 plasmids in the respective clinical isolates, and the tranconjugants were positive for both the IncA/C and IncN type PCR in the PBRT. Southern blot of wild types and transconjugants verified that increased size plasmids in $J53_{RAZ}$ (pKW1/3) and $J53_{RAZ}$ (pKW2/9) were the result of fusion and co-conjugation of these two incompatibility type plasmids (**FIGURE 14**).

bla_{CMY} was amplified from transconjugants $J53_{RAZ}$(pKW1/3), $J53_{RAZ}$(pKW2/9), $J35_{RAZ}$(pKW4/1), $J53_{RAZ}$(pKW6/2), $J53_{RAZ}$(pKW7/9) and $J53_{RAZ}$(pKW8/7), i.e. from those the IncA/C plasmid were conjugated to. PCR did not detect any of the other ß-lactamases, PMQR or ribosomal methylase genes in any of the transconjugants or transformants.

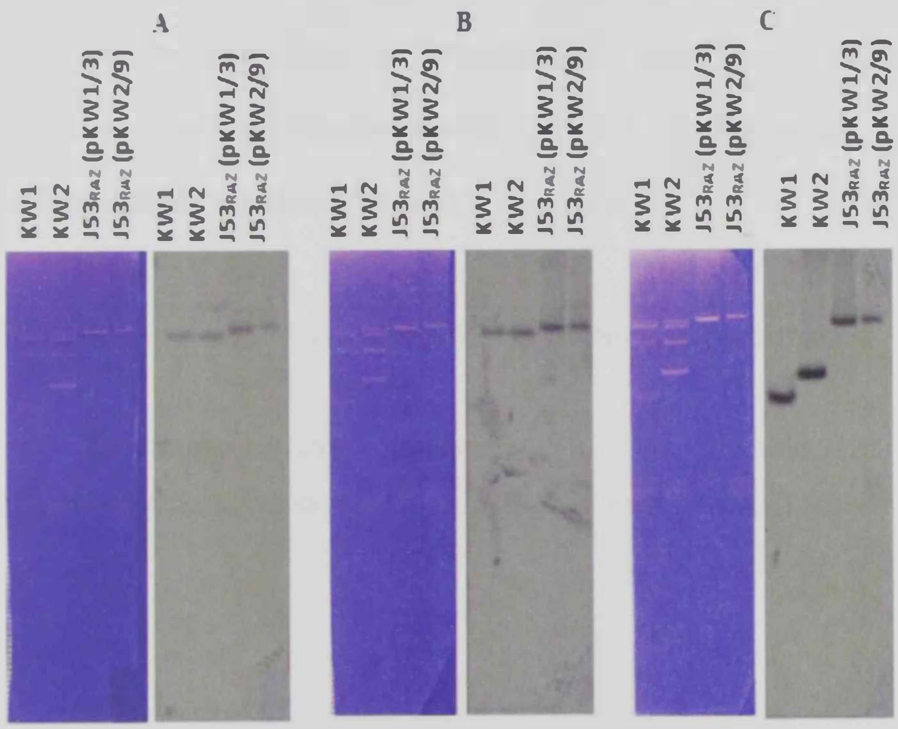

FIGURE 14. KW1 and KW2 wild-type and transconjugants plasmid gel and respective hybridization with VIM-4 (A), IncA/C (B) and IncN probes (C)

IV.2.7. Genetic Surrounding of bla_{VIM-4} Genes

PCR mapping and sequencing of the molecular structure surrounding the bla_{VIM-4} revealed class I integron structures similar to the one of *E. cloacae* ABC104 (GenBank Accession No. JX275775). Comparison of this class I integron is shown in **(FIGURE 15.)**.

The structure marked by (A) corresponds to JX275775 and was found not only in ABC104 but in *E. cloacae* SA4/2 isolated in Saudi Arabia as well. PCR mapping the classical 3' end of class I integrons did not yield an amplicon in any strain isolated in Kuwait (KW1, KW2, KW3, KW4, KW6, KW7, KW8 and KW11), so in these isolates we identified a structure (B) which is identical to In416 described in a *K. pneumoniae* isolated in Italy (GenBank Acc. No. AJ704863). In *E. cloacae* OM63 and OM69 sequencing revealed a class I integron similar to JX275775, but lacked the *dhfrI* and *ΔaadA1* gene casettes (C).

IV.2.8. Comparison of All VIM-4 Producer Strains Investigated

A comprehensive assessment of all features of VIM-producer *Enterobacteriaceae* investigated in this study is given in **TABLE 16.**

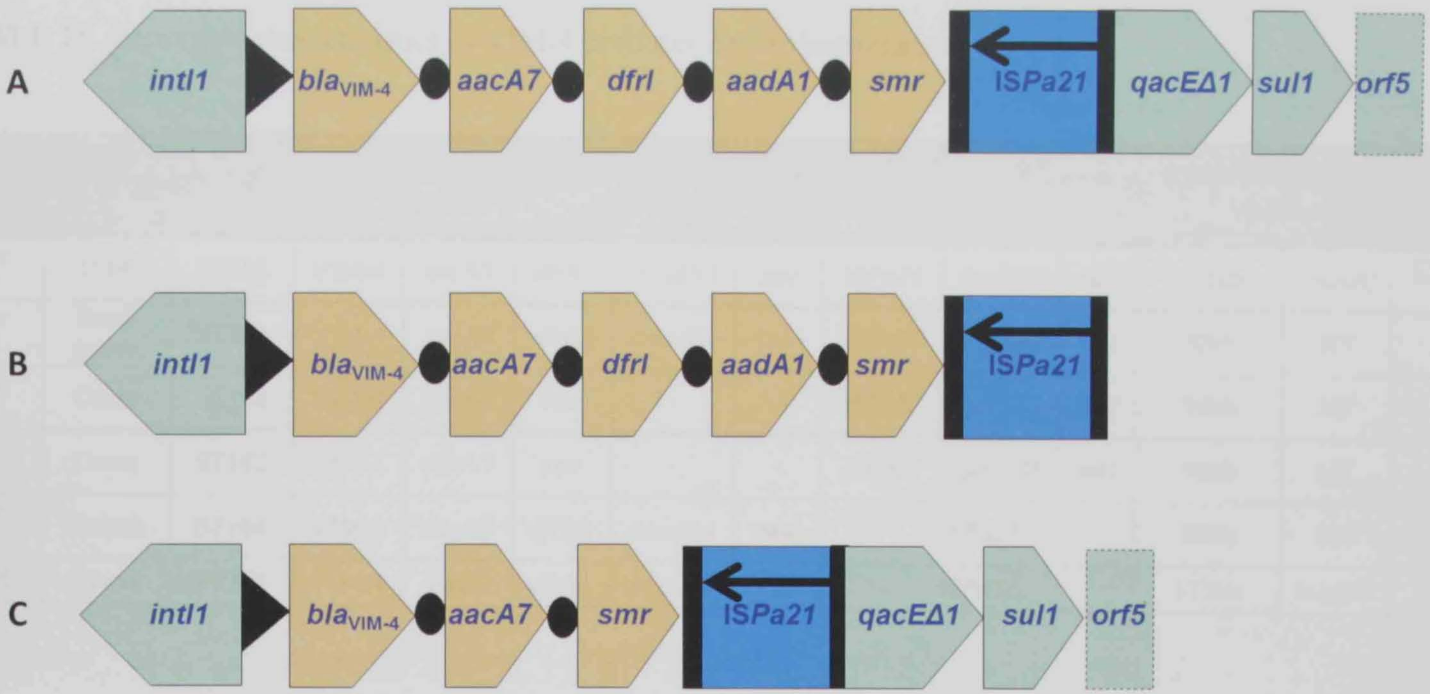

FIGURE 15. *bla*$_{VIM-4}$ bearing class I integrons of *Enterobacteriaceae* from the Arabian Peninsula

See explanation in the text.

(A) Integron structure of ABC104 and SA4/2 (B) Integron structure of all Kuwaiti isolates (C) Integron structure of OM63 and OM69

TABLE 16. Genotypic characteristics of VIM-4 producer *Enterobacteriaceae*

Species	Country	MLST	Class I integron structure								VIM plasmid		Other β-lactamases[a]	PMQR
			Gene cassettes (GC)					3' end						
			GC1	GC2	GC3	GC4	GC5				size	RT		
E. cloacae ABC104	UAE	ST182	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	*qacEΔ1*	*sul1*	175kb	IncA/C	**CMY-4, TEM-1,** CTX-M-15	*aac-6'-Ib-cr*
E. cloacae SA4/2	Saudi Arabia	ST183	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	*qacEΔ1*	*sul1*	50kb	NT	none	*qnrB*
E. cloacae OM63	Oman	ST182	VIM-4	*aac*A7	*smr*	-	-	IS*Pa21*	*qacEΔ1*	*sul1*	50kb	NT	TEM-1, CTX-M-15	*aac-6'-Ib-cr*, *qnrB*
E. cloacae OM69	Oman	ST182	VIM-4	*aac*A7	*smr*	-	-	IS*Pa21*	*qacEΔ1*	*sul1*	50kb	NT	TEM-1, CTX-M-15	*aac-6'-Ib-cr*, *qnrB*
E. cloacae KW3	Kuwait	ST184	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*			80kb	NT	none	none
E. coli KW7	Kuwait	ST167	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*			175kb	IncA/C	**CMY-4**	none

TABLE 16. - *cont.*

Species	Country	MLST	Class I integron structure						VIM plasmid		Other β-lactamases[a]	PMQR
			Gene cassettes (GC)					3' end				
			GC1	GC2	GC3	GC4	GC5		size	RT		
K. pneumoniae KW1	Kuwait	ST1399	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	175kb	IncA/C	**CMY-4**, TEM-1, SHV-12, CTX-M-15	*aac-6'-Ib-cr*
K. pneumoniae KW2	Kuwait	ST1399	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	175kb	IncA/C	**CMY-4**, TEM-1, SHV-12, CTX-M-15	*aac-6'-Ib-cr*
K. pneumoniae KW4	Kuwait	ST138	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	175kb	IncA/C	**CMY-4**	*aac-6'-Ib-cr*
K. pneumoniae KW6	Kuwait	ST1400	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	175kb	IncA/C	**CMY-4**, TEM-1, CTX-M-15	*aac-6'-Ib-cr*, *qnrB*
K. pneumoniae KW8	Kuwait	ST1401	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	175kb	IncA/C	**CMY-4**	none
K. pneumoniae KW11	Kuwait	ST147	VIM-4	*aac*A7	*dfr*A1	*ΔaadA*1	*smr*	IS*Pa21*	>300kb	IncA/C	**CMY-4**, TEM-1, SHV-1, CTX-M-15	*aac-6'-Ib-cr*

NT: non-typable, [a]Enzymes underlined are localized on the VIM-plasmid

V. DISCUSSION

Enterobacteriaceae, harboring metallo-beta-lactamases are considered a serious, globally evolving threat to carbapenem antimicrobials, which represent the last line of antibiotics that are still effective for treating many enterobacterial infections (Nordmann and Cornaglia 2012). Although, VIM-type metallo-beta-lactamases has mostly been described in *P. aeruginosa*, they occurred in *Enterobacteriaceae* (Nordmann et al. 2011a). The expression of bla_{VIM} genes confers resistance to all β-lactams except aztreonam, but their efficiency is dependent on the enzyme variants. VIM-4, a single amino acid variant of VIM-1, hydrolyzes imipenem and meropenem more efficiently than VIM-1 (Lassaux et al. 2011). Despite the enzyme efficiency, VIM-1 producer *Enterobacteriaceae*, especially *K. pneumoniae* has become widespread in some Southern European countries, i.e. Greece, Italy and Spain (Peirano Gisele). VIM-producing *K. pneumoniae* has given rise to major problems both with respect to infection control and treatment in these countries (Daikos et al. 2009).

Since its first description in 2002, VIM-4 has been identified in *K. pneumoniae*, *E. cloacae*, *Acinetobacter* spp., *A. hydrophyla* and *E. coli* (Luzzaro et al. 2004, Ktari et al. 2006, Libisch et al. 2006, Ikonomidis et al. 2007, Figueiredo et al. 2008, Kristof et al. 2010, Juhasz et al. 2012, Shevchenko et al. 2012, Dimude and Amyes 2013, Jamal et al. 2013, Melegh et al. 2014). The majority of these above publications, except two on *Klebsiella pneumoniae* from Tunisia (Ktari et al. 2006) and from Hungary (Melegh et al. 2014), and a further one on *E. cloacae* also from Hungary (Juhasz et al. 2012) report clonally unrelated or sporadic occurrence of VIM-4 producer *Enterobacteriaceae*.

It is noteworthy, that our study proved the presence of VIM-4 producer *E. cloacae* in all the four countries investigated, even though the prevalence of such isolates was low. The only two isolates exhibiting significant similarity by PFGE were isolated from the same hospital in Oman (OM63 and OM69). The isolates from the different countries were clonally unrelated when relatedness was investigated by PFGE; however, the two isolates from Oman (OM63 and OM69) and the one from the UAE (ABC104) carried the bla_{VIM-4} on different plasmids but not identical integrons shared the sequence type. This suggests that applying the newly developed MLST scheme for *E. cloacae* complex may identify further relatedness of multi-drug resistant *E. cloacae*.

Since the MLST scheme was only developed in 2013 it is difficult to compare the clonal relatedness of VIM-4 producing *E. cloacae* reported earlier to the ones studied in the thesis. However, the plasmid carrying the class I integron, as well as the integrons themselves, can be compared to the ones reported earlier (**TABLE 17.**).

Except for SA4/2, none of the bla_{VIM-4} bearing plasmids in our *E. cloacae* isolates were conjugative, unlike the ones reported earlier. Furthermore, most of them were untypable by the extended PBRT used, with the exception of the IncA/C type *bla*$_{VIM-4}$-bearing plasmid of ABC104. *bla*$_{VIM-4}$ bearing plasmids of clonally unrelated strains had different size with the exception of SA4/2 and the OM63, OM69 pair of strains, carried similar sized, but PBRT untypable *bla*$_{VIM-4}$ bearing episomes.

Class I integron carrying *bla*$_{VIM-4}$ in *E. cloacae* KW3 isolated in Kuwait was identical to In416 identified in Italy. *E. cloacae* SA4/2 from Saudi Arabia and ABC104 from the UAE harbored a very similar integron as well, but the integron in these strains possessed the usual 3'-CS downstream of the ISPa21. The clonally related Omani isolates (OM63 and OM69) carried the *bla*$_{VIM-4}$ on class I integron very similar to this latter one, albeit with the lack of gene cassettes of dfrA1 and ΔaadA1 (**TABLE 17.**).

In summary, VIM-4 producer *E. cloacae* isolates with similar, but not identical, genetic features were present in low prevalence in all of the four countries investigated.

Contrary to VIM-4 producer, *E. cloacae, K. pneumoniae* isolates carrying VIM-4 was present in Kuwait only. Earlier, in the 1990s and early 2000, *K. pneumoniae* became the index species for plasmids encoding extended-spectrum-beta-lactamases (ESBLs), along with a variety of genes conferring resistance to drugs other than β-lactams (Tzouvelekis et al. 2012), so it is interesting to note that Kuwait had the highest incidence of VIM-producer *Enterobacteriaceae* (29.6%), which was a clear difference between this country and the other three investigated in this study.

Of the six strains, only two, KW1 and KW2, were clonally related by PFGE and MLST, harboring a unique sequence type ST1399. Another two strains KW4 (ST138) and KW6 (ST1400) belonged to other sequence types not related to any clonal complex. Nevertheless, two of the six strains belonged to international epidemic clones of *K. pneumoniae*, KW11 to ST147 and KW8 to CC18 (ST1401 is a single locus variant of ST18). Both of these clones contributed to the epidemic of VIM-producer *K. pneumoniae* in Greece (Hasan et al. 2014). Furthermore, *K. pneumoniae* ST147 was associated with a small hospital outbreak caused by NDM-1 producer *K. pneumoniae* in China (Wang et al. 2013) and it is one of the highly successful enterobacterial clones spreading CTX-M-type β-lactamases worldwide (D'Andrea et al. 2013).

All VIM-4 producer *K. pneumonia*, with the exception of KW11, carried the gene on an IncA/C plasmid also harboring bla_{CMY-4}, which had a size of 175kb and was conjugative in five of the six isolates. Furthermore, all six strains carried bla_{VIM-4} on similar structured class I integrons (In416). These data, together with the fact that the same size and incompatibility group plasmid with In416 was found in the single *E. coli* KW7 isolate also from Kuwait, suggest of a successful plasmid spread in this geographical location. Moreover, the In416 or similar integron located on conjugative plasmids, which belonged to IncA/C when tested, was reported earlier from Italy spreading between *E. cloacae* and *K. pneumoniae* (Luzzaro et al. 2004) and from an outbreak in Tunisia (Ktari et al. 2006) indicates the dissemination capacity of this genetic structure carrying bla_{VIM-4} and bla_{CMY-4} (**TABLE 18.**) Integration of this episome into successfully spreading MDR clones of *K. pneumoniae*, i.e. ST147 and CC18, is a phenomenon, which warrants not only strict infection control measures, but close monitoring by active surveillance in order to avoid a scenario as emerged in Greece (Hasan et al. 2014) or in Hungary (Melegh et al. 2014).

As far as non-carbapenem susceptibility was concerned the majority of the 12 isolates possessed additional β-lactamases and plasmid-mediated quinolone resistant determinants and were susceptible to few antibiotics only, thus fulfilling the multi-drug resist criteria set by (Magiorakos et al. 2012). Moreover, *K. pneumoniae* KW11 was almost pan-resistant, i.e. resistant to colistin and tigecycline and only intermediate susceptible to amikacin. Considering that this isolate is a member of the ST147 MDR clone of *K. pneumoniae*, further emphasizes the need for active interventions to stop the spread of such organisms.

TABLE 17. Comparison of VIM-4 producer *E.cloacae*

Strain	Country	MLST	Class I integron structure								VIM plasmid			Co-harbored β-lactamases[a]	Ref.
			Gene cassettes (GC)					3' CS							
			GCI	GC2	GC3	GC4	GC5				size	conju-gative	RT		
E. cloacae ABC104	UAE	ST182	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	*qacEΔ1*	*sul1*	175kb	-	IncA/C	**CMY-4,** **TEM-1,** CTX-M-15	This study
E. cloacae SA4/2	Saudi Arabia	ST183	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	*qacEΔ1*	*sul1*	50kb	-	UT	none	This study
E. cloacae OM63	Oman	ST182	VIM-4	*aacA7*	*smr*	-	-	IS*Pa21*	*qacEΔ1*	*sul1*	50kb	-	UT	TEM-1, CTX-M-15	This study
E. cloacae OM69	Oman	ST182	VIM-4	*aacA7*	*smr*	-	-	IS*Pa21*	*qacEΔ1*	*sul1*	50kb	-	UT	TEM-1, CTX-M-15	This study
E. cloacae KW3	Kuwait	ST184	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*			80kb	-	UT	none	This study
E. cloacae	Italy	NT	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*			unknown	+	IncA/C	**CMY-4**	(Colinon et al. 2007)
*E. cloacae**	Hungary	NT	*aacA4*	VIM-4	-	-	-	*qacEΔ1*			unknown	NT	NT	SHV-12	(Juhasz et al. 2012)
E. cloacae	Egypt	NT	VIM-4	*aacA7*	*dfrA1*	*aadA2*	-	*qacEΔ1*	*sul1*		300kb	+	NT	**CTX-M-14**	(Dimude and Amyes 2013)
E. cloacae	Greece	NT	VIM-4	*aacA7*	*dfrA1*	*aadA1*	-	*qacEΔ1*	*sul1*		40kb	+	NT	**SHV-2a**	(Ikonomidis et al. 2007)

* Outbreak isolates NT: not tested, UT: untypable, RT: replicon type, [a] Enzymes underlined are localized on the VIM-plasmid

TABLE 18. Comparison of VIM-4 producer *K. pneumoniae*

Species	Country	MLST	Class I integron structure						VIM plasmid			Co-harbored β-lactamases[a]	Reference
			Gene cassettes (GC)					3' CS					
			GC1	GC2	GC3	GC4	GC5		size	conjugative	RT		
K. pneumoniae KW1 and KW2	Kuwait	ST1399	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	175kb	+	IncA/C	<u>CMY-4</u>, TEM-1, SHV-12, CTX-M-15	This study
K. pneumoniae KW4	Kuwait	ST138	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	175kb	+	IncA/C	<u>CMY-4</u>	This study
K. pneumoniae KW6	Kuwait	ST1400	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	175kb	+	IncA/C	<u>CMY-4</u>, TEM-1, CTX-M-15	This study
K. pneumoniae KW8	Kuwait	ST1401	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	175kb	+	IncA/C	<u>CMY-4</u>	This study
K. pneumoniae KW11	Kuwait	ST147	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	>300kb	-	IncA/C	<u>CMY-4</u>, TEM-1, SHV-1, CTX-M-15	This study
K. pneumoniae	Italy	NT	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	*smr*	IS*Pa21*	unknown	+	IncA/C	<u>CMY-4</u>	(Colinon et al. 2007)
*K. pneumoniae**	Tunisia	NT	VIM-4	*aacA7*	*dfrA1*	*ΔaadA1*	ND	ND	>130kb	+	NT	<u>CMY-4</u>, TEM-1, CTX-M-15	(Ktari et al. 2006)
*K. pneumoniae**	Hungary	ST15	*aacA4*	VIM-4	-	-	-	*qacEΔ1*	unknown	NT	NT	TEM-1, SHV-28, CTX-M-15	(Melegh et al. 2014)
K. pneumoniae	Greece	ST383	VIM-4	-	-	-	-	-	175kb	-	NT	CMY-4, KPC-2	(Papagiannitsis et al. 2010)

* Outbreak isolates NT: not tested, RT: replicon type, [a]Enzymes underlined are localized on the VIM-plasmid

VI. CONCLUSIONS

- VIM-producer *Enterobacteriaceae* are present in the Arabian Peninsula, although their prevalence among carbapenemase producers is low in Saudi Arabia, Oman and the UAE.
- All VIM-producer *Enterobacteriaceae* investigated in this study carried the VIM-4 type, which is more efficient in hydrolyzing the carbapenems than the VIM-1 endemic in Greece and Italy.
- VIM-4 producer *Enterobacter cloacae* carrying the bla_{VIM-4} on similar, but not identical genetic structures, were isolated in all four countries investigated.
- The bla_{VIM-4} bearing class I integron structures identified in the study are similar to those reported from North Africa and Italy suggesting the possibility of the spread these resistance genes from the Mediterranean region to the Gulf.
- The high incidence of VIM-producer *Enterobacteriaceae* encountered in Kuwait was not due to the clonal spread of a strain, but most probably the result of spreading of an IncA/C plasmid, co-harboring bla_{VIM-4} and bla_{CMY-4}, into *Klebsiella pneumoniae* and *E. coli*.

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في جميع الحالات كان جين الVIM-4 يقع على بلازميدات من أحجام مختلفة والتي كانت إمّا من نوع IncA/C او غير محدّدة النوع، معظمها اقترانية و تحمل جينات ß-lactamases مختلفة مثل CTX-M أو CMY-4. و في جميع السلالات كان جين ال VIM-4 موجودا" في class-I integron مع بعض الاختلافات بين تتابع الجينات المتواجدة والذي يشابه لسلالات قد سبق تحديدها في شمال أفريقيا وإيطاليا مما يقترح احتمال وجود إنتشار.

وكشفت دراسة الاستنساخ أنّ ارتفاع نسبة *Enterobacteriaceae* المنتجة ل VIM-4 و الموجودة في الكويت لم يكن بسبب انتشار نسخة معيّنة، ولكن كان على الأرجح نتيجة لنقل بلازميد IncA/C، الذي يحمل جينات ال bla_{VIM-4} وbla_{CMY-4}، في سلالتيْ ال*Klebsiella pneumoniae* و *Escherichia coli*.

و قد أظهرت الدّراسة أنّ نوع الVIM هو الثالث من حيث درجة الشيوع بعد OXA-48 و NDM في شبه الجزيرة العربية لذلك يُطلب المزيد من المراقبة لرصد انتشار هذا النّوع من العيّنات والجينات في المنطقة.

ملخّص عربي

إنّ ظاهرة انتشار سلالة ال*Enterobacteriaceae* المقاومة لمضادات الـcarbapenems أصبحت من الظواهر التي تستدعي الأنتباه العالمي بما في ذلك في منطقة الشرق الأوسط وهذا يعود لقدرتها على الحدّ من خيارات العلاج و زيادة معدّلات الوفيّات. و يعدّ إنتاج إنزيمات الcarbapenemases المختلفة أهمّ آليّات هذه المقاومة.

أمّا بالنّسبة لشبه الجزيرة العربية،لقد تمّ الأفادة حتّى الآن عن وجود أنزيم NDM و OXA-48 في حين أنّ أنزيمات،IMP ،VIM وKPC منتشرة في أماكن أخرى. لذلك كان هدفنا البحث بشكل منهجي عن أنزيم VIM بين العيّنات المحلية و دراسة خصائصهم الوراثية.

في البداية،أدّى فحص العيّنات المأخوذة من مستشفيات أبو ظبي إلى تحديد عيّنة واحدة من سلالة *Enterobacter cloacae* تحمل جين الVIM-4 و التي كانت الأولى من نوعها في منطقة شبه الجزيرة العربية. ومن ثمّ أدّى البحث في مجموعة أخرى من عيّنات تمّ تجميعها من دول الكويت، المملكة العربية السعودية، سلطنة عمان والإمارات العربيّة المتّحدة إلى تحديد المزيد من السلالات التي تحمل نفس الجين وهي عبارة عن عيّنة واحدة من سلالة *E. cloaceae* من المملكة العربية السعودية، إثنتين من سلطنة عمان، وعيّنة واحدة من الكويت. كما تمّ إيجاد عيّنة *Escherichia coli* في الكويت تحمل أيضا" جين ال VIM-4 .

من الجدير بالذّكر أنّه تمّ الكشف عن ست عينات من *Klebsiella pneumoniae* في دولة الكويت كلها تنتج أنزيم ال VIM و قد وجد أنّ كلّ العيّنات تحمل المتغيّرVIM-4 للجين الذي جرى تحديده من خلال دراسة التسلسل الجيني.

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الخصائص الجزيئية لأنزيم VIM في شبه الجزيرة العربيّة

نور يحفوفي

أطروحة مقدمة كجزء لاستكمال متطلبات الحصول على درجة ماجستير العلوم الطبية
في
علم الأحياء الدقيقة الطبية والمناعة

بإشراف
أ.د.تيبور بال

فبراير ٢٠١٤