CHARLES UNIVERSITY
Faculty of Medicine in Pilsen
Department of Microbiology

DISSERTATION THESIS

Molecular epidemiological analysis of carbapenem-resistant isolates of *Enterobacteriaceæ* and *Pseudomonas* spp.

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

In Pilsen, 17.6.2018

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Abstract

Currently, the development of bacterial resistance is one of the major healthcare problems. Especially, the significant continuous increase of carbapenem-resistant Gram-negative bacteria, which most often affected seriously ill hospitalized patients, is a cause for great concern. Worldwide dissemination of carbapenemase-encoding genes is largely associated with mobile genetic elements and the clonal spread of high-risk clones.

The dissertation thesis is focused on a molecular-epidemiological mapping of carbapenemase-positive Gram-negative isolates detected in hospital settings throughout the Czech Republic.

Since 2015, a significant increase of carbapenemase-producing *Enterobacteriaceæ* (CPE) was detected in our country, mainly attributed to the dissemination of OXA-48 and NDM enzymes. Studies focusing on the first big outbreaks as well as sporadic cases of OXA-48 and NDM carbapenemases were performed. Results showed that $bla_{\text{OXA-48}}$-carrying plasmids, which are derivatives of the archetypal IncL plasmid pOXA-48 originally described in Turkey, play a major role in the dissemination of OXA-48 enzymes in Czech hospitals. This finding is in agreement with the data from previous studies reported worldwide. The study of NDM-positive isolates revealed that IncX3 plasmids are the main factor contributing to the spread of NDM-like enzymes in the Czech Republic. The increasing incidence of NDM-positive isolates in Czechia is in concordance with the extremely successful spread of bla_{NDM} genes detected worldwide. *In vivo* horizontal gene transfer of genes encoding for carbapenemases was observed in 4 and 3 patients infected or colonized by OXA-48 or NDM producers, respectively. Moreover, several novel derivatives of mobile genetic elements were identified during both studies.

The GES-5 and IMI-2 carbapenemases are sporadically reported from clinical settings. However, the association with mobile genetic elements should be a warning sign highlighting possible rapid dissemination. The first cases of IMI-2-producing *Enterobacter asburiæ* and GES-5-producing *Enterobacter cloacæ* identified in the Czech Republic in 2016 were described. Both isolates were obtained from patients without previous traveling abroad, lacking the obvious source of origin which indicates possible silent dissemination via unrecognized ways.

Carbapenemase-producing *Pseudomonas æruginosa* isolates have been frequently reported from Czech hospitals. First nationwide surveillance of carbapenemase-positive *P. æruginosa* isolates detected during 2015 throughout the hospitals in the Czech Republic was performed, including deep molecular genetic typing. The vast majority of the isolates harbored $bla_{\text{IMP-7}}$ genes, and sequence type ST357 was the most prevalent. The phylogenetic analysis indicates that IMP-7-producing ST357 *P. æruginosa* isolates, recovered from different hospitals throughout the Czech Republic, were closely related. The study highlighted the importance of dissemination of high-risk clones in the Czech Republic.

Very few antibiotic options are left for patients infected with multidrug-resistant Gram-negative bacilli when resistance to carbapenems is concurrently expressed. Not rarely, bacterial isolates resistant to almost all available antibiotics are detected, which complicate therapy and significantly limit treatment

options. There is an urgent need for comprehensive intervention. Strict adherence to epidemiological precautions and an active approach of individual states, as well as international organizations, is essential. Epidemiological surveillance and molecular genetic typing at a national level can significantly contribute on the deep understanding of the rapid spread of carbapenemases, point out to possible ways of dissemination, and thoroughly map the evolution of carbapenemase-producing isolates frequently contributing to their ongoing successful dissemination.

The results of this dissertation thesis are summarized in 5 manuscripts which have been published in journals with impact factor, one of these studies is the first-author publication.

Abstrakt

Rozvoj a šíření antibiotické rezistence představuje jeden z nejzávažnějších medicínských problémů současnosti. Pokračující vzestup incidence karbapenem-rezistentních gramnegativních izolátů, které často postihují vážně stonající pacienty, je reálným důvodem k obavám. Celosvětová diseminace karbapenemáz je významně spojena s přenosem genů rezistence prostřednictvím mobilních genetických elementů a klonálním šíření epidemiologicky úspěšných kmenů.

Zaměřením této disertační práce je molekulárně-epidemiologická analýza gramnegativních karbapenemáza-pozitivních kmenů zachycených v nemocnicích na území České republiky.

Od roku 2015 byl v České republice zaznamenán signifikantní nárůst incidence karbapenemáza-pozitivních *Enterobacteriaceæ* (CPE), převážně způsobený rozšířením enzymů OXA-48 a NDM. Podařilo se nám zmapovat první velké epidemické epizody a sporadické izoláty detekované v ČR. Výsledky studie zaměřená na enzymy OXA-48-like prokázaly, že plasmidy nesoucí gen $bla_{\text{OXA-48}}$, které byly identifikovány jako deriváty archetypálního IncL plazmidu původně detekovaného v Turecku, hrají hlavní roli v šíření enzymu OXA-48 v českých nemocnicích. Toto zjištění je ve shodě s výsledky publikací z ostatních zemí světa. Studie zabývající se NDM-pozitivními izoláty prokázala, že IncX3 enzymy představují hlavní faktor přispívající k šíření NDM-like enzymů v České republice. Zvyšující se počet NDM-pozitivních izolátů v ČR odpovídá celosvětově úspěšné diseminaci genů bla_{NDM}. *In vivo* horizontální genový přenos byl prokázán v případě 4 pacientů infikovaných nebo kolonizovaných izoláty s produkcí enzymu OXA-48 a u 3 pacientů nesoucích NDM-pozitivní izoláty. Pomocí výsledků celogenomové sekvenace bylo identifikovány několik nových variant mobilních genetických elementů nesoucích geny $bla_{\text{OXA-48}}$ a bla_{NDM}.

Izoláty exprimující karbapenemázy GES-5 a IMI-2 jsou dosud detekovány v klinických vzorcích sporadicky. Nicméně, jejich lokalizace na mobilních genetických elementech by měl být výstražným signálem možné hrozící diseminace. Byly popsány první detekované izoláty *Enterobacter asburiæ* produkujícího enzym IMI-2 a *Enterobacter cloacæ* exprimujícího enzym GES-5, které byly identifikovány v roce 2016 v českých nemocničních zařízeních. Oba izoláty byly získány od pacientů bez předchozí cestovatelské anamnézy. Vzhledem k nejasnému zdroji původu, lze uvažovat o možném dosud neidentifikovaném zdroji těchto izolátů, který může hrát významnou roli v jejich nerozpoznaném šíření.

Karbapenemáza-pozitivní izoláty *Pseudomonas æruginosa* jsou v posledních letech frekventně detekovány z klinických vzorků českých nemocnic. Byla zrealizována první národní surveillance, zahrnující podrobnou molekulárně genetickou typizaci, izolátů *P. æruginosa* s produkcí karbapenemáz, které byly detekovány v roce 2015 napříč nemocnicemi v ČR. Naprostá většina izolátů disponovala genem $bla_{\text{IMP-7}}$ a majoritní část patřila k sekvenčnímu typu ST357. Fylogenetická analýza prokázala blízkou příbuznost IMP-7 pozitivních izolátů detekovaných v různých geografických částech České republiky. Tato studie poukazuje na obrovský význam klonálního šíření epidemiologicky úspěšných kmenů *P. æruginosa* v České republice.

Terapeutické možnosti infekcí způsobených multirezistentními gramnegativními bakteriemi se současnou produkcí karbapenemáz, jsou velmi omezené. Stále častěji jsou detekovány izoláty rezistentní téměř ke všem dostupným antibiotikům, což významně komplikuje léčbu. Je nutná komplexní intervence této problematiky. Striktní dodržování epidemiologických opatření a aktivní přístup jednotlivých státu, stejně tak jako nadnárodních organizací, se jeví jako klíčové. Epidemiologická surveillance a molekulárně genetická analýza izolátů v postižených státech může signifikantně přispět k hlubšímu porozumění rychlého šíření karbapenemáz, poukázat na možné zdroje diseminace a důsledně zmapovat evoluci karbapenemáza-pozitivních izolátů, která může hrát významnou roli v šíření genů karbapenemáz.

Výsledky předkládané disertační práce jsou shrnuty v 5 publikacích, které byly publikovány v impaktovaných časopisech, jedna z uvedených prací je prvoautorská.

List of abbreviations

AMR	Antimicrobial resistance
ATB	Antibiotic
CPE	Carbapenemase-producing *Enterobacteriaceæ*
CPP	Carbapenemase-producing *Pseudomonas æruginosa*
CS	Conserved sequence
DDST	Double-disc synergy test
EARSS	European Antimicrobial Resistance Surveillance System
EDTA	Ethylenediaminetetraacetic acid
ESAC	Extended-spectrum cephalosporinase
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EuSCAPE	European survey on carbapenemase-producing *Enterobacteriaceæ*
GI	Genomic island
ICU	Intensive care unit
IDSA	Infectious disease Society of America
IEF	Isoelectric focusing
IS	Insertion sequence
KPC	*Klebsiella pneumoniæ* carbapenemase
MALDI-TOF MS	Matrix-assisted laser desorbtion ionization-time of flight mass spectrometry
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
MER-S	Meropenem-susceptible
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
N-CPP	Non-carbapenemase-producing *Pseudomonas æruginosa*
NDM	New Delhi metallo-β-lactamase
NRL	National Reference Laboratory for Antibiotics
OMP	Outer membrane protein
ORF	Open reading frame
PBP	Penicillin binding protein
PBRT	PCR-based replicon typing
PCR	Polymerase chain reaction
PDR	Pandrug-resiatnt
PFGE	Pulsed field gel electroforesis
ST	Sequence type
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo-β-lactamase
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant

Summary

1 Introduction

1.1 Clinical significance of antimicrobial resistance

Antibiotics are probably one of the most successful drugs in human history. Before the antibiotic era, incredibly high number of people died as a result of various infectious diseases. These drugs have saved countless lives and have significantly extended expected lifespan. It is hard to imagine how much they influenced our whole society. Antimicrobial resistance (AMR) is a natural process, detected since the first antibiotics came to the clinical practice. Moreover, the genes carrying drug resistance occur even much earlier than the antibiotics started to be an indispensable compound of clinical medicine. An enzyme penicillinase was described by Abrahams and colleagues before the clinical use of penicillin, but its prevalence in *Staphylococcus aureus* was uncommon. Rapid dissemination was subsequently detected after the introduction of penicillin to human medicine, and at the end of the 1940s about 50% of *S. aureus* isolates in Great Britain were penicillin resistant.[1]

Overuse and misuse of antibiotics over the last decades caused, that increasing rate of AMR development and spread has begun to be an emerging worldwide problem. Recently, the term "superbugs", denoting microbes with enhanced morbidity and mortality due to high levels of resistance to the antibiotic classes specifically recommended for their treatment, started to be widely used. The therapeutic options for these microbes are remarkably reduced, and periods of hospital care are extended and costlier.[2] Another alarming fact is that extremely resistant bacteria, which were predominantly associated with the hospital environment, are now detected even in community settings.

The Review on Antimicrobial Resistance chaired by Jim O'Neill calculated, that about 700,000 people die every year from drug-resistant strains of common bacteria, HIV, tuberculosis, and malaria - this number is likely to be underestimated due to poor reporting and surveillance in some parts of the world. According to their propositions, this number will increase to 10 million deaths attributed to AMR by 2050, unless there is a fundamental change in antibiotic policy.[3] The critical data include the situation in Europe and in the United States as well. In Europe 25,000 people die each year related to MDR bacteria, costing the European Union €1.5 billion annually. A similar number of deaths, 23,000, are attributed to the United States, which is the consequence of more than 2 million infections caused by antibiotic-resistant bacteria.[4]

For infections, which were once considered to be well curable, we suddenly miss therapeutic options. However, the impact is even far more complex, the impossibility of treating infectious complications of for example immunosuppressive therapy of cancer, transplantation medicine or chronic disease such as diabetes, can significantly influence the progression of clinical medicine.

Lack of new antibiotics development is another fact playing an essential role. Resistance has eventually been seen to nearly all antibiotics that have been developed. From the 1960s to 1980s, many new antibiotics were introduced to solve the resistance problem, but after that only few new drugs were included in clinical practice[5] Investment of pharmaceutical companies in the development of new antibiotics does not appear to be economically profitable. Antimicrobial drugs are usually used for a

relatively short period and are generally low cost in comparison with drugs for chronic diseases such as diabetes, psychiatric or neuromuscular disorders, and oncology treatment. Moreover, new antibiotics are usually considered as "last-line" drugs used in special cases, which means that they can easily get off-patent before returning expenses of challenging research and clinical trials.[5] However, probably the most important fact is that, the emergence of resistance to new agents is nearly inevitable, and the rate of AMR development and dissemination is unpredictable.[6]

Many studies have demonstrated a direct connection between antibiotic consumption and emergence of bacterial resistance. Misuse and over-prescription of antimicrobial drugs have led to a rise in resistant strains.[7] Up to 50% of all antibiotics prescribed in human medicine of the United States are not correctly indicated or are not optimally effective as prescribed.[8] Many states allow easy access to antibiotics, even without prescription. There is an acute need for global intervention. The setting of a rational stewardship program, focusing on rapid diagnosis detection, changing of prescribing policy, optimizing therapeutic regiments and preventing bacterial transmission should be the core components to improve the situation.

An early effective antibiotic therapy appears to play a key role in sepsis/septic shock management. The Surviving Sepsis Campaign recommends administration of empiric intravenous antimicrobial therapy within one hour for both sepsis and septic shock.[9] Delayed antimicrobial therapy beyond 3 hours was established as an independent risk factor for mortality and prolonged organ failure in pediatric septic patients.[10] Many studies suggest that hospital mortality of septic patients associated with an-inappropriate ATB therapy is 2 to 4 times higher compared to appropriate treatment.[11] Resistance is frequently associated with delay of proper antimicrobial therapy, and moreover, patients with multidrug-resistant (MDR) infections have usually risk factors such as more severe underlying illness, which contribute to worst outcomes.[1]

Bacterial resistance can be intrinsically encoded, acquired by mutations in chromosomal genes, or obtained by horizontal gene transfer. Inefficacy of certain antimicrobial agents can be caused by specific mechanisms, which can be divided into three main categories: first, mechanisms leading to the inability to achieve the necessary intracellular concentration of the drug caused by poor penetration or antibiotic efflux; second, modification of the antibiotic target by genetic mutation or post-translation modification of target structures; third, antibiotic inactivation by hydrolysis or modification.[12] According to international standardized terminology, multidrug-resistant (MDR) is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, extensively drug-resistant (XDR) was characterized as resistance to at least one agent in all but two or fewer antimicrobial agents are remaining, and pandrug-resistant (PDR) as non-susceptibility to all agents in all antimicrobial categories (Figure 1).[13]

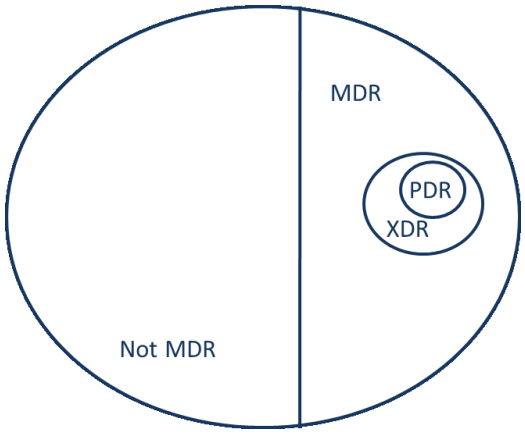

Figure 1. Diagram showing the relationship of MDR, XDR and PDR to each other [according to Magiorakos, 1]

Several epidemiological studies, in Europe as well as in the United States, highlight the importance of Gram-negative pathogens in hospital setting, especially in the critically ill patients.[14] Gram-negative pathogens caused 45–70% ventilator-associated pneumonia, 20–30% catheter-related bloodstream infections, and are frequently associated with other intensive care unit-acquired sepsis, such as surgical site or urinary tract infections.[15,16,17] A dramatic worldwide increase of infections caused by multidrug-resistant Gram-negative bacteria is a reason for great concern because of the ability to become resistant to (nearly) all available antimicrobial agents.[18] The emergence of MDR/XDR/PDR Gram-negative bacteria has significantly affected clinical practice.

Enterobacteriaceæ, *Pseudomonas æruginosa*, and *Acinetobacter* frequently cause the most serious infections. Multiple antimicrobial resistance in non-fermenting Gram-negative bacteria can be caused by chromosomal mutations leading to overproduction of intrinsic β-lactamases, hyper-expression of efflux pumps, permeability alterations, and target modifications. However, especially the dissemination of resistance genes, including the spread of carbapenemases, by mobile genetic elements is becoming a cause for great concern. The accumulation of multiple resistance mechanisms can leads, and nowadays not extraordinary, even up to pan-drug-resistant phenotype.[19]

The successful expansion of MDR resistant *Enterobacteriaceæ* is significantly connected to horizontal gene transfer of resistance genes, which is one of the most efficient ways leading to global dissemination of antimicrobial resistance. Moreover, resistance genes and associated insertion elements localized on plasmids are often concentrated in large multi-resistance regions providing resistance to multiple different antimicrobial agents, so that a single plasmid conjugation may confer to multidrug-resistant phenotype.[19,20] The dissemination of plasmid-borne β-lactamases is the most redoubtable resistance mechanism in *Enterobacteriaceæ*. A significant increase in the resistance to 3rd and 4th generation cephalosporins in *Enterobacteriaceæ* has emerged worldwide, mostly caused by the progressive spread of plasmid-mediated extended spectrum β-lactamases (ESBLs). The number of isolates resistant to 3rd generation cephalosporins in European countries is constantly above 10%, and reach over 70% in certain areas.[21] ESBL producing strains currently represent 15–25% of *Enterobacteriaceæ* isolates obtained from clinical samples of intensive care unit (ICU) patients.[19] In a situation, where carbapenems are often the

last remaining option is the alarming spread of carbapenemases even more worrisome. The prevalence of resistance to carbapenems is 2–7% in ICUs in Europe, Asia, and the United States. The situation is especially alarming for *Klebsiella pneumoniæ*, with overall carbapenem resistance of invasive isolates counting 33.9% in Italy, or 66.9% in Greece.[21] According to European survey on carbapenemase-producing *Enterobacteriaceæ* (EuSCAPE), in 2015, 13 out of 38 participating countries reported interregional spread or an endemic situation for carbapenemase-producing *Enterobacteriaceæ* (CPE), compared with 6 countries in 2013 (Figure 2,3).[22] CPE infections and colonization are generally health-care associated, although increasing evidence of community spread is beginning to emerge.[23,24]

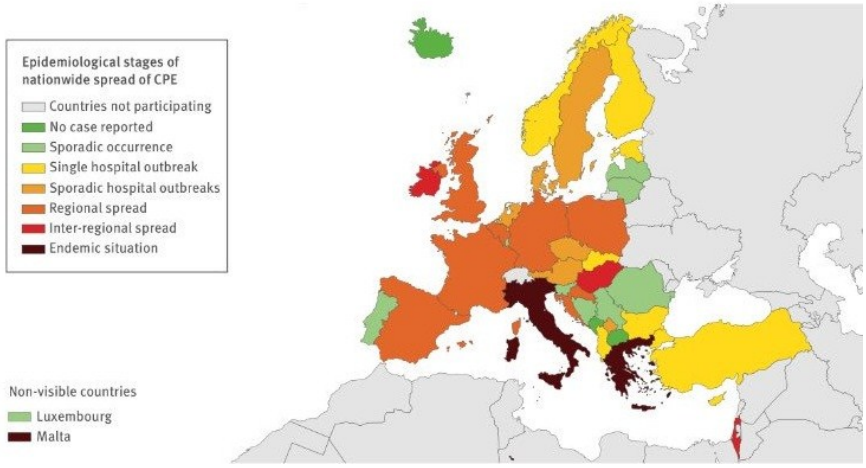

Figure 2. Occurrence of carbapenemse-producing *Enterobacteriaceae* in 38 European countries based on self-assessment by the national experts, March 2013 [2]

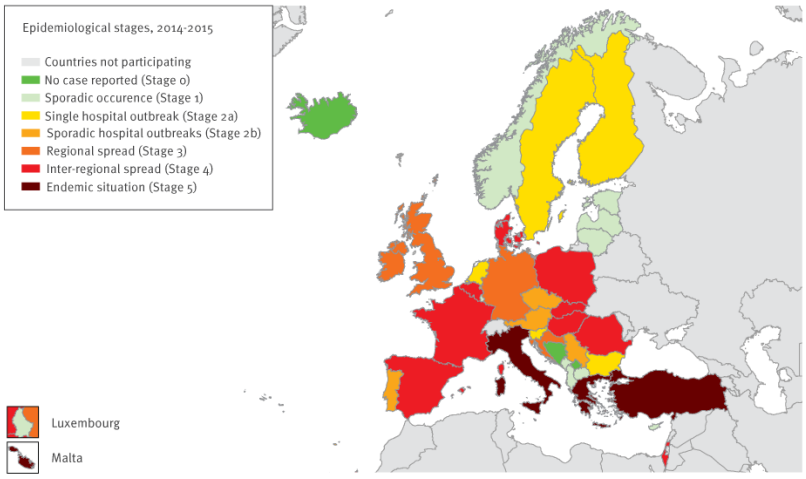

Figure 3. Occurrence of carbapenemse-producing *Enterobacteriaceae* in 38 European countries based on self-assessment by the national experts, May 2015 [3]

Zildberger et al. showed that sepsis caused by MDR Gram-negative pathogens was the strongest predictor of inappropriate treatment, which is an essential risk factor for short-term mortality among patients with Gram-negative sepsis/septic shock.[25] According to Lautenbach et al., patients with ESBL-producing *K. pneumoniæ* and *E.coli* infection were treated with appropriate antibiotics on average for 72 hours after infection was suspected, compared to 11.5 hours attributed to non-ESBL-producers.[26] The

initial response to antimicrobial therapy reveals that treatment failure rates for ESBL-producing *K. pneumoniæ* infection are almost twice as high as for non-ESBL strains.

Infections caused by CPE have approximately a 2- to 5-fold higher risk of death compared to carbapenem susceptible strains, and in-hospital mortality of CPE infections is unacceptable high, counting 48–71%.[27,28] Prospective cohort study of Dautzenberg et al. demonstrated an association of CPE colonization with 1.79 times higher overall hazard of mortality in ICU patients, primarily caused by the increased length of stay.[29]

Few treatment options are remaining for carbapenemase-producing Gram-negative bacteria, due to the ability to hydrolyze most other β-lactam antibiotics, as well as the frequent coexistence of additional mechanisms of resistance against other antimicrobial classes such as fluoroquinolones and amino-glycosides.[30] As the continuous spread of MDR Gram-negative bacteria throughout the world is being observed, and the lack of effective antibiotic treatment complicates daily patient care, we can still more frequently hear the question: "Are we in post-antibiotic era"? Unfortunately, it seems that we are not so far.

1.2 The hypothesis of the dissertation thesis

- Antimicrobial resistance of Gram-negative bacteria is currently one of the most serious medical problems. Carbapenems are considered as "last resort" drugs possessing activity against many MDR Gram-negative bacteria. The rapid spread of carbapenemases (enzymes capable of hydrolyzing carbapenems) significantly affected their medical use. Treatment options of infections caused by carbapenem-resistant isolates are alarmingly limited.
- Consistent and proper surveillance of carbapenem resistant isolates can help us to reveal possible risk factors associated with dissemination of carbapenem-resistance mechanisms and systematically work on the improvement of current epidemiological situation.
- One of the most successful ways leading to the spread of carbapenem-resistance is horizontal gene transfer of genes encoding for carbapenemases. Deeper understanding of genetic aspects associated with carbapenemase genes can be beneficial for better understanding of their successful spread and mechanisms of bacterial evolution.

1.3 The aim of the dissertation thesis

- Systematic diagnostic, analysis, and surveillance of carbapenem-resistant strains of *Enterobacteriaceæ* and *Pseudomonas æruginosa* detected in the Czech Republic, focusing on clinical isolates.
- Molecular-genetic analysis, including whole-genome sequencing, of carbapenemase-producing isolates, focusing on the most disseminated carbapenemase variants, mapping of the hospital outbreaks, and first cases of rare carbapenemases detected in the Czech Republic.
- Identification of novel genetic features associated with carbapenemase genes.
- Comparison with molecular-epidemiological data from other countries, monitoring of possible epidemiological link.

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2 Mechanisms of carbapenem-resistance in Gram-negative bacteria

2.1 Carbapenems

Carbapenems, the most broad-spectrum β-lactam antibiotics, play a critically important role in the treatment of infections caused by multidrug-resistant bacteria. These antimicrobial agents possess a great activity against many Gram-negative, Gram-positive and anaerobic bacteria. Consequently, they should be used as "last-line antibiotics" dedicated to seriously ill patients suspected of harboring resistant bacterial isolates. However, ongoing dissemination of multidrug-resistant pathogens seriously threatens this class of lifesaving drugs.[1] Significant continuous increase in carbapenem resistance is obvious throughout the world.

Carbapenems enter Gram-negative bacteria through outer membrane proteins (OMPs) – porins. In the periplasmic space, they irreversibly acylate the penicillin binding proteins (PBSs). This irreversible inhibition of the PBSs prevents the final transpeptidation of the nascent peptidoglycan layer in the bacterial cell wall. Under normal circumstances, peptidoglycan precursors signal a reorganization of the bacterial cell wall and consequently trigger the activation of autolysis hydrolases. An affection of cross-linking by β-lactam antibiotics results in the creation of peptidoglycan precursors triggering the autolysis of existing peptidoglycan without the formation of the new one.[2] A major fact resulting in the broad-spectrum activity of carbapenems is the ability to bind to multiple different PBPs.[3] Moreover, carbapenems can also work as a "slow-substrates" or inhibitors of non-carbapenemase β-lactamases, providing them a unique feature of "dual function" (PBPs and β-lactamase inhibition).[1,4]

2.2 Carbapenem resistance in *Enterobacteriaceæ* and *Pseudomonas æruginosa*

Enterobacteriaceæ are Gram-negative rod-shaped bacteria, which are part of normal intestinal flora, and also one of the most common human pathogens, able to cause a variety range of infections. This bacterial family represents one of the most common causative agents of hospital- as well as community-acquired infections. *Enterobacteriaceæ* spread easily between humans by e.g., hand carriage or contaminated food and water. Moreover, the ability of acquisition of different resistance genes via horizontal gene transfer, mostly mediated by plasmids and transposons, is a reason for great concern.

Two main mechanisms are responsible for carbapenem resistance in *Enteobacteriaceæ*: first, reduced antibiotic uptake caused by changes in porin expression associated with overexpression of β-lactamases possessing a weak carbapenemase activity; second, acquisition of carbapenemase genes encoding for enzymes causing carbapenem degradation (Figure 1).[2]

Pseudomonas æruginosa has become one of the most clinically important opportunistic pathogens, possessing intrinsic resistance to a wide variety of antimicrobial agents. Although carbapenem resistance in *P. æruginosa* is usually caused by changes in porins and e fflux pumps expression, production of carabapenemases is increasingly reported worldwide.

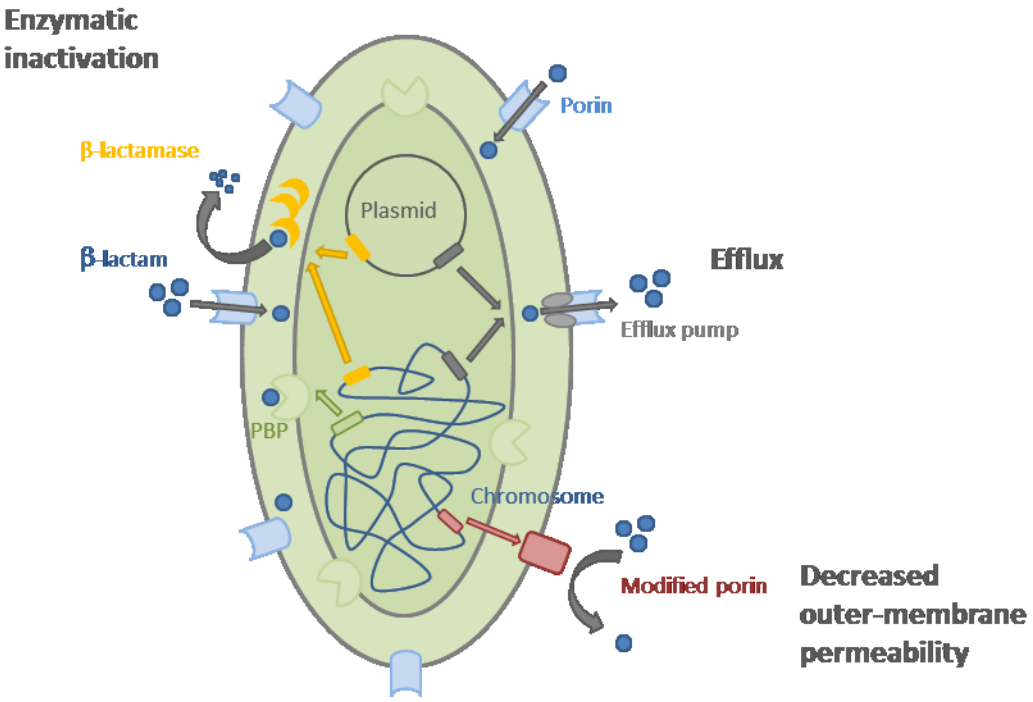

Figure 1. Primary mechanisms of β-lactam resistance in *Enterobacteriaceae* [according to Nordmann, 1]

2.2.1 Non-carbapenemase carbapenem resistance in *Enterobacteriaceæ*

2.2.1.1 Porins

All nutrients or antibiotics (hydrophilic, as well as hydrophobic) have to cross the outer membrane of Gram-negative bacteria, which constitute a hydrophobic barrier protecting the cell against external agents. The outer membrane contains various proteins forming hydrophilic channels, called porins, enabling an influx of essential nutrients and other molecules, including antibiotics. Reduction of influx through porins, caused by changes in porins number or activity, can strongly affect antimicrobial resistance. The efficiency of certain antibiotics, e.g., β-lactams and fluoroquinolones, is significantly dependent on the bacterial influx.

Several types of porins have been found in Gram-negative bacilli, classified according to their activity (specific or non-specific), functional structure (monomeric or trimeric), and regulation and expression.[5,6] Studies of three major *E. coli* trimeric porins (OmpF, OmpC, PhoE) formed current knowledge of many other porins, hence the designation as "classical porins". Most porins that participate in antibiotic transport in *Enterobacteriaceæ* belong to the OmpF or OmpC subfamilies.[5] These porins have general preferences for charge and size of the solute, favoring cations slightly over anions.[7]

The bacterial sensitivity to antibiotics can be decreased by a shift in the type of outer membrane porins, a change in the level of porin expression, and mutation or modification that impairs the functional attributes of porin channels (Figure 2).[5] Porin synthesis can be influenced by antimicrobial compounds or aromatic products via several cascades including the multiple antibiotic resistance (*mar*)operon,

causing subsequent decrease of number of outer membrane porins.[7,8] This locus is able to modulate efflux pump and porin expression by two encoded transcription factors, MarR and MarA, conferring resistance in enteric bacteria. The mar locus was identified as a cross-resistance to tetracyclines, fluoroquinolones, and β-lactams.[8]

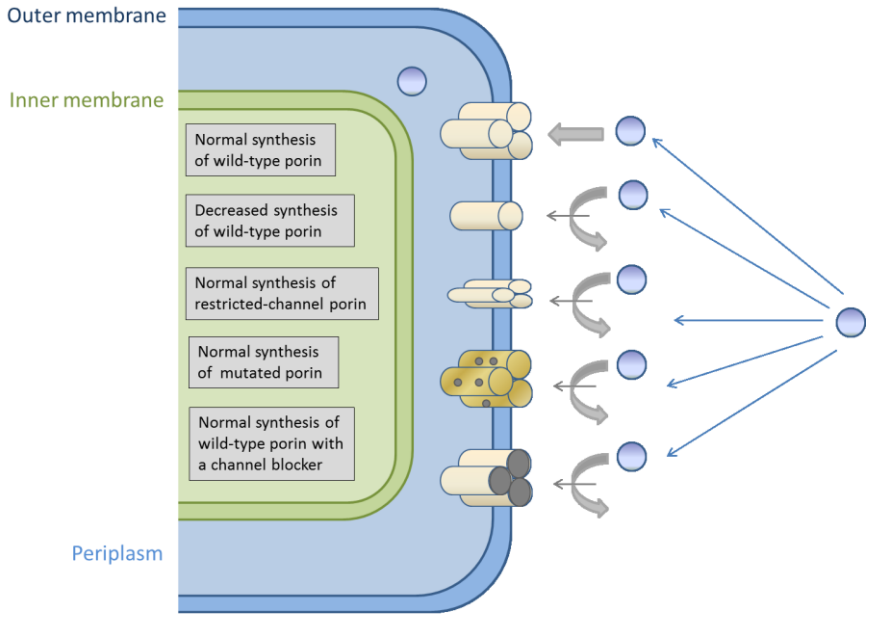

Figure 2. Multidrug resistance mechanisms associated with porin modification [according to Pages, 2]

Several studies have indicated a radical alteration of porin phenotype occurring during antibiotic therapy.[6,9,10] Prolonged exposure to sub-inhibitory antibiotherapy can selects porin-expression modifications, resulting in reduced influx.[6] Hasdemir et al. demonstrated changes in outer membrane permeability of *K. pneumoniæ,* caused by porin group expression in patients undergoing antibiotic treatment. OmpK35, a large size channel which belongs to OmpF porin group, was replaced by OmpK36 in most of the isolates during antibiotic therapy. OmpK36 is a member of OmpC porin group, characterized by significantly smaller channel size.[11] Doménech-Sanchéz et al. observed 4-8 times increased level of susceptibility to certain β-lactams (e.g., cefepime or cefotaxime) in strains expressing OmpK35, compared to OmpK36.[12] A study of Bornet et al. proved the effect of imipenem therapy on decreased porin expression and subsequent imipenem resistance in clinical isolates of *Enterobacter aerogenes*. Moreover, restoration of imipenem susceptibility was observed in isolates recovered a few days after the termination of treatment. [13]

The study of carbapenem-nonsusceptible isolates of *K. pneumoniæ* detected in Czech Republic from January 2007 to June 2008 showed lack of OmpK36- and reduced OmpK35-expression combined with ESBL and/or AmpC production. Carbapenem-susceptible isolates from the same patients, included in the study for comparative reasons, differed from their carbapenem-nonsusceptible counterparts only by porin expression profile.[14] Results of this publication are in agreement with study reported by Martínez-

Martínez indicating the role of OmpK35 deficiency alone or concomitant with that of OmpK36 in increasing the resistance of ESBL- or AmpC-producing *K. pneumoniæ* isolates.[15]

The correlation between antibiotic susceptibility and outer-membrane permeability is clearly proven. The diffusion of antibiotic agents through channels is significantly affected by porin quantity and structure, which has severe consequences on the intracellular concentration of the drug and subsequent clinical effect.

The modification of porin profile is also frequently associated with expression of degradative enzymes, such as AmpC or ESBLs. In addition to the amount and intrinsic activity of β-lactamase, the quantity of substrate which reaches the enzyme is another important determinant affecting the resistance spectrum. The concentration of β-lactam antibiotics in the periplasmic space is strongly influenced by the permeability of bacterial cell membrane, which is connected to porin phenotype.[16] Mentioned combination of different resistance mechanisms efficiently confer a high-level of β-lactam resistance, including carbapenems.[5]

2.2.1.2 Resistance to carbapenems caused by AmpC and Extended spectrum β-lactamases (ESBLs)

β-lactamases belong to the most heterogeneous group of resistance enzymes, counting more than 700 distinct variants.[17] β-lactamases are classified according to two general schemes: the Ambler molecular classification and the Bush-Jacoby-Medieros functional classification.[18,19,20] The Ambler classification divides β-lactamases into four classes (A to D) based on protein homology. Enzymes belonging to classes A to C are characterized as serine β-lactamases, while class B enzymes are metallo-β-lactamases (MBLs). In contrast, Bush-Jacoby-Medieros system is sorted according to functional similarities (substrate and inhibition profile), dividing β-lactamases into four main groups (1 to 4) and multiple subgroups. This system is more clinically focused, based on considering clinically relevant β-lactam substrates and inhibitors.

2.2.1.2.1 The AmpC enzymes

Resistance to carbapenems in *Enterobacteriaceæ* was detected in isolates (over)expressing AmpC enzymes in combination with altered outer membrane permeability. The AmpC enzymes are classified as class C in the structural Ambler system and group 1 according to Bush-Jacoby-Medieros classification.[18,20] Most of the AmpC β-lactamases are cephalosporinases, but are capable of hydrolyzing all β-lactams to some extended-spectrum.[21] Chromosomally determined AmpC β-lactamases may be expressed at a high level following exposure to certain β-lactams, either by induction or selection of derepressed mutants with potential risk of consequent clinical treatment failure. Reporting of *in vitro* susceptibility can be challenging for such isolates. The minimal inhibitory concentrations (MICs) may not correlate with supposed clinical efficiency as resistance can emerge by selection of mutants expressing high levels of AmpC.[22] This aspect has been observed in the context of severe infection caused by *Enterobacter* spp. which had been treated by third-generation cephalosporins.[23] The MIC is significantly affected by the level of enzyme induction and the stability of the drug against hydrolysis.

The induction can be reversed when the β-lactam stimulus is removed.[22] However, there is a risk of constitutive production caused via mutations in the regulatory *ampD* or *ampR* genes (a member of the LysR transcriptional regulator family), leading to derepression of normally low-level expressed AmpC. Overexpression of chromosomally encoded inducible AmpC gene in combination with OmpC and OmpF porin modification is another important cause of carbapenem resistance in enterobacterial isolates, observed frequently e.g., among *Enterobacter* spp. even developed during antimicrobial therapy. Several other Gram-negative bacteria disposal such inducible β-lactamase genes with the potential risk of derepression: *Serratia* spp., *Citrobacter freundi*, *Providentia* spp., and *Morganella morganii*.[2,22] However, numerous of other bacteria possess chromosomally determined AmpC enzymes. The clinical significance and consequent risk of resistance-development depends on concrete bacterial strain and specific genetic features related to *ampC* carriage. Moreover, the ability of different β-lactams to induce *ampC* expression is variable.[24] Certain species (e.g., *E. coli*) express chromosomally mediated *ampC* genes at levels which are not clinically important. This fact is caused by lack of appropriate repressor gene so the expression is not inducible or occurs in negligible amount, however mutation in the *ampC* promoter region can result in constitutive overexpression.[22,25]

Consequently, the therapeutic options for isolates disposing of derepressed/overexpressed AmpCs are limited – usually aminoglycosides, quinolones, carbapenems, trimethroprim/sulfamethoxazol and nitrofurantoin (for urinary tract infections) are remaining.[22] Carbapenems are considered highly effective therapy for species possessing a high probability of AmpC derepression and are frequently chosen due to specific limitations of alternative drugs.[22,26]

Furthermore, emerging increase of transmissible plasmid-harbored AmpC genes has been detected over the past years, particularly in *E.coli* and *Klebsiella* spp. Isolates possessing acquired AmpCs have been observed in hospital as well as community settings. Nevertheless, despite their worldwide dissemination, the overall occurrence still remain far below that of ESBLs.[1,27,28] Plasmid-mediated genes are derived from a small number of chromosomal *ampC* genes, which are usually not associated with repressor genes (such as *ampR*) and are therefore not-inducible. Nevertheless, inducible plasmid-mediated AmpC enzymes, has been described (e.g., DHA).[22,29-31] The acquired AmpCs are usually expressed constitutively, conferring the resistance similar to that of derepression or hyperproducing mutants of natural AmpC producers.[32] Moreover, plasmids carrying AmpC genes commonly associate multiple other antimicrobial resistance determinants.[31] There are several lineages of AmpC genes encoded on mobile genetic elements, originating from natural producers such as *Enterobacter* group (MIR, ACT), the *C. freundi* group (CMY-2-like, LAT, CFE), the *M. morganii* group (DHA), the *Hafnia alvei* group (ACC), the *Aeromonas* group (CMY-1-like, FOX, MOX) and the *Acinetobacter baumannii* group (ABA). The most widespread are the CMY-2-like enzymes, although an extensive spread of the inducible DHA-like β-lactamases and some others have been detected.[32] European Committee on Antimicrobial Susceptibility Testing (EUCAST) proposed several recommendations for AmpC detection, however the difficulties associated with AmpC monitoring in

routine clinical labs caused the lack of information regarding the epidemiology, risk factors and clinical features.[32]

In 2006, the first report focusing on DHA-1 dissemination in nosocomial settings in the Czech Republic was described.[33] Subsequently, in 2009, the first cases of *Proteus mirabilis* expressing CMY-2 AmpC β-lactamase were reported.[34] Thereafter, AmpC enzymes have rapidly spread in Czech hospitals. Moreover, AmpC positive isolates was reported even from community settings.[35]

2.2.1.2.2 The ESBL enzymes

Similarly, ESBLs genes expression combined with altered outer membrane permeability can be responsible for carbapenem resistance in *Enterobacteriaceæ*.[2] The ESBLs are capable of hydrolyzing penicillins, cephalosporins of the first, second, third and fourth generation and the monobactam aztreonam. They are inhibited by clavulanic acid, which serves as an important phenotypic test widely used for ESBL detection.[20,36] ESBLs belong to Ambler´s class A or D serine β-lactamases, in functional Bush-Jacoby-Medieros classification are located in two subgroups of group 2: 2be (extended spectrum β-lactamases, Ambler's class A) and 2d (cloxacillin-hydrolyzing β-lactamases, Ambler's class D), sharing most of the fundamental properties of the 2be subgroup. The first large part of 2be enzymes was derived by amino acid substitution in TEM-1, TEM-2, and SHV-1 β-lactamases, resulting in a broader spectrum of activity. These enzymes have been joined by functionally similar but more rapidly proliferating CTX-M enzymes that are related to chromosomally determined β-lactamases of the species *Kluyvera*. Finally, less common ESBLs (e.g., BEL-1, BES-1, SFO-1, TLA-1, TLA-2, members of the PER and VEB families) unrelated to TEM-, SHV-, or CTX-M are included. The functional group 2 represents the largest group of β-lactamases, mainly due to the increasing identification of new ESBLs variants.[18,20] The total number of ESBLs characterized until now exceed 200. Enzymes are sorted into distinct structural families based on their deduced amino acid sequence (e.g., TEM, SHV, CTX-M, PER, VEB, GES, TLA, BES, OXA).[37]

ESBLs, now disseminated worldwide and detected in most genera of *Enterobacteriaceæ*, was firstly described in Germany (1983) and France (1985) among *Klebsiella* spp.[36,38] The first ESBL isolates were detected in western Europe, possibly in connection with the start of expanded clinical use of extended-spectrum β-lactam antibiotics. Nevertheless, soon after that, ESBLs were detected in the United States and Asia.[38]

The third-generation cephalosporins were introduced in the clinical practice as a reaction on increasing prevalence of certain β-lactamases. One of the main reasons was the dissemination of TEM-1, TEM-2, and SHV-1 β-lactamases, possessing the ability to hydrolyze penicillin, ampicillin, and to a lesser degree carbenicillin and cephalothin. TEM-1 is considered as the most common plasmid-mediated β-lactamase conferring ampicillin resistance in *Enterobacteriaceæ*, whereas SHV-1 is predominantly found in *Klebsiella pneumoniæ*. The only genetic change of the first detected ESBL enzyme (designated SHV-2), compared to the gene encoding SHV-1, constituted a single nucleotide mutation (leading to the replacement of glycine by serine at the 238 position). This change results in a profound shift in the enzymatic activity, providing the ability to hydrolyze extended-spectrum cephalosporins and

aztreonam. Other enzymes with such enzymatic properties, closely related to TEM-1 and TEM-2 β-lactamases, were subsequently identified. The ESBLs differ from their progenitors by as few as one amino acid, hence the extension of the antimicrobial spectrum.[36] Fifteen years after the discovery of SHV-2, these enzymes had spread in every inhabited continent. The selection pressure caused by the wide use of extended-spectrum cephalosporins is considered to be one of the reasons for their successful spread.[36,39]

Data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), formerly European Antimicrobial Resistance Surveillance System (EARSS), indicate continuous increase since 2000 in invasive *E. coli* and *K. pneumoniæ* isolates resistant to third generation cephalosporins. The number of invasive *E. coli* isolates resistant to third generation cephalosporins in 2016 exceed 10% in most of the participating countries. The situation is even worst for invasive isolates of *K. pneumoniæ*, ranging between 10 to >50% in the majority of European countries. Data showed significant geographical differences, ranging from percentage of 4,2% (Iceland) to 41,6% (Bulgary) for *E. coli* and from 0% (Iceland) to 72,5% (Greece) for *K. pneumoniæ*. The prevalence of resistance to extended-spectrum cephalosporins remains low in most northern European countries compared to southern and eastern Europe. Although these proportions are generally associated with ESBL production, the data might be somewhat overestimated due to isolates with AmpC overproduction, which is considered to represent about 1–2% of isolates resistant to third generation cephalosporins.[40]

The dissemination of specific clones, clonal groups and epidemic plasmids in nosocomial, as well as in community settings, has caused the alarming worldwide expansion of ESBLs enzymes belonging especially to the TEM, SHV, and CTX-M families. Outbreaks associated with ESBL infection have been reported from virtually every European country.[36] In the 1990s the most common ESBLs were the TEM and SHV variants, mainly among isolates of *K. pneumoniæ*. However, a dramatic increase of CTX-M enzymes was subsequently noticed in Europe. Now, CTX-M enzymes present the most prevalent ESBLs detected in clinical isolates, with *E. coli* joining *K. pneumoniæ* as a major host, and with increasing number of strains isolated from community settings.[17,41] Nevertheless, the prevalence of predominant ESBL type strongly varies among different geographical locations, enterobacterial species and a source of isolation.

The clonal expansion of epidemiologically successful clones has emerged throughout Europe. Highly virulent *E.coli* O25:H4-ST131 is responsible for pandemic dissemination of the CTX-M-15 enzymes. Moreover, this enzyme has started to be frequently isolated from the community setting, predominantly in association with urinary tract infections (UTIs).[40,41] Other ESBLs variants are detected locally, e.g., CTX-M-3 in eastern countries, CTX-M-9 and -14 in Spain, SHV-12 is one of the most prevalent enzymes connected with nosocomial *K. pneumoniæ* strains in Italy, Poland, and Spain, or TEM-3 and -4 frequently reported from *K. pneumoniæ* isolates from France and Spain. Worryingly, some of such enzymes are being increasingly detected from community settings.[40–42]

The plasmid-mediated dissemination plays an essential role in accelerating the worldwide spread of ESBLs. Plasmids harboring ESBLs genes are highly diverse, generally large (between 50 to 200 kb) and contain many mobile genetic elements enabling the transfer of genes within plasmids or chromosome and plasmid. For instance, *E. coli* plasmids carrying CTX-M genes were found to belong to distinct plasmid families, e.g., IncF, IncN, IncN2, IncI1, IncHI2, IncL/M, IncA/C, IncK, IncX4, IncU.[43] ESBL-producing *E. coli* is frequently associated with IncF plasmids carriage. These plasmids are mobile genetic elements of large size with low copy numbers, narrow host range (disseminating among *Enterobacteriaceæ*), often encoding resistance genes (highly associated with $bla_{\text{CTX-M}}$) and many virulence genes.[44] Compared to other plasmid types, they display higher diversity regarding plasmid size, number of replicons and ability to conjugate due to extensive recombination. Successful spread of *E. coli* sequence type (ST) 131 can be certainly partially attributed to connection with IncFII plasmids.[43] The emergence of epidemic strains harboring multiple plasmids encoding distinct ESBLs, AmpC, and even carbapenemases is a cause of great concern. Such isolates are increasingly reported in many European countries.[40] Moreover, plasmids coding for ESBLs that express a low level of resistance to β-lactams or contain multiple silenced antibiotic resistance genes has been detected. These findings are considerably worrisome in the context of potentially undetected and silent spread of such isolates which can constitute a hidden reservoir of antibiotic resistance, that cannot be detected by phenotype.[45]

Data from the Czech Republic indicate that 15.1% of *E. coli* and 51.8% of *K. pneumoniæ* invasive isolates are resistant to third-generation cephalosporins.[40] Moreover, the study comparing gastrointestinal carriage of ESBL-positive bacteria in hospital and community settings reported increased prevalence in both hospitalized patients (from 3% to 8%) and community subjects (from 1% to 3%) within 3 years (2007 compared to 2010).[46] Molecular-genetic analysis of ESBL-producing clinical isolates obtained in 2017 in hospital in Pilsen (Czech Republic) indicate that CTX-M-15 is the most common variant (personal data). Worryingly, and in concordance with publications reported worldwide, ESBLs and AmpC isolates were detected in czech samples from livestock.[45,47,48]

Production of ESBL enzymes in combination with outer membrane permeability can be responsible for carbapenem-resistance in *Enterobacteriaceæ*. Widely disseminated strain of ESBL-producing *K. pneumoniæ* carrying a novel OmpK36 porin variant (OmpK36V), leading to ertapenem resistance and reduced susceptibility to meropenem has been firstly described in Italy in 2009.[49] A study monitoring carbapenem resistance due to porin loss and ESBL production in *K. pneumoniæ* strain during meropenem therapy has been documented by Webster et al.[50] Experiment of Mena et al. demonstrated the development of *in vivo* resistance to carbapenems in CTX-M-1 producing *Enterobacteriaceæ* due to selection of mutations leading to lack of porin expression.[51] Carbapenems have the most consistent activity against ESBL-producing organisms and often represent the last possible choice of antibiotic treatment. Carbapenem-resistance caused by ESBL enzymes in combination with changes in outer membrane permeability is especially alarming when we consider the pandemic spread of ESBLs throughout the world.

2.2.2 Non-carbapenemase resistance to carbapenems in *Pseudomonas æruginosa*

Pseudomonas æruginosa is an important nosocomial pathogen, frequently affecting seriously ill and immunocompromised patients. This species is noted for its intrinsic resistance to antibiotics, the ability to acquire genes encoding resistance determinants, or accumulation of heterogeneous mutations leading to the development of multiresistant or even pan-resistant phenotype. Carbapenems are among the most important antimicrobials used for the treatment of infections caused by multidrug-resistant strains of *P. æruginosa*, and the development of carbapenem resistance may significantly compromise the therapeutic outcome of affected patients. In the absence of carbapenem-hydrolyzing enzymes, carbapenem resistance is usually caused by multifactorial mechanisms – hyperproduction of certain β-lactamases, reduction in outer membrane permeability caused by reduced porin expression and efflux pumps systems.

Carbapenems get into the periplasmic space of *P. æruginosa* through a 54-kDa outer membrane protein (OMP), usually known as the OprD or D2 porin. The mutational loss of this porin, likely due to inactivation of the OprD gene, is the most common mechanism of resistance to imipenem.[52,53] Moreover, isolates with lost OprD have reduced susceptibility to meropenem, while other β-lactams are not affected.[52] Furthermore, mutational loss of OprD was frequently registered during imipenem therapy.[54] Whereas inactivation of OprD alone can cause resistance to imipenem, the mechanisms conferring the resistance to meropenem is more likely complex and multifactorial including over-production of AmpC-type β-lactamases or changes in efflux pumps systems.

Efflux pumps represent an extremely important mechanism of multidrug resistance in *P. æruginosa*. The multidrug efflux systems serve as an extrusion system for toxic molecules presented in the cytoplasm, cytoplasmic membrane or periplasm. They are tripartite systems composed of an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin, and a linker protein connecting the two membrane components. The most common pump system MexAB-OprM comprises a pump MexB, a linker lipoprotein MexA, and an exit portal OprM.[52] Up-regulation of the MexAB-OprM efflux system caused the resistance to quinolones, the antipseudomonal penicillins, and antipseudomonal cephalosporins, and decreased efficacy of meropenem, while imipenem and aminoglycoside susceptibility are usually not affected. The MexXY-OprN efflux pump system is coregulated with OprD protein. Mutants possessing up-regulation of this efflux system and with reduced OprD are being resistant to multiple antimicrobial agents, including meropenem and imipenem, quinolones, antipseudomonal penicillins and cephalosporins, and aztreonam.[52] Similarly, MexEF-OprN is coregulated with OprD. Thus, mutants of a gene upstream of the MexEF-OprN efflux operon (designated *mexT*), that are occasionally selected by fluoroquinolones (not carbapenems) leads to up-regulation od MexEF-OprN and OprD reduction causing the resistance to fluoroquinolones and imipenem and reduced susceptibility to meropenem.[55] In addition, the MexCD-OprJ and MexXY-OprM efflux systems may also play a significant role in reduced susceptibility to meropenem.[53]

Extended-spectrum cephalosporinases (ESACs) with broadened hydrolytic spectrum toward imipenem have been reported in *P. æruginosa*. Those chromosome-encoded AmpC-type β-lactamases have an alanine residue at position 105, which confer them a slight carbapenemase activity compromising the efficiency of carbapenems in case of enzyme overexpression.[53] The study of Rodríguez-Martínez et al. on a collection of imipenem- and meropenem-nonsusceptible *P. æruginosa* demonstrates a high prevalence of ESAC encoding genes, which probably play an additive role toward reduced susceptibility or resistance to imipenem.[53]

Multidrug resistance, including resistance to carbapenems, was common in *P. æruginosa* in many European countries in 2016. According to EARSS reports the distribution of carbapenem resistance range from the percentage of 2.4% (Denmark) to 51.6% (Romania) (Figure 3).[40] The extensive intrinsic resistance combined with acquired resistance to multiple antimicrobial agents further complicate the treatment of serious infections.

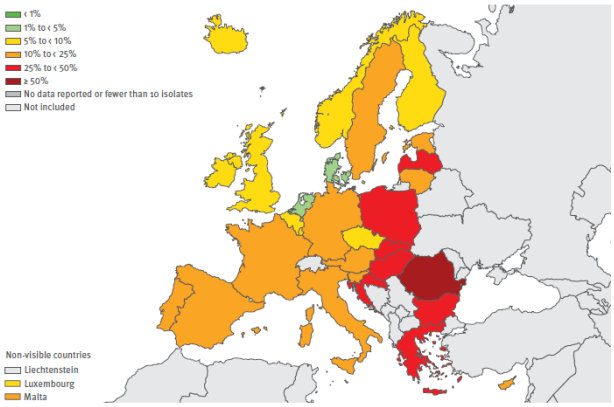

Figure 3. The percentage of invasive isolates of *P. æruginosa* with resistance to carbapenems, 2016 [3]

2.2.3 Carbapenemases

The emergence of resistance to carbapenems in Gram-negative bacteria, including *Enterobacteriaceæ*, *Pseudomonas*, and *Acinetobacter* species has become a major worldwide public health problem. Their rapid spread and lack of development of new antimicrobial agents is cause for great concern. Carbapenemases, enzymes possessing the ability to inhibit almost all β-lactam antibiotics including carbapenems, have been mainly detected in mentioned species. Until the early 1990s, carbapenemases were considered as species-specific, chromosomally encoded β-lactamases.[56] Identification of genes encoding for carbapenemases on mobile genetic elements emerged the possible horizontal spread of these enzymes. The findings of plasmid-encoded IMP-1 metallo-β-lactamase in *P. æruginosa*, OXA-23 serine carbapenemase in *A. baumannii*, and KPC-1 serine carbapenemase in *K. pneumoniæ*, pointed on alarmingly dangerous interspecies dispersion.[57–59] Since then, carbapenemase-producing strains have been disseminated and reported worldwide, and a large variety of carbapenemases have been described.

Classification, based on amino acid homology, sort carbapenemases into three different groups: A, B and D. Classes A and D include enzymes that hydrolyze the substrate by forming an acyl-enzyme through an

active site serine, whereas class B are metalloenzymes utilizing at least one active-site zinc ion to facilitate β-lactam hydrolysis.[60] According to functional classification, carbapenemases are found primarily in groups 2df, 2f and 3. Subgroup 2df β-lactamases include OXA enzymes of molecular class D, with carbapenem-hydrolyzing activities which are frequently detected as chromosomally-mediated enzymes among *A. baumannii*. However, plasmid-harbored enzymes (e.g., OXA-48 or OXA-23) have been identified in *Enterobacteriaceæ*. The characterized OXA carbapenemases disposal of weak hydrolytic activity for carbapenem antibiotics, although the producing strains are usually highly resistant to carbapenems because of additional resistance mechanisms (e.g., ESBLs coproduction). The OXA enzymes are typically unresponsive to inhibition by clavulanic acid.[19] Serine carbapenemases of molecular class A belong to functional group 2f. Carbapenems are the distinctive substrates for this enzymatic group and inhibition is more efficient by tazobactam than clavulanic acid. One of the most worrisome members included in this subclass represents the plasmid-mediated KPC enzyme. In group 3 belong the metallo-β-lactamases (MBLs), structurally and functionally unique enzymes, distinguishing from the other β-lactamases by a presence of zinc ion at their active site. Unlike serine β-lactamases, the MBLs possess a poor hydrolytic activity for monobactams and are not inhibited by tazobactam or clavulanic acid, whereas inhibition by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) is characteristic. MBLs have been subdivided into several subgroups based on either structure (subclasses B1, B2, and B3) or function (subgroups 3a, 3b, 3c).[19]

2.2.3.1 Class A carbapenemases

Class A serine carbapenemases of functional group 2f can be divided into six main groups formed by members of the GES, KPC, SME, IMI/NMC-A enzymes, and SHV-38 and SFC-1 enzymes, which each constitute a separate group.They possess a hydrolytic ability for aztreonam and a broad variety of β-lactams, including carbapenems, cephalosporins, penicillines, and are inhibited by clavulanate and tazobactam. Various level of carbapenem-resistance varying from reduced susceptibility to full resistance has been detected across this group. SME, NMC, IMI, SHV-38 and SFC-1 enzymes are usually chromosomally encoded, whereas KPC and GES are plasmid-mediated.[56] IMI/NMC-A, SME, KPC and SFC-1 enzymes share a common origin, which differentiates them from the ancestors of GES and SHV-38 enzymes.[61] SME enzymes are usually restricted to *Serratia marcescens*, IMI and NMC-A enzymes are being detected in *Enterobacter* spp. The more frequent chromosomal location of genes encoding for these enzymes probably explain why they are more rarely reported worldwide. Contrary, genes for KPCs located on mobile genetic elements are being successfully spread by transferable plasmids, leading to their high prevalence, mainly in *K. pneumoniæ*, but even to other *Enterobacteriaceæ*, *P. æruginosa* and *A. baumannii*. Genes encoding for GES enzymes are mainly detected in integrons on transferable plasmids of *P. æruginosa* and *K. pneumoniæ*.[60]

The origin of class A carbapenemases is probably connected with environmental microbiota carrying genes encoding for carbapenem-hydrolyzing enzymes. These enzymes provide them the ability to survive in an environment containing a carbapenem compound thienamycin (imipenem is a N-formidiol

derivative of thienamycin), which is produced by soil organism *Streptomyces cattleya*.[61] Interestingly, the first enzyme of class A carbapenemases, SME-1, was identified in 1982 in *S. marscescens* strain originated from London, before the introduction of imipenem in clinical practice.[62] Similarly, the *E. cloacæ* isolate producing IMI-1 was detected in 1984 in the USA.[63]

Moreover, a novel class A carbapenemase, designated PAD-1, from recently identified new species *Paramesorhizobium desertii* coming from a soil sample of the Taklimakan Desert in China have been described. Because the location in which was the isolate detected is not affected by human activity, PAP-1 is unlikely to be associated with the selective pressure caused by extended use of antibiotics.[64]

2.2.3.1.1 The KPC (*K. pneumoniæ* carbapenemase) enzymes

The first identified KPC carbapenemase was detected in 1996 in a clinical isolate of *K. pneumoniæ* in North Carolina.[59] The strain was resistant to all tested β-lactams, however, the MICs for carbapenems decreased in the presence of clavulanic acid. Subsequently, enzyme designated KPC-1, localized on a large plasmid was identified. The discovery of the first KPC carbapenemase was soon after followed by the description of novel variants, named in the sequential numeric order. Nevertheless, later correction of KPC-1 gene sequence clarified, that KPC-1 and KPC-2 variants were, in fact, identical enzymes, so the designation KPC-1 is no longer in use. KPC-2 enzyme, harbored on a transferable plasmid, was soon after detected in the east coast of the United States. The dissemination of KPC-2 enzymes in the New York subsequently followed. This finding was especially alarming in the context of a large outbreak of ESBL-producing organisms, for which carbapenems remain as one of the few therapeutic options.[65] Concurrently, KPC-3, a single amino acid variant of KPC-2, was reported.[66] Soon after the rapid expansion of KPC in the United States, worldwide reports started to appear, e.g., from France, Scotland, Colombia, Israel, Greece, and China.[56] The first KPC isolate detected in Europe was described in France in 2005 by Naas et al. The isolate harbored KPC-2 variant and was obtained from a patient who was recently hospitalized in New York City hospital, suggesting the import of resistant isolate from the USA.[67] The first outbreak outside the USA was detected in Israel in 2004 to 2006 and was mainly caused by KPC-3-producing clone together with several KPC-2-producing clones.[68] Molecular-genetic analysis indicated genetically linkages between strains from Israel and those reported from the USA, suggesting strain exchange between patients from Israel and USA.[69] The first KPC-2 positive isolate of *K. pneumoniæ* in the Czech Republic was reported by Hrabák et al. in 2011 from a patient previously hospitalized in Greece.[70] In May 2015, KPC carbapenemases had the widest dissemination in Europe compared to other types of carbapenamases, but carbapenem-hydrolyzing oxacillinase-48(OXA-48)-producing *Enterobacteriaceæ* had almost reached the same spread. Concurrently, eight European countries reported regional or inter-regional spread, while Greece and Italy reported an endemic situation (Figure 4).[71]

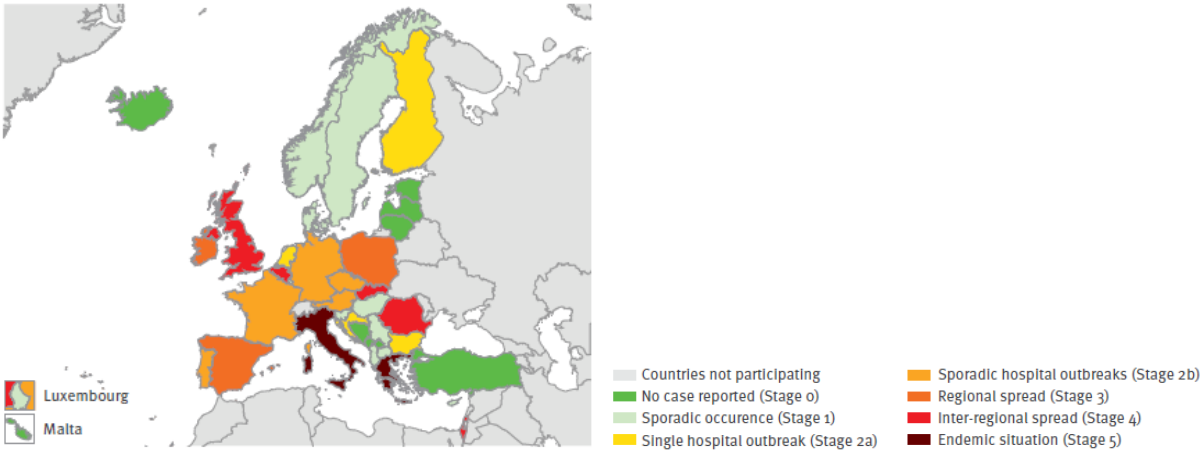

Figure 4. Geographical distribution of KPC-producing *Enterobacteriaceae* in Europe, May 2015 [4]

Although the KPCs are predominantly detected in *K. pneumoniæ* isolates, they have been found in a variety of *Enterobacteriaceæ*. Kukla and Chudejova et al. described recently an outbreak of KPC-2 producing enterobacteria in the Czech Republic, including *Citrobacter freundii*, *K. pneumoniæ*, *E. coli* and *Morganella morganii* species.[72] KPCs are also increasingly reported in other genera such as *Proteus*, *Serratia*, or *Salmonella*.[73] Worryingly, plasmid-mediated KPC-2 was detected in *P. aueruginosa*, providing a further cause for concern.[74] In 2008 KPC-2 enzyme was detected in a clinical isolate of *P. putida* in Texas, suggesting the possibility to extend the host range of KPCs into another *Pseudomonas* species.[75]

KPC enzymes possess a great potential for spread due to its location on plasmids. This ability multiplies the fact that they are most frequently found in *K. pneumoniæ*, an organism famous for the capability to accumulate and transfer resistant determinants. About 20 variants of KPC enzymes have been described until now.[76] Further analysis showed, that KPC-2 was probably the ancestor from which other KPC variants derived.[73] KPC-2 now represents the most common KPC type disseminated worldwide in Gram-negative bacteria, presented on a wide variety of plasmids varying in size, nature, and structure.[77] Especially successful single *K. pneumoniæ* clone ST-258, has been spread throughout the world, which significantly contributes to global expansion of KPC-2 enzymes.

Studies focusing on the genetic structures surrounding KPC genes identified a Tn-3-like transposon, designated Tn*4401*, that is capable of a high frequency of transpositions. Tn*4401* transposon is 10kb length, delimited by two 39-bp inverted repeat sequences, possessing transposase (*tnpA*) and resolvase (*tnpR*), and two insertion sequences, ISkpn6 and ISKpn7, in addition to bla_{KPC-2}. At least three isoforms of Tn*4401*, differing by a 100- to 200-bp sequence upstream of bla_{KPC-2}, have been identified. Studies describing the different genetic environment of bla_{KPC} have reported findings of other insertion sequences upstream of the bla_{KPC} gene but with downstream sequences similar to those of Tn*4401*, suggesting that these ISs have been inserted in Tn*4401*.[77]

The first identified cases of KPC- producing *E. coli* were detected as early as 2004–2005 in Cleveland, New York City, New Jersey, and Tel Aviv. Nevertheless, these isolates was considered rare as recently as

2010.[78] Subsequently, detection of *bla*$_{KPC}$ in *E. coli* ST131, a globally disseminated and clinically very successful strain associated with various resistance mechanisms, provided cause for concerns. Considering the high occurrence of ST-131 in community and healthcare setting, a stable association of ST-131 with *bla*$_{KPC}$ could have important clinical consequences.[78] Nevertheless, the study of Stoesser et al. focusing on the genetic features in KPC-producing *E. coli* isolates obtained from global surveillance schemes found diversity in genetic structures associated with *bla*$_{KPC}$ at all genetic levels: bacterial strain, plasmid type, associated transposable mobile genetic elements, and *bla*$_{KPC}$ alleles. This study demonstrated the particular association with IncN and Col-like plasmids, previously connected with the successful spread of antimicrobial resistance. The well-known association of *bla*$_{KPC}$ with Tn*4401* has not been in *E. coli* so obvious (complete Tn*4401* structure was missing in 50% of strains), in contrast to most worldwide descriptions of KPC-*K. pneumoniæ* strains largely associated with intact Tn*4401* isoforms.[78]

The chromosomal location of the KPC genes has been previously described in *K. pneumoniæ* from the USA, *P. æruginosa* from Colombia and *A. baumannii* from Puerto Rico.[79] The bla$_{KPC-2}$ in *A. baumannii* isolate from Puerto Rico, described by Martines et al., was identified on a novel truncated version of Tn*4401e* (255 bp deletion upstream of the KPC gene), located in the chromosome within an IncA/C plasmid fragment derived from an *Enterobacteriaceæ*. The gene was flanked by IS*Kpn6* and IS*Kpn7* but lacks the transposase and resolvase genes.[79]

Infections due to KPC-producing strains are usually healthcare-associated, however, rare community-acquired isolates have been detected.[80] The level of carbapenem-resistance among KPC producers may vary markedly. In the absence of other resistance mechanisms, KPC-positive isolates may not confer high resistance to carbapenems, but only reduced susceptibility, which leads to difficulties connected with detection.[61] However, KPC-producing bacteria often dispose of other mechanisms of resistance affecting the function of the remaining antimicrobial agents. Plasmids carrying KPC genes frequently harbored genetic determinants leading to quinolone and aminoglycoside resistance.[73] As a result, KPC-producing organisms may confer resistance to almost all β-lactam and non-β-lactam antibiotics. Thus, infections caused by KPC-positive isolates are associated with high rates of treatment failure and mortality. The meta-analysis performed by Ramos-Castaneda et al. indicated that overall KPC-infection-related mortality was 41%.[81] Limited number of possible antimicrobial choices presents an immense clinical problem. The optimal therapeutic regiment remains undefined. Usually only several isolates remain susceptible to amikacin or gentamicin. However, most isolates are susceptible to colistin and tigecycline. Nevertheless, to achieve the desired concentration of colistin or tigecycline in certain compartments could be difficult, possessing a risk of treatment faiulure. In a review published by Lee et al., significantly more treatment failure were observed in patients treated with monotherapy compared to combination therapy (49% vs. 25%).[78]

2.2.3.1.2 The GES (Guiana Extended-Spectrum β-lactamase) enzymes

The GES enzymes dispose of the ability to hydrolyze broad-spectrum cephalosporins, but the carbapenemase activity is limited to isolates with specific amino acid substitution inside the active site,

causing an extension of their spectrum of activity towards carbapenems. GES enzymes have been mainly found in *P. æruginosa*, but they are increasingly reported from *Enterobacteriaceæ* and *A. baumannii* as well. The majority of genes encoding the GES family are located in integrons on transferable plasmids. However, chromosomally located bla_{GES} genes have been described.[60,61]

The first member of GES enzymes, variant GES-1, lacking a carbapenemase activity, was reported in France in 2000 from *K. pneumoniæ* isolate obtained from an infant previously hospitalized in French Guiana.[82] Since that time, more than 30 variants of GES enzymes have been identified. Carbapenemase activity has been detected in several variants including GES-2, -4, -5, -6, -14, -15, -16, -18, -20, -21, and -24.[76] Even though enzymes from GES family remain quite rare, they have been detected worldwide. The GES enzymes have been most frequently reported as single occurrences, however, less extensive nosocomial outbreaks have been documented. GES-2 enzyme possessing a carbapenemase activity, differing from GES-1 by a glycine-to-asparagine substitution in position 170 located in the omega loop of class A β-lactamases, was detected in 2001 in *P. æruginosa* isolate originating from South Africa.[83] Subsequently, a nosocomial outbreak of GES-2-producing *P. æruginosa* isolates occurred in South African teaching hospital.[84] A glycine-to-serine change in position 170 was identified in several GES enzymes, e.g., GES-4, GES-5, GES-6, GES-14, and GES-18, resulting in the ability of carbapenem hydrolysis.[85-87] GES-4 enzyme, differing from GES-2 by three amino acids and located in class 1 integron, was described by a Japanese research group in 2004.[88] GES-5 and GES-6 were firstly detected in Greece.[85] GES-5 variant, possessing significant carbapenemase activity, has widely disseminated throughout the world. The first isolate carrying bla_{GES-5} was recovered in 2004 from a clinical *E. coli* strain obtained from a patient in Greece. Consistent with previous findings, the enzyme was localized on class 1 integron.[89] Subsequently, another GES-5 isolates have been reported from Korea, Brazil, and China.[85] Soon after, an outbreak of *K. pneumoniæ* producing GES-5 was detected in Bundang City, Republic of Korea.[90] Nowadays, GES-5-producing bacteria are increasingly reported from European countries. In 2015, several GES-5-positive isolates of *P. æruginosa* were reported from the first nationwide surveillance study on carbapenemase-positive *P. æruginosa* isolates. The first case of GES-5-producing *E. cloacæ* in the Czech Republic was reported in 2016 from a patient with anamnesis of repeated hospitalization but without known travel history.[91] The bla_{GES-5} gene occurred in a novel class 1 integron (In*406*) carried by a ColE2-like plasmid, previously repeatedly reported in association with antibiotic resistance dissemination.[91,92] The GES-5 is considered as the main carbapenem-hydrolyzing GES-type enzyme detected in *Enterobacteriaceæ*. In 2012, novel GES-14 enzyme from a clinical isolate of *A. baumannii* and GES-18 isolated from a clinical strain of *P. æruginosa* were detected in Belgium.[93] Until now, many other carbapenem-hydrolyzing GES-type β-lactamases have been described.

Although currently quite rare, GES enzymes, frequently associated with mobile genetic elements, may rapidly become major concerns, as was repeatedly exemplified in the past on a variety of other β-lactamases.

2.2.3.1.3 The IMI/NMC-A (imipenemase/ not metalloenzyme carbapenemase – A) enzymes

The IMI/NMC-A enzymes are capable of hydrolyzing carbapenems, whereas they remain fully susceptible to extended-spectrum cephalosporins.[56] IMI carbapenemases, together with closely related NMC-A β-lactamase, are mainly found in *Enterobacter* spp. and have remained overall uncommon in clinical settings.[94] NMC-A was first detected in 1992 from a clinical isolate of carbapenem-resistant *Enterobacter* sp. in France.[95] The $bla_{\text{NMC-A}}$ was identified as chromosomally located gene, which was inducible due to LysR-type regulatory gene, similar to those found upstream of chromosomally encoded AmpC-type enzymes, located upstream of $bla_{\text{NMC-A}}$ gene.[96] Rasmussen et al. described in 1996 the first IMI carbapenemase, designated IMI-1, which was obtained from two *E.cloacæ* clinical strains isolated in Southern California in 1984. IMI-1 shared greater than 95% amino acid identity with NMC-A enzyme and was inducible due to upstream-located LysR-type regulatory gene.[97] Until now eight variants of IMI-type enzymes has been described.[76] Since the first description, NMC-A enzyme has been sporadically reported in *E. cloacæ* isolates from Europe, the United States, and South America. IMI-type enzymes have been reported from isolates of *E. cloacæ*, *E. asburiæ*, and *E. coli* from the United States, Europe, the Far East and South Africa.[98] In 2005 Aubron et al. reported the gene encoding IMI-2, a point-mutant derivative of IMI-1, located on plasmids from clonally-related *E. asburiæ* strains obtained from several rivers in the United States. The $bla_{\text{IMI-2}}$ gene was located on a self-transferable 66-kb plasmid. Moreover, LysR-type regulatory gene was detected upstream, explaining inducibility of IMI-2 expression. Identification of clonally related *E. asburiæ* isolates from distant rivers points on a possible environmental reservoir of mentioned carbapenemase genes.[99] Subsequently, plasmid harbored IMI-2 enzyme was reported from a clinical isolate of *E. cloacæ* from China.[100]

The first case of IMI-2-producing *E. asburiæ* in the Czech Republic was identified in 2016.The isolate was obtained from a patient with no history of traveling abroad or previous hospitalization, indicating the potential silent spread of carbapenemases via unknown roads. The $bla_{\text{IMI-2}}$ gene was located on a conjugative plasmid and was linked to mobile elements. This fact suggests the possibility of the potential spread of this emerging resistance mechanism to other members of *Enterobacteriaceæ*.[94]

2.2.3.1.4 The SME (*Serratia marscescens* enzyme) enzymes

The first SME enzyme, designated SME-1, was identified from two *S. marscescens* clinical isolates collected in London in 1982.[101] The strains were recovered before carbapenems were approved for general medical use. Even though SME-1 variant shares only about 68% amino acid identity with NMC-A, it expresses a very similar hydrolysis profile. Similarly to NMC-A, the expression of $bla_{\text{SME-1}}$ is regulated by Lys-R type regulatory gene.[96] Subsequently, the SME-1 along with its nearly identical derivatives, SME-2 and SME-3, has been sporadically reported in *S. marscescens* strains in the United States, Canada, Argentina, and Switzerland.[56,96,102] Until now, five SME variants have been identified (SME-1 to SME-5) and seem to be restricted to *S. marscescens* species.[76] Carbapenem resistance in *S. marscescens* due to SME enzymes continues to be a sporadic hospital problem. Chromosomal location of these enzymes could be an explanation of their rare occurrence. However, as with all other resistance genes, the

theoretical possibility of an acquisition by mobile genetic elements exists. Moreover, a study of Canadian SME-producing isolates, reported by Mataseje et al., identified the *bla*$_{SME}$ gene located on a novel genomic island (SmarGI1-1), that can be excised and circularized, which may significantly contribute to its further dissemination.[102]

2.2.3.1.5 The SHV-38 and SFC-1 enzymes

The SHV-38 enzyme was identified in a clinical strain of *K. pneumoniæ*, possessing a reduced susceptibility to several extended-spectrum cephalosporins and imipenem, in 2001 in France.[103] Enzyme SHV-38 is a point-mutation of the narrow spectrum penicillinase SHV-1, differentiating by alanine-to-valine substitution in position 146, resulting in carbapenemase activity. The *bla*$_{SHV-38}$ gene, as like *bla*$_{SHV-1}$ which is naturally presented on chromosome of *K. pneumoniæ*, was also chromosomally encoded. The SHV-38 carbapenemase is considered to be the first example of SHV-1 derivatives with the ability to hydrolyze carbapenems.[103] Worryingly, SHV-type enzymes, apart from being naturally chromosomally located, may also be localized on plasmids, and moreover, *K. pneumoniæ* is known for the ability of different resistant genes transmission. This facts, may potentially easily contribute to the dissemination of SHV-38 carbapenemases.

In 2003, Henriques et al. described the SFC-1 enzyme obtained from a carbapenem-resistant environmental isolate of *Serratia fonticola* from Portugal.[104] The *bla*$_{SFC-1}$ gene was chromosomally encoded. Above that, the mentioned strain was carrying another chromosomally located carbapenemase, metallo-enzyme Sfh-I. Those enzymes were not reported in other *S. fonticola* strains, indicating the possibility of resistant genes acquisition by horizontal gene transfer.[104]

2.2.3.2 Class B metallo-β-lactamases

Class B metallo-β-lactamases (MBLs) of functional group 3, exhibit a broad spectrum of hydrolytic activity including virtually all β-lactam antibiotics except monobactam. They are not inhibited by clavulanic acid or tazobactam. However, in contrast to serine β-lactamases, MBLs are inhibited by metal chelators such as EDTA or dipicolinic acid. Their hydrolytic activity is dependent on the interaction of the β-lactam with zinc ion/ions in their active site, explaining the inhibition of their activity by metal chelators. The serine β-lactamases belong to a large superfamily of acyltransferases (SxxxK superfamily) and are structurally and mechanistically related to the penicillin-binding proteins, whereas the MBLs belong to separate superfamily including proteins with several other functions. The structures of several class B enzymes, which vary considerably from the serine-β-lactamases indicate an independent evolutionary origin.[105,106]

The MBLs were firstly identified in the 1960s (about 25 years after the serine-β-lactamases). Initially, MBLs were not considered as a serious problem with the potential to significantly affect antibiotic treatment, since they were mainly found chromosomally located and in species with low pathogenic potential. Since the 1990s, dissemination of genes encoding metallo-β-lactamases on mobile genetic elements have emerged in Gram-negative bacilli (*Enterobacteriaceæ*, *P. æruginosa*, *A. baumannii*).[105,106]

This continuous alarming spread have significantly contributed to recent crisis resulting from the worldwide dissemination of carbapenem-resistant Gram-negatives.

MBLs have been subdivided based on either structure (subclasses B1, B2, B3) or function (subgroups 3a, 3b, 3c).[19] Based on the more extensive biochemical characterization of the increasing number of MBLs, it is now being proposed that only two functional subgroups be described (3a, 3b).[19] Members of different subclasses exhibit a substantial diversity in sequence identity (only about 20% identity between some enzymes) and also the structure of their active sites.[105,106] The active site of classes B1 and B3 contains two zinc ions, while the members of class B2 have only one, explaining the narrower substrate specificity of this subclass.[105] Each subclass includes several different variants of metallo-β-lactamases, and many of them have several allelic variants. The cutoff for classification of a new metallo-β-lactamase was established as at least 30% aminoacid diversity.[107]

The subclass B1 contains the largest number of known MBLs, including the clinically most important and transferable IMP-, VIM-, NDM-type enzymes. Further, transferable MBLs: SPM-, SIM-, AIM-, GIM-, KHM-,TBM-, DIM-type enzymes, also belong to B1 subclass. Moreover, several different enzymes are included: the extensively studied BcII enzyme from *Bacillus cereus* or other *Bacillus* sp. (the first MBL for which an amino acid sequence was determined), the CcrA enzyme of *Bacteroides fragilis*, the BlaB proteins from *Chryseobacterium meningosepticum*, the CGB-1 from *Chryseobacterium gleum*, the IND-1 enzyme from *Chryseobacterium indologenes*, the EBR-1 from *Empedobacter brevis*, the SFB-1 protein of *Shewanella frigidimarina*, the SLB-1protein obtained from *Shewanella livingstonensis,* the JOHN-1 of *Flavobacterium johnsoniæ*, the TUS-1 and MUS-1 from *Myroides* sp., and Uvs123 enzyme from uncultured bacterium.[108-110]

The subclass B2, possessing only 11% identity with subclass B1, contains the enzymes CphA and ImiS found in various species of *Aeromonas* and the Shf-1 isolated from *Serratia fonticola*.[108,109]

Finally, subclass B3 includes the L1 protein from *Stenotrophomonas maltophilia*, the GOB enzymes of *Chryseobacterium meningosepticum*, the FEZ-1 enzyme from *Legionella gormanii*, and THIN-B, Mblıb, and BJP-1 enzymes produced by environmental bacteria (*Janthinobacter lividum*, *Caulobacter crescentus*, *Bradyrhizobium japonicum*, respectively).[108,109]

Subsequently, several novel MBLs variants belonging in different subclasses have been continuously detected.

MBLs are encoded either by chromosomally localized genes or by heterogeneous genes acquiredby horizontal gene transfer. Only a few chromosomally-encoded metallo-β-lactamases occur in species of clinical significance, such as *Bacillus* sp., *Stenotrophomonas maltophilia*, *Aeromonas* sp., *Bacteroides fragilis*, various flavobacteria, and *Pseudomonas otitidis*, contributing the intrinsic profile of resistance to β-lactams. Acquired MBLs have been mainly identified in *Enterobacteriaceæ* and in the genera *Pseudomonas* and *Acinetobacter*. Majority of the acquired MBLs belong to subclass B1, indicating a high ability of this subclass to be disseminated by mobile genetic elements compared to subclasses B2 and B3.

At least ten different variants of acquired metallo-β-lactamases have been described (IMP-, VIM-, NDM-, SPM-, GIM-, SIM-, AIM-, KHM-, TBM-, DIM-variants).[105] Alarming spread with important clinical consequences has been reported especially for IMP-, VIM-, and NDM-enzymes.

The origin of acquired MBLs genes is most likely environmental bacteria, sharing with clinically important strains several common environmental niches. The majority of acquired MBL genes are carried on mobile gene cassettes inserted in integrons, and can thus exploit the system of integron recombination and the mobility of DNA elements (transposons and plasmids) associated with integrons.[105] Moreover, most integrons carrying gene cassettes encoding for MBLs contain another additional gene cassettes possessing resistance to other antibiotic classes (e.g., aminoglycosides), further limiting the therapeutic options.

The rapid spread of MBLs among major Gram-negative pathogens is alarming throughout the world. Especially the dissemination in Asian and European countries is a matter of particular concern (Figure 5). Europe was one of the first continents where the emergence of acquired MBLs has been reported. However, the data may be significantly distorted by the incompleteness of reliable epidemiological reporting from some geographical regions.[107] Available data indicate a country-specific epidemiological patterns.[105]

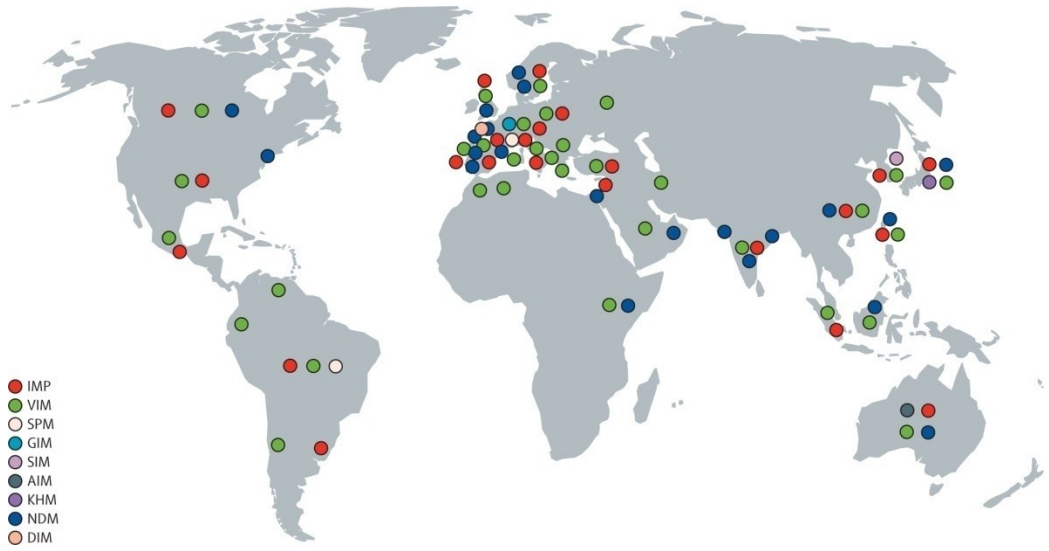

Figure 5. Worldwide dissemination of different types of MBLs in 2011 [5]

2.2.3.2.1 The IMP (IMiPenemase) enzymes

The IMP-type metallo-β-lactamases possess a broad substrate specificity with a high affinity for cephalosporins and carbapenems, contrary they exhibit only little activity against temocillin (6α-methoxy-penicillin).[105]

The IMP enzyme was firstly detected in Japan in 1988 from a clinical isolate of *P. æruginosa*.[111] The bla_{IMP} gene was localized on transferable conjugative plasmid, thus IMP enzyme was among the first acquired MBLs identified. The enzyme was characterized biochemically, but the authors did not sequence or

name it, nevertheless, it was almost certainly IMP-1.[112] In *Enterobacteriaceæ*, IMP-1 was first found in *S. marscescens* isolate from Japan in 1991.[2] Subsequently, during the early 1990s, another IMP-producing isolates of *S. marscescens* and *P. æruginosa* were reported from Japanese hospitals localized in different geographical areas, and also IMP-1-producing isolate of *K. pneumoniæ* was identified. The study of Senda et al. focusing on a survey of IMP-1-positive strains of *P. æruginosa* isolated between 1992 and 1994 found that the host strains belonged to diverse lineages, implying possible horizontal gene transfer.[113] However, the level of carbapenem-resistance vary significantly among the isolates, suggesting that the acquisition of MBL genes alone does not necessary confer a high level resistance to carbapenems.[112,114] Soon afterwards, another variants of IMP-1: IMP-3, IMP-6, and IMP-10 have been identified in *Shigella flexneri*, *S. marscescens*, *P. æruginosa* and *Alcaligenes* sp. IMP-3 enzyme possess two amino acid changes compared to IMI-1, including glycine-to-serine substitution on position 196 leading to the reduction of activity against penicillin.[115] The same amino acid substitution was detected in IMP-6, conferring to reduced activity against penicillin and piperacillin, however, higher level of meropenem hydrolysis compared to imipenem (opposite to IMP-1) was observed.[116] IMP-10 MBL was detected to be either plasmid-harbored in *P. æruginosa* or chromosomally located in *P. æruginosa* and *Achromobacter xylosoxidans*. The $bla_{\text{IMP-10}}$ differs from $bla_{\text{IMP-1}}$ by a phenylalanine-to-valine substitution in position 49, leading to reduced activity against penicillins, but with no changes in carbapenems hydrolysis.[114]

The IMP-producing isolates, which from the beginning seemed to be limited to Japan, started to be subsequently reported from other distant countries. The first IMP enzyme identified in 1997 in Europe, IMP-2 variant (sharing about 85% identity with IMP-1) located on the first cassette on class 1 integron, was obtained from *A. baumannii* isolate detected in Italy.[56] Subsequently, IMP-5 variant was detected in 1998 in Portugal. IMP-2 possess 36 and IMP-5 17 amino acid differences compared to IMP-1. Concurrently, both enzymes were chromosomally located and differ in terms of their genetic context. The $bla_{\text{IMP-2}}$ gene was found next to two aminoglycoside resistance genes (*aacA4*, *aadA1*), whereas the $bla_{\text{IMP-5}}$ was identified as a sole gene cassette.[114] Soon after, other variants, like IMP-12 localized on non-transferable plasmid from *P. putida* and IMP-13 chromosomally encoded in *P. æruginosa*, have been detected in Italy. The differences between the European and Asian isolates could not be satisfactorily explained by global dissemination of IMP alleles from Japan. It is more likely that these alleles represented local emergency. Nevertheless, in 2002 two IMP-1-producing isolates of *A. junii* and *A. baumannii* was detected in the UK. Interestingly, one mentioned isolate was obtained from a patient previously hospitalized in Spain.[117,118] Retrospective study of carbapenem-resistant isolates collected as early as 1995 in Canada identified IMP-7 variant in *P. æruginosa*. Subsequently two outbreaks caused by IMP-7-producing *P. æruginosa* were reported from Canada. The $bla_{\text{IMP-7}}$ was localized on integron in the third gene cassette with other gene cassettes encoding for aminoglycoside resistance. Another retrospective analysis, including isolates collected from 1994 to 1998 in Hong Kong, was performed by Chu et al. Novel IMP-4 metallo-β-lactamase harbored on plasmid and integron-encoded was identified.[119] Few years later, IMP-4 variant was reported in Australia from *E. coli*, *K. pneumoniæ*, and

P. æruginosa, suggesting a possible import from South Asia.[120,121] Soon after, IMP-type enzymes have been described in Taiwan, Singapore and Korea.[114]

The first MBL-producing strains of *P. æuruginosa* have been reported in Czech Republic in 2008 from two patients hospitalized in the same hospital unit. The enzyme was identified as IMP-7 variant and was localized on class 1 integron. The bla_{IMP-7} gene cassette was found together with other resistance genes (aac(6')-Ib).[122] A recent comprehensive study focused on molecular-characterization of MBL producing isolates of *P. æruginosa* detected in Czech hospitals reported an emerging clonal spread of ST357 *P. æruginosa* carrying the IMP-7 enzyme. Among 136 carbapenemase-positive isolates of *P. æruginosa* collected in 2015, 117 isolates was IMP-positive (IMP-7 [n = 116], IMP-1 [n = 1]). The bla_{IMP-7} gene was located in class 1 integron, predominantly in In-p110-like integron identified in most of the isolates.[123]

Global analyses of the genetic environment of IMP enzymes indicated that the bla_{IMP} genes most frequently occur as gene cassettes in class 1 integrons or, more rarely, integrons of class 2 or 3.[123] Mentioned integrons often harbored another gene cassettes conferring resistance to other classes of antibiotics (e.g., aminoglycosides, trimethoprim, sulphonamides, chloramphenicol), which can be coexpressed from a single promoter. Integrons cannot mobilize themselves, however, they are frequently found within transposons localized on plasmids or chromosome, thus allowing their spread.[123]

Nowadays, IMP enzymes have been reported worldwide mostly in *P. æruginosa*, *Acinetobacter baumannii* and *Enterobacteriaceæ*. More than 50 different variants, belonging to various sublineages, have been described.[76] Among them, more than 30 variants have been reported from *P. æruginosa*.[124] Different IMP variants frequently possess predominant geographical distribution, however, some variants (e.g., IMP-1, IMP-4, IMP-7) have been detected across the continents, showing their potential for successful spread.[105] The IMP-enzymes detected in Europe have been mainly identified in *P. æruginosa* isolates, whereas IMP-producing *Enterobacteriaceæ* remain in European countries quite rare.[71]

2.2.3.2.2 The VIM (Verona Integron-encoded Metallo-β-lactamase) enzymes

The VIM-type metallo-β-lactamases possess even broader substrate specificity compared to IMP-type enzymes. They are able to hydrolyze temocillin, and moreover, among the group of metallo-β-lactamases, they exhibit a uniquely high affinity for carbapenems.[105,125] The VIM MBLs are associated with clinically important pathogens such as *Pseudomonas* spp., *Acinetobacter* spp., and the family of *Enterobacteriaceæ*, of which *P. æruginosa* is the most important reservoir of VIM genes.[126]

The first VIM enzyme, designated VIM-1, was first described in Italy (Verona) from a clinical isolate of *P. æruginosa* identified in 1997. The gene bla_{VIM-1} was integrated as a gene cassette into a chromosomally located class 1 integron, and besides the bla_{VIM-1} gene cassette, a gene cassette encoding for aminoglycoside resistance (*aacA4*) was detected.[127] Identification of bla_{VIM-1} in *Achromobacter xylosoxidans* isolated from the same hospital subsequently followed. The bla_{VIM-1} gene was harbored by

30-kb nonconjugative plasmid carrying a class 1 integron, containing three different aminoglycoside genes (*aacA4*, *aphA15*, *aadA1*) located downstream of the bla_{VIM-1} gene cassette.[128] Additionally, nosocomial infections caused by VIM-1-producing *P. putida* was detected in Italy. The bla_{VIM-1} gene was plasmid encoded.[129] Soon after, the VIM-1 enzymes started to be widely reported from other European countries (e.g., Greece, France, Spain, Germany) and in different members of *Enterobacteriaceæ* (e.g., *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Proteus mirabilis*, *Morganella morganii, Citrobacter freundi*, *Providentia stuartii*) and non-fermenting Gram-negative bacilli (*Pseudomonas* spp., *A. baumannii*).[126] A report from a Greek surveillance study has showed how rapid and alarming could be the dissemination of VIM enzymes. The number of imipenem-resistant *K. pneumoniæ* isolates increased from less than 1% in 2001 to 20% in isolates from hospital wards and to 50% in intensive care units in 2006. In 2002, these strains were detected in only three hospitals, whereas in 2006, 25 of the 40 participating hospitals reported such isolates. The spread of bla_{VIM-1} cassette among rapidly evolving multiresistant plasmids was identified to be the dominant determinant harbored by the resistant strains.[130]

The VIM-2 enzyme was first reported from a clinical isolate of *P. æruginosa* in France in 1996. The VIM-2 variant, possessing 90% amino acid identity with VIM-1, was localized on a gene cassette as the only resistant gene identified in the bla_{VIM-2}-positive class 1 integron localized on plasmid.[131] Subsequently, two *P. æruginosa* isolates harboring the same bla_{VIM-2} gene cassette but embedded in different class 1 integrons (possessing a sulfonamide-resistance gene and a variety of aminoglycoside-resistance genes) was describe in France.[132] Moreover, a retrospective study of isolates obtained from a hospital in Marseille (France) from 1995 to 1999 identified 10 other VIM-2 positive isolates. Additionally, outbreaks caused by VIM-2-producing isolates from Italy and Greece followed. Soon after, VIM-2-positive isolates started to be identified from different geographical areas (e.g., Japan, South Korea, Portugal, Spain, Poland, Croatia, Chile, Venezuela, Argentina, Taiwan Belgium, and the United States) and, besides to *Pseudomonas* spp., started to be increasingly report from *Enterobacteriaceæ* (e.g., *Citrobacter freundi, Serratia marscescens*).[114] The VIM-2 enzyme, is at this time the most-reported MBL worldwide and the most important VIM-type MBL in clinical practice.[114,126] The VIM-1 enzyme has been frequently detected among *Enterobacteriacea*, whereas VIM-2 have been predominantly found in non-fermenting Gram-negative bacteria, such as *P. æruginosa* and *A. baumannii*.[133]

Subsequently, VIM-3 variant, differing from VIM-2 by two amino acid substitutions, was detected in Taiwan. The VIM-4 variant, differing from VIM-1 by a single amino acid substitution, was reported from *P. æruginosa* in Greece, Sweden, and Poland and from *K. pneumoniæ* and *E. cloacæ* in Italy. The VIM-5, possessing five amino acid changes compared to VIM-1, was identified in *P. æruginosa* and *K. pneumoniæ* in Turkey, and VIM-6, differing from VIM-2 by two amino acid changes, from *P. putida* isolated in Singapore. The VIM-7 enzyme, sharing only 77% identity with VIM-1 and 74% with VIM-2, was identified in Texas.[114]

Until now, 48 different VIM variants have been identified.[76] According to the phylogenetic tree and the number of different amino acid residues, VIM enzymes may be divided into three main subgroups (Figure 6).[126]

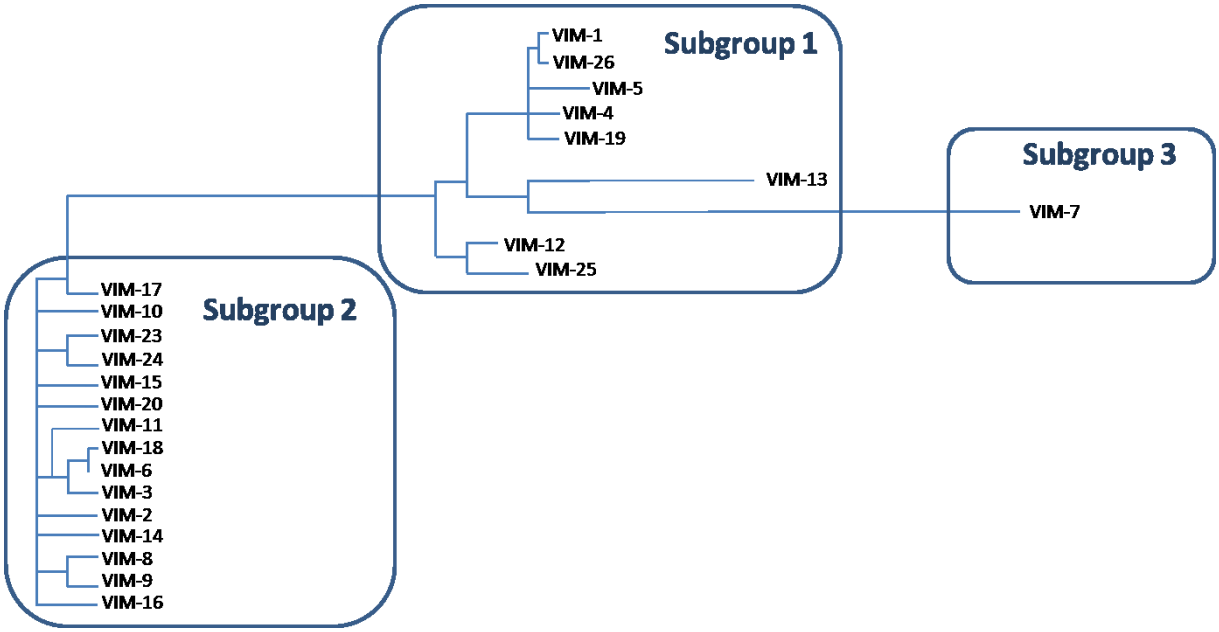

Figure 6. Phylogenetic tree of the VIM family based on the amino acid sequences with examples of concrete enzymes [6]

The *bla*$_{VIM}$ genes are frequently located as gene cassettes in class 1 integrons, which are usually embedded in transposons harbored by plasmids of different replicon types (e.g., IncN, IncA/C, IncI). Hundreds of structures of class 1 integrons have been associated with the spread of VIM enzymes among Gram-negatives. Moreover, *bla*$_{VIM}$ genes are frequently associated with aminoglycoside-resistance genes, such as *aacA4*, *aacA7*, *aadA2*, *aadB*, and *aaC1*, of which *aacA4* is the dominant.[126,133]

Similarly to IMP enzymes, the majority of VIM metallo-β-lactamases have defined geographical distribution. The VIM-1 and VIM-4 variants are frequently reported from Europe, the VIM-3 enzyme from Taiwan, the VIM-6 protein is predominantly identified in Asia and the VIM-7 enzyme in the USA. Of particular interest is the VIM-2 variant, which has been detected worldwide, emphasizing on its high ability to spread, with alarming dissemination in Southern Europe (Greece, Spain, Italy) and Southern Asia (South Korea, Taiwan) (Figure 7).[40,126]

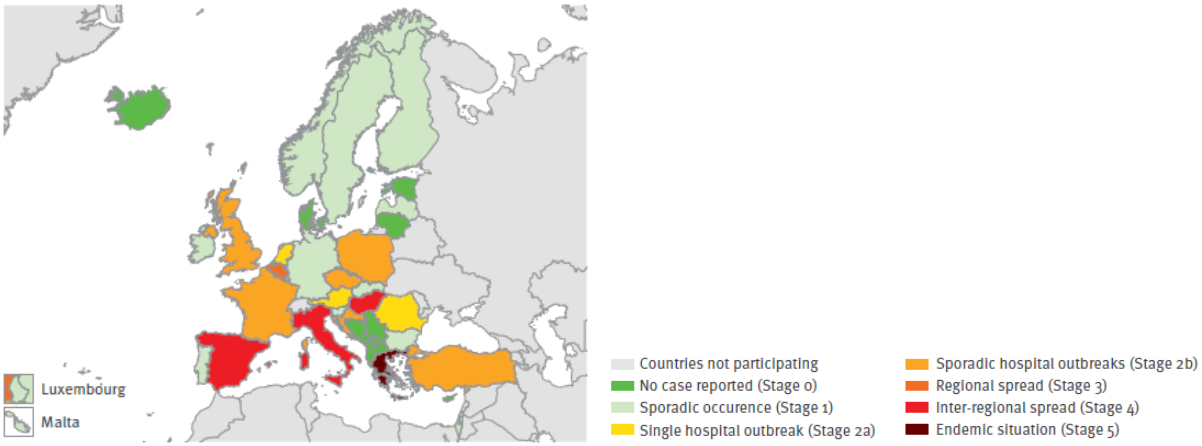

Figure 7. Geographical distribution of VIM-producing *Enterobacteriaceae* in Europe, May 2015 [4]

The study focused on molecular characterization of MBLs-producing *P. æruginosa* isolates obtained in Czech hospitals from 2009 to 2011 reported 8 VIM-positive and 3 IMP-positive isolates. The MBL-positive isolates was classified into two sequence types (STs), 357 and 111. The VIM-encoding integrons from *P. æruginosa* ST 357 possess the bla_{VIM-2} as a sole gene cassette of a class 1 integron identical to In56 previously described in France. The same bla_{VIM-2} cassette was identified in VIM-encoding integrons of ST111, designated Inp385, possessing an aac29a cassette, similar to that in In59 previously reported from another French isolate.[134] Moreover, clinical isolate of *Serratia marscescens* coproducing ESBL (TEM-6), AmpC (inherent and acquired DHA-1) and VIM-1 metallo-β-lactamases was reported from the Czech Republic in 2009.[135] Recent study including 136 carbapenemase-producing isolates of *P. æruginosa* recovered from Czech hospitals in 2015 identified, besides to 117 IMP-positive isolates, fifteen VIM-2 producing strains. The bla_{VIM-2} genes were found to be localized on class 1 integrons including In59-like, In56, and a novel element In1391.[123]

2.2.3.2.3 The NDM (New Delhi Metallo-β-lactamase) enzymes

The NDM enzymes efficiently hydrolyze almost all β-lactam antibiotics including penicillins, cephalosporins, and carbapenems, with the exception of aztreonam, resulting in considerably limited therapeutic options. The main reservoir of NDM producers is localized in the Indian subcontinent, whereas secondary reservoirs seem to have established in the Balkan regions and the Middle East.[136] The spread of NDM enzymes is mainly connected with the dissemination of conjugative plasmids among *Enterobacteriaceæ*, however, NDM carbapenemases have also been reported from and *Acinetobacter* spp. and *P. æruginosa*. [136]

The NDM-1 enzyme was first identified in 2008 in *K. pneumoniæ* isolate in Sweden from a patient with urinary tract infection, previously hospitalized in New Delhi, India. In addition, bla_{NDM-1} gene was detected in *E. coli* strain detected in patient's feces. Occurrence of the same novel resistant gene in two genera suggested possible gene transfer. The subsequent molecular analysis confirmed that the bla_{NDM-1} gene was located on a transferable plasmid of 180 and 140 kb in the *K. pneumoniæ* and *E. coli* isolates, respectively.[137] Since then, NDM carbapenemases have disseminated worldwide. Frequently, obvious

connection of NDM-positive isolates with the Indian subcontinent or Balkan countries can be observed.[138]

Soon after the first isolation of NDM-1-producing isolates, possessing an evident epidemiological link to Indian Subcontinent, a study of Kumarasny et al. documented NDM-1-positive isolates from clinical samples obtained in different areas of India, Pakistan, Bangladesh, and the United Kingdom.[139] The first NDM-1-positive isolate in the UK was reported in 2008. Subsequently, a growing number of NDM metallo-β-lactamases started to be reported from this area. Of particular interest was the fact that at least 17 of the first 29 UK patients who were identified to carry NDM-1-positive isolates had a history of travel to India or Pakistan within the previous year, of which 14 were hospitalized in mentioned countries.[140] A study focusing on *Enterobacteriaceæ* carbapenem-resistant isolates obtained in 2006-2007 in India reported NDM-1-positive isolates from several Indian hospitals, pre-dating the first described NDM-1 isolate.[141] According to several studies, the overall prevalence of NDM-1-positive isolates among *Enterobacteriaceæ* range from 5 to 18,5% in Indian and Pakistan hospitals.[139,142] The NDM-1-positive isolates of *Pseudomonas* spp. and *Acinetobacter* spp. from Indian hospitals subsequently followed.[140] In addition, the NDM-1-producing isolates have been repeatedly reported from community settings.[139] Moreover, the bla_{NDM} genes were detected in environmental samples from sources of drinking water and seepage samples in New Delhi, highlighting the need of improvements in sanitary conditions as a key public health intervention.[143] Interestingly, Isozumi et al. described environmental contamination of water samples from a river in Vietnam, which is known by strong cultural and economics link to India.[144] Similarly to the spread of NDM-1-positive *Enterobacteriaceæ*, NDM-producing isolates of *Acinetobacter* has been reported from environmental samples obtained in China.[145] Currently, most of the NDM-positive isolates of *Acinetobacter* spp. are identified in China and the Middle East.[136]

In addition to rapid dissemination of NDM-1 metallo-β-lactamases in the Indian subcontinent, subsequent reports from distant geographical areas started to be documented, usually with a detectable epidemiological link to the Indian subcontinent (hospitalization/travel). Such reports were published from Australia, the Far East, the United States, Canada, the Middle East, and many European countries. In 2015 five European countries reported sporadic hospital outbreaks, and seven countries regional or inter-regional spread of NDM-producing *Enterobacteriaceæ* (Figure 8).[71]

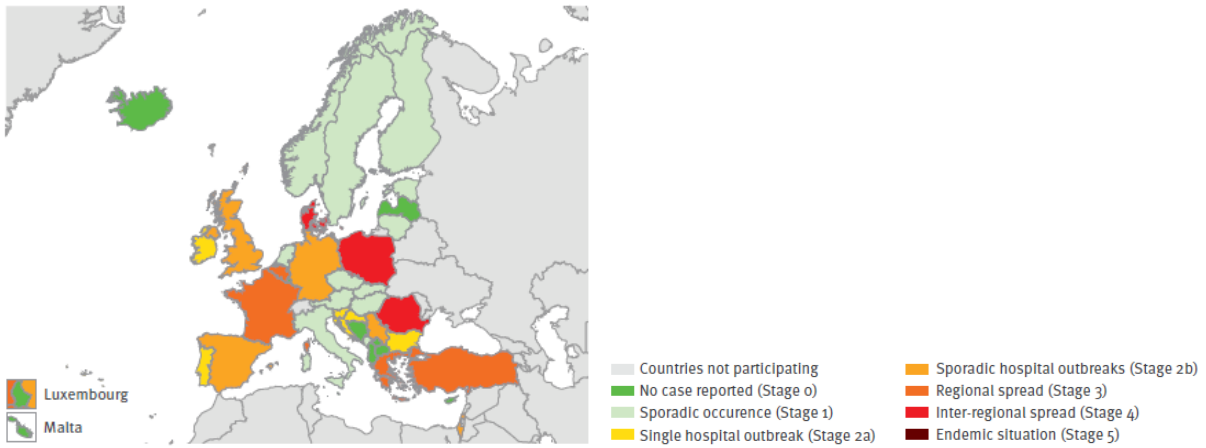

Figure 8. Geographical distribution of NDM-producing *Enterobacteriaceae* in Europe, May 2015 [4]

The Balkan countries have been highlighted as a possible second reservoir of NDM enzymes, based on the considerable number of NDM-positive isolates from patients repatriated from this area.[140] A study focused on carbapenemase-producing Gram-negative isolates from Serbia in 2010 identified seven NDM-1-positive isolates of *Pseudomonas æruginosa* from patients with no history of travel to Indian subcontinent or Europe.[146] This fact has led to speculations about the possible endemic area of NDM-positive isolates in Serbia. However, some investigators highlight the importance of medical tourism (commercial kidney transplantation), when Balkans traveled to Pakistan.[147,148] Further isolates of NDM-producing bacteria have been reported from a range of countries of the Middle East and North and Central Africa.[140]

Since the first description of NDM-1, 16 variants of NDM enzymes have been identified.[76] The NDM-2 variant, differing from NDM-1 by a proline-to-alanine substitution at position 28, was reported from *A. baumanni* strain obtained from a patient repatriated from Egypt to Germany in 2011.[149] The NDM-2 enzyme is predominantly disseminated among *Acinetobacter* spp. The NDM-3 enzyme, differing from NDM-1 by a single nucleotide change leading to aspartate-to-asparagine substitution at position 95, was described from *E. coli* isolate from Australian patient who previously traveled to India.[150] The NDM-4 variant, differing from NDM-1 by a methionine-to-proline substitution in position 154, possess an increased hydrolytic activity towards carbapenems compared to NDM-1. The NDM-5 metallo-β-lactamase shares mentioned substitution in position 154 (Met→Leu) conferring enhanced hydrolytic activity against carbapenems, and a second amino acid substitution of valine-to-leucine at position 88 was detected.[136] Subsequently, a series of further variants have been reported.

In the Czech Republic, the NDM enzymes were rare, with only three sporadic cases detected from 2011 to 2013. The first NDM-1-positive isolate, obtained from *A. baumannii* strain, was recovered from a patient repatriated from Egypt in 2011.[151] The NDM-4 positive *E. cloacæ* was identified in 2012 from a patient previously hospitalized in Sri Lanka.[152] Isolate of *K. pneumoniæ* ST11 harboring two plasmids encoding the NDM-1 variant was detected from a patient previously hospitalized in Slovakia.[153] However, increased occurrence of NDM-positive isolates have been reported since 2016. A recent study focusing

on 18 NDM-positive *Enterobacteriaceæ* isolates obtained from Czech hospitals during 2016 identified 12 NDM-4-, 3 NDM-5-, and 3 NDM-1-producing isolates.[154]

In *Enterobacteriaceæ*, the *bla*$_{NDM}$ genes is most frequently harbored by conjugative plasmids belonging to several incompatibility groups.[136] Investigation of NDM-1-positive isolates of worldwide origin obtained from unrelated countries reported by Poirel et al. have suggested that the dissemination of *bla*$_{NDM-1}$ genes is not connected to the spread of specific clones, specific structures, or single genetic structure.[155] However, in recent years, an increasing number of publications regarding the dissemination of IncX3 plasmids harboring *bla*$_{NDM}$ genes have been reported.[156-158] These findings are in concordance with the results of the study focusing on Czech isolates, indicating that IncX3 plasmids play a major role in the spread of NDM-like enzymes in the Czech Republic.[154] In *Acinetobacter* spp. the *bla*$_{NDM}$ genes are either plasmid or chromosomally localized. In less common NDM-positive isolates of *P æruginosa* the *bla*$_{NDM}$ genes were detected on chromosome.[136] Studies focusing on the genetic structures surrounding NDM genes identified the presence of a conserved structure that always associated the complete or truncated insertion sequence IS*Aba125* at the 5′-end and the *ble*$_{MBL}$ gene encoding resistance to bleomycin (anticancer drug and also an antibacterial substance occurring in environment) at the 3′-end of the *bla*$_{NDM}$ genes. In NDM-producing isolates of *Acinetobacter baumannii*, the *bla*$_{NDM}$ gene was found to be located between two copies of the IS*Aba125* element, forming a composite transposon Tn*125*. Molecular-genetic analysis of the truncated form of the composite transposon in *Enterobacteriaceæ*, while it was described in its entire form in *A. baumannii*, suggest that *Acinetobacter* spp. has been a reservoir of the *bla*$_{NDM}$ genes before targeting *Enterobacteriaceæ*.[136]

The rapid spread of a new type of resistant bacteria with the potential to cause severe infections in humans is exemplified by NDM metallo-β-lactamases. Moreover, besides the risk of colonization/infection of patients hospitalized in most affected countries, travelers to high risk areas can get asymptomatically colonized by NDM-positive bacteria and thus can constitute undetected reservoir. Interestingly, studies focusing on the duration of colonization by NDM-positive isolates showed a prolonged persistence of such isolates for periods of 10 and 13 months in case of NDM-producing *E. coli* gut carriage.[140]

2.2.3.2.4 Other acquired metallo-β-lactamases: SPM-, GIM-, SIM-, KHM-, DIM-, TBM-, AIM-variants

Acquired metallo-β-lactamases hydrolyze almost all β-lactams except monobactams. Genes encoding these MBL variants are localized in various bacterial mobile genetic elements.

The SPM-1 (Sao Paolo MBL) enzyme was reported in 2002 from *P. æruginosa* isolated in 1997 from a clinical sample in Brazil. Interestingly, the *bla*$_{SPM-1}$ gene possess a unique genetic context, since it is immediately associated with common region elements and not with transposons or integrons.[114,159]

The GIM-1 (German imipenemase) enzyme was identified in 2002 from 5 clinical samples of *P. æruginosa* recovered from Germany (Düsseldorf). The GIM-1-positive isolates were resistant to all antibiotics, including carbapenems, remaining susceptible only to colistin. Similarly to the majority of

MBL genes, bla_{GIM-1} was localized on a class 1 integron harbored by a plasmid.[114] Since that, GIM enzymes have been sporadically reported from Germany in *Pseudomonas* spp., *Acinetobacter pittii*, and *Enterobacteriaceæ*. Subsequently, in 2014, novel variant designated GIM-2 was reported from a clinical isolate of *E. cloacæ* in Germany. The patient had an anamnesis of a previous hospitalization in Germany and Saudi Arabia.[160]

The enzyme SIM-1 (Seoul imipenemase) was reported from seven clinical isolates of *Acinetobacter baumannii* from South Korea in 2005. The bla_{SIM-1} gene cassette was encoded on a class 1 integron. Moreover, the same integron was detected in isolates belonging to different clonal lineages, indicating a horizontal gene transfer of bla_{SIM-1} gene.[161]

The KHM-1 (Kyorin Health Science MBL) metallo-β-lactamase was identified in Japan in 2008 from *Citrobacter freundii* clinical isolate recovered in 1997.[162]

The DIM-1 (Dutch imipenemase) enzyme was detected in 2009 from a clinical sample of *Pseudomonas stutzeri* obtained in the Netherlands. The bla_{DIM-1} gene was found in a class 1 integron, possessing two other gene cassettes encoding resistance to aminoglycosides and disinfectants, located on a plasmid.[163] Later, in 2015, novel DIM-2 variant was reported from a clinical sample of *Pseudomonas putida* detected in China.[164]

The TBM-1 (Tripolli MBL) enzyme was first identified in 2011 in Libya from *Achromobacter xylosoxidans* strain obtained from hospital ward swab. After that, in 2014, the first clinical sample of *Acinetobacter* spp., isolated in Japan, was detected to possess bla_{TBM-1} gene localized in class 1 integron.[165,166]

Finally, the AIM-1 (Adelaide imipenemase) enzyme was detected on a mobile genetic element from a clinical sample of *P. æruginosa* in Australia in 2012.[167]

2.2.3.3 Class D carbapenemases

Class D β-lactamases, also named OXAs (for "oxacillin-hydrolyzing") include about 455 enzymes, among which only some variants are considered to be carbapenemases. The hydrolytic spectrum of OXA carbapenemases includes penicillins, they usually possess a quite weak carbapenemase activity, they do not hydrolyze aztreonam, and with the exception of one variant (OXA-163), they do not hydrolyze extended-spectrum cephalosporins. However, even if they generally exhibit weak carbapenemase activity, they significantly contribute to carbapenem resistance if other carbapenem resistance mechanisms are presented (decreased permeability of bacterial cell wall, efflux pumps, co-expression of other β-lactamase). This group of enzymes is very poorly inhibited by clavulanic acid or EDTA, but *in vitro* inhibition by NaCl has been documented. There are 12 major subgroups of OXA-type carbapenemases, based on amino acid homologies.[168] The majority of OXA carbapenemases have been identified among *Acinetobacter* spp. However, the epidemiologically most important variants have frequently been detected among *Enterobacteriaceæ*.

The first OXA β-lactamase hydrolyzing carbapenems was described in 1993 from a clinical sample of multidrug-resistant *A. baumannii* isolated in 1985 in Scotland. The enzyme, initially designated ARI-1

(*Acinetobacter* resistant to imipenem) and later renamed to OXA-23, was harbored by a large plasmid.[169] Subsequently, by 1998, OXA-carbapenemases in *Acinetobacter* spp. isolates had emerged worldwide. The OXA-23 carbapenemase was identified to cause nosocomial outbreaks in the UK, Brazil, Korea and Tahiti. The OXA-24 and OXA-40 variants, differing by two amino acids, were isolated in clinical samples of *Acinetobacter* spp. from Spain and Portugal.[56] The OXA-40 enzyme was also the first OXA carbapenemase identified in the United States.[170] Long persistence of OXA-40-producing *A. baumannii* has been detected in Portugal hospitals.[171] Vast majority of OXA-23- and OXA-40-positive strains identified worldwide possess a high level of carbapenem-resistance due to combination with additional resistance mechanisms. The OXA-58 enzyme was first identified in France from *A. baumannii* isolate obtained during a nosocomial outbreak.[172] The $bla_{\text{OXA-58}}$ gene was localized on a plasmid and subsequently detected worldwide, including many European countries (Belgium, the UK, Romania, Italy, Greece, Sweden, and Turkey).[96] Moreover, it was found that *A. baumannii* naturally possesses a chromosomally-encoded OXA β-lactamases (OXA-51-like), some of which could confer resistance to carbapenems in case of their overexpression. In addition, *Acinetobacter* species closely related to *A. baumannii* possess their own chromosomally encoded OXA β-lactamases potentially transferable to *A. baumannii*. [168]

The majority of oxacillinase genes are part of gene cassettes in class 1 integrons, whereas OXA-type carbapenemases exhibit different genetic features. The $bla_{\text{OXA-23}}$ genes were described in association with transposons/composite transposons. The $bla_{\text{OXA-40}}$-like genes are frequently chromosomally located, however, plasmid location was reported from *A. baumannii* and *Pseudomonas* spp., suggesting a possible spread among unrelated Gram-negative species.[96] The $bla_{\text{OXA-58}}$ gene seems to be predominantly plasmid-harbored, usually associated with specific insertion sequences influencing its expression.

Insertion sequences (ISs), encoding transposases, are often crucial for the mobilization and expression of OXA β-lactamases. In *A. baumannii*, the ISs are frequently identified in association with the bla_{OXA} genes. The most prevalent is the IS*Aba1* insertion sequence, which has been frequently reported in connection with a number of OXA enzymes, such as OXA-23-like, OXA-51-like, OXA-58-like, and OXA-235-like. The IS*Aba1* located upstream of certain bla_{OXA} genes leads to increase of gene expression to levels conferring resistance to carbapenems. The location of IS*Aba1* 25bp upstream of the $bla_{\text{OXA-23}}$ gene provides a promoter determining the transcription of the gene.[173] Similarly, IS*Aba1* located 7bp upstream of the $bla_{\text{OXA-51}}$ gene provides a promoter increasing the $bla_{\text{OXA-51}}$ expression up to 50-fold.[168] The MICs for carbapenems of *A. baumannii* strains overexpressing the $bla_{\text{OXA-51}}$-like gene due to IS*Aba1* exhibit the same values as those for isolates possessing acquired OXA-type carbapenemases, indicating that all *A. baumannii* isolates could become carbapenem-resistant through the insertion of this promiscuous IS element.[168,174] The IS*Aba1* certainly plays an important role in the bla_{OXA} gene expression and moreover, in their mobilization. Many different transposons containing the IS*Aba1* in a single or multiple copies have been identified.[168] Beyond the IS*Aba1*, the insertion sequence IS*Aba3* have been repeatedly reported in association with $bla_{\text{OXA-58}}$-like genes, forming a composite transposon. Similarly to IS*Aba1*, the IS*Aba3* provides a promoter leading to $bla_{\text{OXA-58}}$-like genes expression.[168]

Besides OXA-type carbapenemases identified predominantly among *A. baumannii* species, especially the OXA-48-like enzymes, identified with increasing frequency among *Enterobacteriaceæ*, are of particular interest constituting an alarming public threat.

2.2.3.3.1 The OXA-48-like carbapenemases

The OXA-48 enzyme was first described from carbapenem-resistant *K. pneumoniæ* isolated in 2001 in Turkey.[175] Series of reports, including sporadic cases as well as nosocomial outbreaks from Turkish hospitals, followed. Soon after, the $bla_{\text{OXA-48}}$ genes were identified in the Middle East and North African countries.[176,177] Currently, mentioned geographical areas constitute an important reservoir of OXA-48-producing isolates. Subsequently, OXA-48 producers started to be sporadically reported from European countries, including the UK, Belgium, France, Germany, Italy, Ireland, Slovenia, Spain, Switzerland and the Netherlands. The reports of OXA-48 producers in these countries had been frequently attributed to patients transferred from North Africa and Turkey. Shortly after that, France, the UK, Germany, and Belgium reported emergence of OXA-48-positive isolates from hospital settings.[178] The spread of OXA-48 enzyme has proven to be much more alarming than was previously thought. In 2015, eight European countries reported regional or inter-regional spread and two countries an endemic situation (Figure 9).[71]

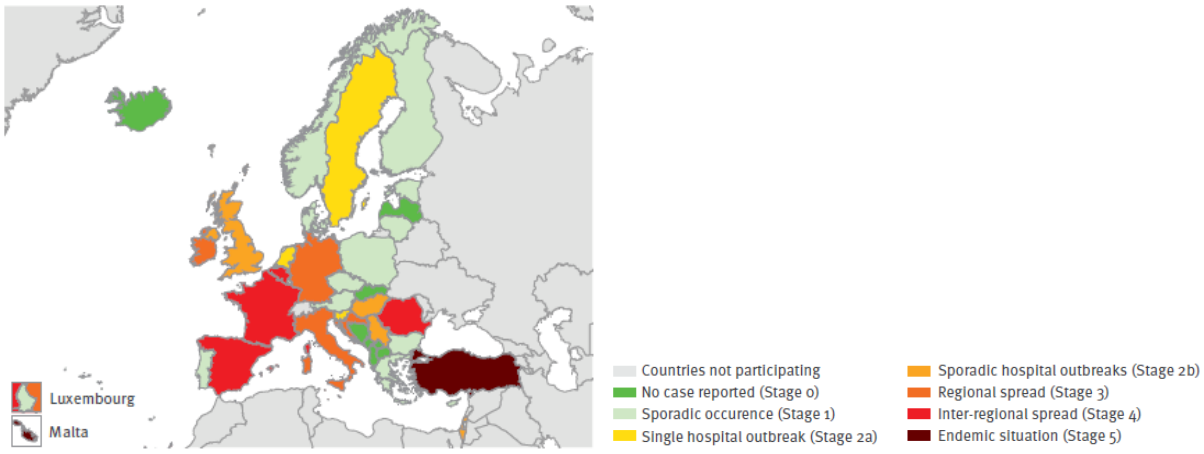

Figure 9. Geographical distribution of OXA 48 producing *Enterobacteriaceae* in Europe, May 2015 [4]

Difficulties associated with OXA-48 detection, since the carbapenem-resistance may remain quite low, significantly contribute to the rapid and successful spread. The expression of the bla_{OXA48} gene in the absence of additional resistance mechanisms possess only low-level hydrolysis of carbapenems. Moreover, there is no reliable inhibitor-based phenotypic test for recognition of OXA-48 enzymes. However, the vast majority of reported outbreaks caused by OXA-48-producing *K. pneumoniæ* is associated with strains exhibiting multidrug resistance patterns, including high-level resistance to carbapenems.

The genes encoding for OXA-48-like enzymes are currently widespread among *K. pneumoniæ* and other *Enterobacteriaceæ*. However, the $bla_{\text{OXA-48}}$-like genes have been reported also in *A. baumanii*.[179]

OXA-48-positive isolates have been reported even from community settings of different geographical areas, e.g., from Mrocco or Switzerland.[180,181] Nevertheless, it is highly probable, that in endemic countries OXA-48 enzymes have spread in the community.

Since the first description of OXA-48 enzyme, several variants of OXA-48-like enzymes have been reported. The OXA-162 variant, differing by a single amino acid substitution, was detected in *K. pneumoniæ* isolates from Turkey.[182] Subsequently, OXA-162 was identified in Germany from nosocomial isolates of various species (e.g., *E. coli*, *C. freundii*, *Raoultella ornithinolytica*).[183] The enzyme OXA-163, possessing unique hydrolytic features among OXA β-lactamases, was reported from Argentina. The OXA-163 enzyme differs from OXA-48 by a single amino acid substitution and four amino acid deletion.[184] The OXA-163 hydrolyzes extended-spectrum cephalosporins, but very weakly carbapenems. Moreover, in contrast to the other class D β-lactamases, its activity is partially inhibited by clavulanic acid and tazobactam. The spread of OXA-181 variant, differing from OXA-48 by 4-amino-acid substitution, has been reported from several countries including India, France, the Netherlands, New Zeland and Sultanate of Oman. Moreover, the $bla_{\text{OXA-181}}$ gene has been found associated with other carbapenemase-resistant genes, such as the $bla_{\text{NDM-1}}$ and $bla_{\text{VIM-5}}$, especially in isolates with an epidemiological link with the Indian subcontinent. The OXA-204 variant, differing by two amino acid substitutions compared to OXA-48, was obtained from clinical isolates recovered from patients with epidemiological link to Algeria and Tunisia.[178] The OXA-232, point mutation of OXA-181, was recovered from clinical strains of K. *pneumoniæ* isolated from patients who had been transferred from Mauritius and India. Subsequently, several other OXA-48-like variants have been described.

The origin of OXA-48-like genes seems to be connected with waterborne species *Shewanella oneidensis*, which possess an intrinsic $bla_{\text{OXA-54}}$ gene encoding a β-lactamase sharing 92% amino acid identity with OXA-48. Moreover, sequence identical to $bla_{\text{OXA-181}}$ was identified to be chromosomally located in *Shewanella xiamenensis*, suggesting that this species could be a progenitor of OXA-181 genes. The predominant hypothesis is that the chromosomal genes get mobilized by insertion sequencesonto plasmids, which disseminated among clinically important species. This way of transmission supported the finding of plasmid-mediated $bla_{\text{OXA-48}}$ gene in *Serratia marscescens* strain isolated from an aquatic environment in Marocco, which can played the role of intermediate reservoir.[178]

The $bla_{\text{OXA-48}}$ gene was originally identified as a part of composite transposon Tn*1999*, flanked by two insertion sequences IS*1999*. Subsequently, composite transposon Tn*1999.2* was reported, differing by the insertion of IS*1R* upstream of $bla_{\text{OXA-48}}$, thus enhancing its expression by providing strong promoter sequences. The isolates harboring Tn*1999.2* composite transposon thus exhibit higher MICs of carbapenems compared to isolates possessing Tn*1999*.[178,185] Third isoform of Tn*1999*, designated Tn*1999.3*, was described in *E. coli* isolate from Italy.[186] Another variant of composite transposon associated with the $bla_{\text{OXA-48}}$ gene was characterized in France from a patient previously hospitalized in Algeria. The $bla_{\text{OXA-48}}$ gene was a part of novel variant, designated Tn*1999.4*, consisting of Tn*1999.2* truncated by another transposon. In this mosaic structure, the transposon carrying the $bla_{\text{OXA-48}}$ gene

was associated with transposon possessing the *bla*$_{CTX-M-15}$ gene, giving rise to a complex genetic structure capable of hydrolyzing all available β-lactam antibiotics.[187] The variant Tn*1999.5* was reported from *K. pneumoniæ* isolated in the Czech Republic.[188]

Nevertheless, genetic features associated with the *bla*$_{OXA-181}$ gene seem to be totally different, lacking the IS*1999*. Instead, insertion sequence IS*Ecp*, frequently associated with acquisition of broad-spectrum β-lactamases, was identified upstream of the *bla*$_{OXA-181}$ gene.[178]

The *bla*$_{OXA-48}$-like genes have been mainly identified among *Enterobacteriaceæ*, which could be explained by a narrow host range of plasmids possessing OXA-48-like enzymes. The current worldwide dissemination of *bla*$_{OXA-48}$ enzymes is mainly connected with the spread of a single IncL-type self-transferable plasmid of approximately 60kb, which does not carry any additional genes of resistance.[178] However, the OXA-181 enzyme was found on different plasmid types (ColE2, IncT, IncX3).[178,188]

The first two OXA-48-producing isolates in the Czech Republic was detected in 2013 in clinical strains of *K. pneumoniæ*. The first one, chromosomally-located, was obtained from an infant with no history of previous hospitalization. The second one, plasmid-mediated, was detected from a patient repatriated from Romania.[188] Recent comprehensive study, including all OXA-48-like producers from 2013 to 2015 detected in the Czech Republic, identified 26 isolates of *K. pneumonia*, *E. coli*, and *E. cloacæ*. Vast majority of the isolates (n = 22) carried the *bla*$_{OXA-48}$ gene on IncL plasmids of a similar size (approximately 60kb). Whereas, two remaining isolates possess the *bla*$_{OXA-181}$ and the *bla*$_{OXA-232}$ genes, located on IncX3 and ColE2-like plasmids, respectively. The findings confirm, consistently with the results of other studies reported worldwide, that the dissemination of OXA-48 genes in the Czech Republic is mainly associated with the spread of single self-transferable plasmid.[188]

2.3 Occurrence of carbapenemases in *Enterobacteriaceæ* and *Pseudomonas æruginosa* in the Czech Republic

In the Czech Republic, the occurrence of carbapenemase-producing Gram-negative bacteria was rare until 2011, with only sporadic cases of carbapenemase-producing *Klebsiella pneumoniæ* (VIM-1, KPC-2), *Serratia marcescens* (VIM-1) and metallo-β-lactamase-producing *Pseudomonas æruginosa* (VIM-2, IMP-7).[70,135,189] However, in 2011, the incidence of carbapenem-resistant bacteria significantly increased attributed mainly to the spread of KPC and MBL enzymes. To control this increase, the national surveillance program recommended an active screening focused on carbapenem resistant isolates as part of its surveillance scheme. The Ministry of Health issued, in 2012, official national guidelines for the control of CPE covering both infected and colonized cases.[190] No further increase in the occurrence of CPE was observed from 2012 till 2014.[153]Nevertheless, in 2015, significant increase of CPE isolates was detected, due to the spread of OXA-48 enzymes.[188] Moreover, in 2016, NDM producers started to be increasingly reported from Czech hospitals.[154] In 2017, a significantly alarming increase of CPE was detected, mainly attributed to the dissemination of OXA-48 and NDM enzymes. The epidemiological data regarding CPE isolates are summarized in Figure 10.

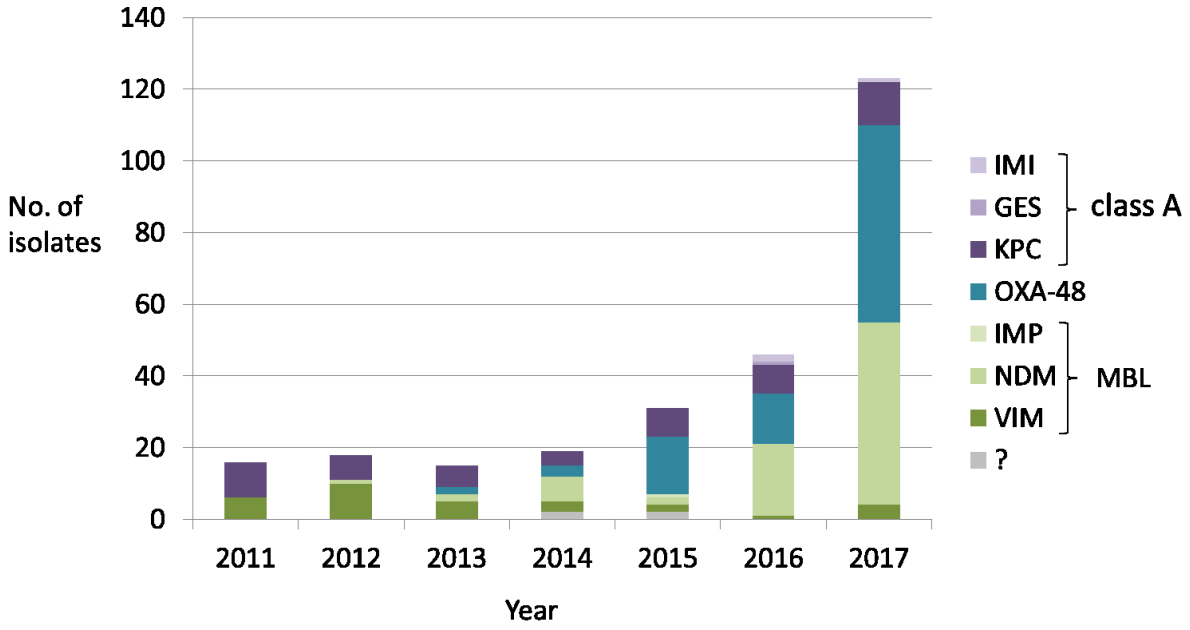

Figure 10. CPE isolates detected in the Czech Republic from 2011 to 2017 [7]

In 2016, altogether 46 CPE isolates were detected in the Czech Republic, including 8 KPC-, 14 OXA-48-, 20 NDM-, 1 VIM-, 1 IMP-, and 2 GES-producers. Moreover, 275 MBL-positive *Pseudomonas æruginosa* isolates were identified throughout the country.

In 2017, 123 CPE isolates were identified, counting 12 KPC-, 55 OXA-48-, 51 NDM-, 4 VIM-, and 1 IMI-positive isolates. Further 167 MBL-producing isolates of *Pseudomonas æruginosa* were reported, including 121 IMP- and 46 VIM-producers.

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3 Methods

Phenotypic and genotypic methods for bacterial typing were performed for all the isolates included in presented studies. Species was identified by matrix-assisted laser desorbtion ionization-time of flight mass spectrometry (MALDI-TOF MS). Antimicrobial susceptibility testing was determined by the broth dilution method and data were interpreted according to the criteria of European Committee on Antimicrobial Susceptibility Testing (EUCAST).[1,2] All isolates were tested for carbapenemase production by MALDI-TOF MS meropenem hydrolysis assay.[3,4] Isolates that were positive by MALDI-TOF MS meropenem hydrolysis assay were subjected to carbapenemase detection using double-disc synergy test (DDST) with EDTA, the phenylboronic acid disc test and the temocillin disc, respectively.[5,6] Subsequently, carbapenemase genes were detected by PCR amplification. Integron analysis and detection of virulence-associated genes was performed for certain *Pseudomonas* sp. isolates. PCR products were sequenced by Sanger sequencing technique. Selected isolates were typed by multilocus sequence typing (MLST).[7,8,9] The β-lactamase content of certain isolates was determined by isoelectric focusing (IEF). Conjugation or transformation were used for transfer of genes encoding for carbapenemases.[10,11] Plasmid analysis was performed using S1-pulsed-field gel electrophoresis (PFGE) followed by Southern blot hybridization with the DIG-labeled probes.[12] Plasmid incompatibility (Inc) groups were determined by PCR-based replicon typing (PBRT) method.[13] Plasmid DNAs from transconjugants and transformants were extracted using the Qiagen Large-Construct Kit (Qiagen, Hilden, Germany). The genomic DNAs were extracted using the DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy). Selected plasmids and chromosomes were sequenced using the Illumina MiSeq platform. Additionally, selected Illumnina data were used for phylogenetic analysis.

All methods performed during work on the dissertation thesis are described in detail in presented publications.

Species identification by matrix-assisted laser desorbtion ionization-time of flight mass spectrometry (MALDI-TOF MS)	See publication no.: 1, 3, 4
Antimicrobial susceptibility testing by broth microdilution method[1]	See publication no.: 1, 3, 4
Testing for carbapenemase production by the MALDI-TOF MS meropenem hydrolysis assay[3,4]	See publication no.: 1, 3, 4
The double-disc synergy test (DDST) with EDTA, the phenylboronic acid disc test, and the temocillin disc test[5,6]	See publication no.: 1, 3, 4
PCR amplification of genes encoding for selected β-lactamases	See publication no.: 1, 3, 4
Typing of the isolates by multilocus sequence typing (MLST)[7,8,9]	See publication no.: 1, 3, 4
Sanger sequencing of the PCR products	See publication no.: 1, 3, 4
Detection of β-lactamases by isoelectric focusing (IEF)	See publication no.: 1,3
Conjugal transfer of genes encofing for carbapenemases[10]	See publication no.: 1, 3, 5
Plasmid DNA extraction	See publication no.: 1, 2, 3, 4, 5
Transformation of genes encoding for carbapenemases[11]	See publication no.: 1, 2, 3, 4, 5
Genomic DNA extraction	See publication no.: 1, 3, 4
S1- PFGE, Southern blot hybridization with the DIG-labeled probes[12]	See publication no.: 1, 3, 4

Plasmid incompatibility group determination by PCR-based replicon typing (PBRT) method[13]	See publication no.: 1, 3
Whole-genome sequencing by Illumina MiSeq platform	See publication no.: 1, 3, 4
Detection of virulence-associated genes	See publication no.: 4
Integron analysis	See publication no.: 4
Bayesian analysis	See publication no.: 4

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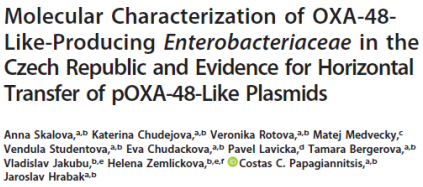
4 List of publications included in dissertation thesis

Selected publications focused on molecular-epidemiological analysis of carbapenem-resistant isolates of *Enterobacteriaceæ* and *Pseudomonas* sp. detected in the Czech Republic are included in the dissertation thesis. Concluding remarks of the studies are summarized in discussion.

1. **Skalova A**, Chudejova K, Rotova V, Medvecky M, Studentova V, Chudackova E, Lavicka P, Bergerova T, Jakubu V, Zemlickova H, Papagiannitsis CC, Hrabak J. Molecular characterization of OXA-48-like-producing Enterobacteriaceæ in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids. Antimicrobial Agents and Chemotherapy. 2017 Feb 1;61(2):e01889-16.
2. Chudejova K, Rotova V, **Skalova A**, Medvecky M, Adamkova V, Papagiannitsis CC, Hrabak J. Emergence of sequence type 252 Enterobacter cloacæ producing GES-5 carbapenemase in a Czech hospital. Diagnostic Microbiology and Infectious Disease. 2018 Feb 1;90(2):148-50.
3. Paskova V, Medvecky M, **Skalova A**, Chudejova K, Bitar I, Jakubu V, Bergerova T, Zemlickova H, Papagiannitsis CC, Hrabak J. Characterization of NDM-encoding plasmids from Enterobacteriaceæ recovered from Czech hospitals, Frontiers in Microbiolgy. 2018; Article in press.
4. Papagiannitsis CC, Medvecky M, Chudejova K, **Skalova A**, Rotova V, Spanelova P, Jakubu V, Zemlickova H, Hrabak J. Molecular Characterization of Carbapenemase-Producing Pseudomonas æruginosa of Czech Origin and Evidence for Clonal Spread of Extensively Resistant Sequence Type 357 Expressing IMP-7 Metallo-β-Lactamase. Antimicrobial Agents and Chemotherapy. 2017 Dec 1;61(12):e01811-17.
5. Rotova V, Papagiannitsis CC, Chudejova K, Medvecky M, **Skalova A**, Adamkova V, Hrabak J. First description of the emergence of Enterobacter asburiæ producing IMI-2 carbapenemase in the Czech Republic. Journal of Global Antimicrobial Resistance. 2017 Oct 10;11:98.

4.1 Publication no. 1: Molecular characterization of OXA-48-like-producing *Enterobacteriaceæ* in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids



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Abstract

The aim of this study was to characterize the first cases and outbreaks of OXA-48-like-producing *Enterobacteriaceæ* recovered from hospital settings, in the Czech Republic. From 2013-2015, 22 *Klebsiella pneumoniæ* isolates, 3 *Escherichia coli* isolates, and 1 *Enterobacter cloacæ* isolate producing OXA-48-like carbapenemases were isolated from 20 patients. Four of the patients were colonized or infected by two or three different OXA-48-like producers. The *K. pneumoniæ* isolates were classified into nine sequence types (STs), with ST101 being the predominant (n = 8).The *E. coli* isolates were of different STs, while the *E. cloacæ* isolate belonged to ST109. Twenty-four isolates carried bla_{OXA-48}, while two isolates carried $bla_{OXA-181}$ or $bla_{OXA-232}$.Almost all isolates (n = 22) carried bla_{OXA-48}-positive plasmids of similar size (~60kb), except the two isolates producing OXA-181 or OXA-232. In an ST45 *K. pneumoniæ* and an ST38 *E. coli* isolate, S1 nuclease profiling plus hybridization indicated a chromosomal location of bla_{OXA-48}. Sequencing showed that the majority of bla_{OXA-48}-carrying plasmids exhibited high degrees of identity with the pOXA-48-like plasmid, pE71T. Additionally, two novel pE71T derivatives, pOXA-48_30715 and pOXA-48_30891, were observed. The $bla_{OXA-181}$-carrying plasmid was identical to the IncX3 plasmid pOXA181_EC14828, while the$bla_{OXA-232}$-carrying plasmid was a ColE2-type plasmid, being a novel

derivative of pOXA-232. Finally, sequencing data showed that ST45 *K. pneumoniæ* and ST38 *E. coli* isolates harbored the IS*1R*-based composite transposon, Tn*6237*, containing bla_{OXA-48}, integrated into their chromosomes. These findings underlined that the horizontal transfer of pOXA-48-like plasmids has played a major role in the dissemination of bla_{OXA-48} in the Czech Republic. In combination with the difficulties with their detection, OXA-48 producers constitute an important public threat.

Introduction

Since the beginning of the 2000s, carbapenemases of the Ambler class A KPC type or class B type, including IMP- and VIM-like enzymes, were considered to be the most important carbapenemases in *Enterobacteriaceæ*. In 2001, the class D β-lactamase OXA-48, which possesses weak but significant carbapenemase activity, was first detected from a carbapenem-resistant *Klebsiella pneumoniæ* isolate that had been recovered in Instabul, Turkey.[1] Soon, a series of sporadic cases, but also hospital outbreaks, was reported in the main cities of Turkey.[2,3] At about the same time, the bla_{OXA-48} gene, most often in *K. pneumoniæ* isolates, was also identified in other Middle Eastern and North African countries.[4,5] All those countries can be considered important reservoirs of OXA-48 producers.

Additionally, OXA-48 producers have been identified sporadically in several European countries, including the United Kingdom, Belgium, France, Germany and the Netherlands.[3] The emergence of OXA-48 producers in these countries has been attributed mainly to colonized patients who transferred from North Africa and Turkey.[6] These data indicated that the spread of the bla_{OXA-48} gene was limited to Turkey, the Middle East and North Africa. However, in countries such as the United Kingdom, France, Belgium and Germany, recent studies revealed the emergence of OXA-48-producing *Enterobacteriaceæ* in hospital settings, supposing a much more important spread than was previously thought.[7-10] Notably, concern was raised by the occurrence of OXA-48 producers in the community in the countries of North African and Europe.[3,11] Indeed, the fact that their detection is difficult might have played a significant role in the spread of OXA-like producers, which have somehow been silent. Actually, the expression of bla_{OXA-48} gene in the absence of additional resistance mechanisms (e.g., low levels of expression of porins), confers only a low level of resistance to carbapenems. Also, there is no inhibitor-based phenotypic test that canrecognize the production of OXA-48-type enzymes. Thus, these two main points do not contribute to the easy recognition of OXA-48-like producers.

In the Czech Republic, the occurrence of carbapenemase-producing *Enterobacteriaceæ* (CPE) was rare, with only a total of two cases being detected in 2009 and 2010.[12] In 2011, the occurrence of CPE increased, and this was mainly due to two hospital outbreaks.[13] To contain this increase, in 2012, the Ministry of Health issued national guidelines for the management of patients infected and colonized with CPE.[13] In 2012 and 2013, only an outbreak of VIM-producing isolates and four sporadic cases were reported.[14] The Sporadic cases included two NDM-producing *Enterobacteriaceæ* and the first two OXA-48-producing *K. pneumoniæ* isolates identified in the Czech Republic.[15,16] These data supposed the success of the

national guidelines. However, an increase in the occurrence of CPE was observed, during 2014 and 2015, and this was mainly due to the spread of OXA-48-like-producing *Enterobacteriaceæ* in Czech hospitals.

The aim of the present study was to characterize the OXA-48-like producers detected in Czech hospitals, in 2014 and 2015.

Materials and methods

Bacterial isolates and confirmation of carbapenemase production

In 2014 and 2015, Czech hospitals referred a total of 630 *Enterobacteriaceæ* isolates with a meropenem MIC >0.125 µg/ml to the National Reference Laboratory for Antibiotics.[17] Species identification was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). All isolates were tested for carbapenemase production by the MALDI-TOF MS meropenem hydrolysis assay.[18] Isolates that were positive by the MALDI-TOF MS meropenem hydrolysis assay were subjected to metallo-β-lactamase, KPC and OXA-48 detection using the double disc synergy test with EDTA, the phenylboronic acid disc test and the temocillin disc, respectively.[9,19,20] Additionally, carbapenemase genes (bla_{KPC}, bla_{VIM},bla_{IMP},bla_{NDM} and bla_{OXA-48}-like) were detected by PCR amplification.[1,21-23] PCR products were sequenced as described below. Isolates positive for bla_{OXA-48}-like genes were further studied. Moreover, the two OXA-48-producing *K. pneumoniæ* isolates, recovered at the University hospital Pilsen (Pilsen, Czech Republic) during 2013, were included in this study for comparative epidemiological purposes.

Susceptibility testing

The MICs of piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, imipenem, meropenem, ertapenem, co-trimoxazole, ciprofloxacin, gentamicin, amikacin, colistin and tigecycline were determined by the broth dilution method.[24] Data were interpreted according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org).

Typing

All bla_{OXA-48}-like-positive isolates were typed by multilocus sequence typing (MLST).[25-27] The databases at:

- http://pubmlst.org/ecloacæ,
- http://mlst.warwick.ac.uk/mlst/dbs/Ecoliand,
- http://bigsdb.web.pasteur.fr/klebsiella

were used for assigning STs.

Detection of β-lactamases

The β-lactamase content of all bla_{OXA-48}-like-positive isolates was determined by isoelectric focusing (IEF). Bacterial extracts were obtained by sonication of bacterial cells suspended in 1% glycine buffer and clarified by centrifugation. Sonicated cell extracts were analyzed by IEF in polyacrylamide gels containing ampholytes (pH 3.5-9.5; APBiotech, Piscataway, NJ). The separated β-lactamases were

visualized by covering of the gel with the chromogenic cephalosporin, nitrocefin (0.2 mg/ml; Oxoid Ltd., Basingstoke, United Kingdom).[28]

On the basis of the IEF data, PCR detection of various *bla* genes was performed by the use of primers specific for $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{CMY}, as reported previously.[29-32] Both strands of the PCR products were sequenced using an ABI 377 sequencer (Applied Biosystems, Foster City, CA).

Transfer of $bla_{\text{OXA-48}}$-like genes

Conjugal transfer of $bla_{\text{OXA-48}}$-like genes from the clinical strains was carried out in mixed broth cultures, using the rifampin-resistant *E. coli* A15 laboratory strain as a recipient.[33] Transconjugants were selected on MacConkey agar plates supplemented with rifampin (150μg/ml) and ampicillin (50 μg/ml). Plasmid DNA from clinical isolates which failed to transfer $bla_{\text{OXA-48}}$-like by conjugation, was extracted using a Qiagen maxikit (Qiagen, Hilden, Germany) and used to transform *E. coli* DH5α cells. The preparation and transformation of competent *E. coli* cells were done using calcium chloride, as described by Cohen et al.[34] Transformants were selected on Luria-Bertaniagar plates with ampicillin (50 μg/ml). Transconjugants or transformants were confirmed to be OXA-48-like producers by PCR and MALDI-TOF MS meropenem hydrolysis assay.[1,18]

Plasmid analysis

To define the genetic units of the $bla_{\text{OXA-48}}$-like genes, the plasmid contents of all OXA-48-producing clinical and recombinant strains were analyzed by pulsed-field gel electrophoresis (PFGE) of total DNA digested with S1 nuclease (Promega, Madison, WI, USA).[35] Following PFGE, the DNA was transferred to BrightStar-Plus positively charged nylon membrane (Applied Biosystems, Foster City, CA) and hybridized with digoxigenin-labelled $bla_{\text{OXA-48}}$-like probes.

Plasmid incompatibility (Inc) groups were determined by the PCR-based replicon typing (PBRT) method, using total DNA from transconjugants and transformants.[36] $bla_{\text{OXA-48}}$-like-carrying plasmids were further characterized by a specific IncLPCR assay, using L-FW and L/M-RV primer pair.[37] The forward primer targeted the *excA* gene of the IncL plasmid type, while the reverse primer targeted the highly conserved *repA* gene of the IncL and IncM plasmid types.[37]

Plasmid and chromosome sequencing

Plasmid DNAs from transconjugants and transformants were extracted using the Qiagen large-construct kit (Qiagen, Hilden, Germany). Additionally, the genomic DNAs of *K. pneumoniæ* Kpn-82929/13 and *E. coli* Eco-32005/15 were extracted using the DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy). Plasmids and chromosomes were sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Initial paired-end reads were quality trimmed using Trimmomatic tool.[38] For assembly of the plasmids, reads were mapped to the reference *E. coli* K-12 substrain MG 1655 genome (GenBank accession no. U00096) using the BWA-MEM algorithm, in order to filter out the chromosomal DNA.[39] Then, all the unmapped paired-end reads were assembled by use of the de Bruijn graph-based *de novo* assembler SPAdes.[40] The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For

sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISFinder database (www-is.biotoul.fr/), and open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using the Mauve (version 2.3.1) program.[41]

Accession number(s)

One nucleotide sequence representing each different plasmid type was submitted to the GenBank. The nucleotide sequences of the pOXA-48_4963 (type A0), pOXA-48_30715 (type A1), pOXA-48_30891 (type A2), pOXA-181_29144 (type B) and pOXA-232_30929 (type C) plasmids have been deposited in GenBank under accession numbers KX523900, KX523901, KX523902, KX523903 and KX523904, respectively.

Results and Discussion

Carbapenemase-producing *Enterobacteriaceæ*

A total of 52 *Enterobacteriaceæ* isolates showing carbapenemase activity on MALDI-TOF MS meropenem hydrolysis assay were recovered from Czech hospitals, during 2014 (n = 17) and 2015 (n = 35).PCR screening showed that 50 of the isolates were positive for one carbapenemase gene (17 isolates from 2014 [bla_{KPC}, n = 4; bla_{VIM}, n = 4; bla_{NDM}, n = 7; bla_{OXA-48}-like, n = 2] and 33 isolates from 2015 [bla_{KPC}, n = 8; bla_{VIM}, n = 2; bla_{NDM}, n = 2; bla_{OXA-48}-like, n = 21]), while the remaining 2 isolates were positive for the presence of two carbapenemase genes (bla_{VIM} and bla_{IMP}, n = 1; bla_{OXA-48}-like and bla_{NDM}, n = 1).

OXA-48-like-producing isolates

Altogether, 24 nonrepetitive isolates producing OXA-48-like carbapenemases were isolated from 18 patients, in 2014 and 2015. Among them, 20 of the isolates were identified to be *K. pneumoniæ*, 3 were identified to be *Escherichia coli* and 1 was identified to be *Enterobacter cloacæ*. Four of the patients were colonized or infected by two or three different OXA-48-like producers (Table 1). Additionally, the two OXA-48-like-producing *K. pneumoniæ* isolates identified in 2013were studied.

OXA-48-like producers were collected from seven Czech hospitals, located throughout the Czech Republic. Hospital B was the setting with the highest occurrence of OXA-48 producers. In June 2013, the first OXA-48 producer (Kpn-82929), identified in the Czech Republic was isolated from a newborn. The second OXA-48-producing isolate (Kpn-63870) was recovered from a patient who was directly repatriated from Romania. From April 2014 till March 2015, three further patients colonized or infected with OXA-48-producing *K. pneumoniæ* were identified. Additionally, in hospital B, an outbreak that included six patients diagnosed with OXA-48-producing *K. pneumoniæ* lasted from August to December of 2015. Only two cases of OXA-48-producing *K. pneumoniæ* isolates. were reported in hospital A1. The first case, a 1-year-old child, who was directly repatriated from a Russian hospital, was colonized or infected by three OXA-48-producing isolates: *K. pneumoniæ* Kpn-04976 and Kpn-04963 and *E. cloacæ* Ecl-04292. One month later, the transmission of an OXA-48-producing

Table 1. Characteristics of OXA-48-like-producing *Enterobacteriaceae*

Isolate[a]	Isolation year (hospital)	ST	β-Lactamase content	Size of bla_{OXA-48}-like-carrying plasmid[b] (kb)	Type of plasmid sequence (replicon)	MICs (μg/ml)[d]											
						Ctx	Caz	Fep	Imp	Mer	Etp	Gen	Amk	Sxt	Cip	Col	Tgc
K. pneumoniæ																	
Kpn-82929	2013 (B)	45	OXA-48, CTX-M-14	chr[c]	–	8	1	8	2	≤0.12	1	≤0.12	≤0.5	1	≤0.06	≤0.25	1
Kpn-63870	2013 (B)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	>16	16	>16	>16	16	1	>8	8	1
Kpn-74996	2014 (B)	461	OXA-48	**63.566**	A0 (IncL)	0.5	1	0.5	1	1	2	≤0.12	≤0.5	2	≤0.06	≤0.25	4
Kpn-81700	2014 (B)	461	OXA-48, TEM-1	**63.566**	A0 (IncL)	0.5	1	0.5	4	1	8	≤0.12	≤0.5	2	≤0.06	0.5	2
Kpn-04976■	2015 (A1)	1520	OXA-48	**63.566**	A0 (IncL)	0.25	0.5	≤0.12	2	0.25	4	≤0.12	1	1	≤0.06	≤0.25	1
Kpn-04963■	2015 (A1)	395	OXA-48, CTX-M-15, OXA-1, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	>16	8	>16	0.5	8	>32	>8	≤0.25	4
Kpn-05159	2015 (A1)	395	OXA-48, CTX-M-15, OXA-1, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	>16	16	>16	>16	8	>32	>8	≤0.25	4
Kpn-29097	2015 (B)	461	OXA-48	**63.566**	A0 (IncL)	0.25	0.5	0.25	1	0.25	2	0.25	≤0.5	1	1	≤0.25	4
Kpn-17153•	2015 (B)	461	OXA-48	**63.566**	A0 (IncL)	0.5	0.25	0.25	0.5	0.25	4	0.25	1	0.12	0.12	0.5	4
Kpn-18921•	2015 (B)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	0.5	0.25	4	>16	8	1	>8	1	0.25
Kpn-20382	2015 (B)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	2	0.25	4	>16	4	0.5	>8	≤0.25	0.25
Kpn-23770○	2015 (B)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	>16	8	>16	>16	32	8	>8	≤0.25	0.5
Kpn-23495	2015 (B)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	2	0.25	2	>16	1	2	>8	≤0.25	1
Kpn-23482	2015 (B)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	2	0.25	2	>16	8	1	>8	≤0.25	0.5
Kpn-24100	2015 (B)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	2	0.25	2	>16	4	0.5	>8	≤0.25	0.5
Kpn-29144	2015 (C)	18	OXA-181, CTX-M-15, OXA-1, TEM-1	**51.478**	B (IncX3)	>8	>16	>16	>16	>16	>16	>16	4	>32	>8	≤0.25	1
Kpn-30715▲	2015 (D)	11	OXA-48, CTX-M-15	**65.488**	A1 (IncL)	>8	>16	>16	>16	>16	>16	>16	>64	>32	>8	≤0.25	1
Kpn-30891▲	2015 (D)	891	OXA-48, CTX-M-15, TEM-1	**66.059**	A2 (IncL)	>8	>16	16	1	0.5	4	>16	>64	>32	>8	2	2
Kpn-30890	2015 (D)	11	OXA-48, CTX-M-15	**65.488**	A1 (IncL)	>8	>16	>16	2	1	16	>16	>64	>32	>8	8	1
Kpn-31329	2015 (D)	15	OXA-48, CTX-M-15, OXA-1, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	>16	1	2	>16	2	1	>8	0.5	0.5
Kpn-31569	2015 (D1)	101	OXA-48, CTX-M-15, TEM-1	**63.566**	A0 (IncL)	>8	>16	>16	2	8	>16	>16	8	1	>8	≤0.25	1
Kpn-30929	2015 (E)	15	OXA-232, NDM-1, CTX-M-15, OXA-1	12.531	C (ColE2-like)	>8	>16	>16	>16	8	>16	>16	>64	>32	>8	1	0.5
E. coli																	
Eco-32005	2015 (A2)	38	OXA-48, TEM-1	chr[c]	–	0.25	≤0.12	≤0.12	0.25	0.12	1	>16	2	0.12	≤0.06	≤0.25	>32
Eco-17646•	2015 (B)	4956	OXA-48	**63.566**	A0 (IncL)	0.5	0.25	≤0.12	0.5	0.12	2	1	2	0.06	8	≤0.25	0.25
Eco-26031○	2015 (B)	216	OXA-48	**63.566**	A0 (IncL)	0.12	≤0.12	≤0.12	1	0.12	0.25	0.25	1	0.03	≤0.06	≤0.25	0.25
E. cloacæ																	
Ecl-04292■	2015 (A1)	109	OXA-48, CTX-M-15, OXA-1, TEM-1	**63.566**	A0 (IncL)	>8	16	16	4	0.5	>16	0.25	1	4	≤0.06	≤0.25	1

[a] Black squares, black circles, white circles and black triangles indicate OXA-48-like-producing isolates recovered from the same patient.
[b] Data for plasmids found in transconjugants are shown in bold; data for plasmids observed in transformants are underlined.
[c] chr, chromosomal location of a bla_{OXA-48} gene.
[d] Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Imp, imipenem; Mer, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin; Col, colistin; Tgc, tigecycline.

K. pneumoniæ Kpn-05159 to an infant who stayed in the same department was found. An OXA-48 outbreak restricted to three patients occurred in hospital D. A patient that had recently traveled to Ukraine was diagnosed with two OXA-48-producing isolates of *K. pneumoniæ* (Kpn-30715 and Kpn-30891) in August of 2015. Two further patients colonized or infected with OXA-48-producing *K. pneumoniæ* were identified until September. The remaining four cases were detected in four different hospitals. Two of those cases had recently traveled abroad (to India [Kpn-30929] and Tunisia [Kpn-

31569]), while no data on whether the other patients had traveled abroad or had previously been hospitalized were available.

All, 26 OXA-48-like producers exhibited resistance to piperacillin and piperacillin-tazobactam (data not shown), while the variations in the MICs of cephalosporins and carbapenems that were observed (Table 1) might reflect the presence of additional resistance mechanisms in some of the isolates. Seventeen of the OXA-48-like producers also exhibited resistance to ciprofloxacin, 16 were resistant to gentamicin, 6 were resistant to tigecycline, and 5 were resistant to amikacin, whereas 2 isolates were resistant to colistin.

The population structure of OXA-48-like-producing isolates studied by MLST is shown in Table 1. *K. pneumoniæ* isolates comprised nine STs. ST101 was the most prevalent, accounting for eight isolates. The majority of ST101 isolates (7/8) was recovered from patients hospitalized in hospital B. Ten of the isolates were distributed in STs 461 (n = 4, from hospital B), 11 (n = 2, from hospital D), 15 (n = 2) and 395 (n = 2, from hospital A1). The remaining isolates belonged to distinct STs. STs 11, 15, 45, 101, 395 and 461 have previously been associated with OXA-48-like-producing isolates from several geographical areas.[42-44] All three *E. coli* isolates were of different STs, including the pandemic ST38.[44-46] The *E. cloacæ* isolate was assigned to ST109, previously associated with the production of CTX-M-15 or SHV-12 enzymes.[47]

Sequencing of the PCR products revealed three *bla*$_{OXA-48}$-type genes encoding the OXA-48, OXA-181, and OXA-232enzymes (Table 1).[1,48,49] Twenty-four of the isolates were found to produce the OXA-48 β-lactamase, while the ST18 *K. pneumoniæ* isolate produced the OXA-181 enzyme. The remaining *K. pneumoniæ* isolate, which belonged to ST15, coproduced the OXA-232 and NDM1carbapenemases. Additionally, most of *bla*$_{OXA-48}$-like-positive isolates were confirmed to coproduce the extended-spectrum β-lactamase CTX-M-15 (n = 17), either alone or along with TEM-1 (n = 16) and/or OXA-1 (n = 5), whereas the ST45 OXA-48-producing *K. pneumoniæ* isolate coproduced the CTX-M-14 β-lactamase.

bla$_{OXA-48}$-like-carrying plasmids

The *bla*$_{OXA-48}$-like genes from 24 out of 26 clinical strains were transferred by conjugation (n = 23) or transformation (n = 1) (Table 1). Neither the ST45 *K. pneumoniæ* isolate nor the ST38 *E. coli* isolate was capable of transferring the *bla*$_{OXA-48}$ gene by either conjugation or transformation. All *bla*$_{OXA-48}$-like-positive recombinants exhibited similar resistance phenotypes; showing resistance to piperacillin and piperacillin-tazobactam, and decreased susceptibility or resistance to imipenem and ertapenem, while they remained susceptible to cephalosporins and meropenem. Additionally, all *bla*$_{OXA-48}$-like-positive recombinants were susceptible to non-β-lactam antibiotics.

Plasmid analysis of OXA-48-producing donor and transconjugant strains revealed the transfer of plasmids, all of which were ~60 kb (Table 1). The OXA-181-producing transconjugant carried a *bla*$_{OXA-48}$-like-positive plasmid with a size of ~50 kb, while the OXA-232-producing transformant harbored a plasmid of ~10 kb that hybridized with a *bla*$_{OXA-48}$-like probe. Moreover, in the S1 nuclease

profiles of the OXA-48-producing ST45 *K. pneumoniæ* and ST38 *E. coli* isolates, the bla_{OXA-48}-like probe hybridized only with largest DNA bands corresponding to the chromosomal material.

Replicon typing showed that all plasmids carrying bla_{OXA-48} were positive for the IncL allele, whereas the $bla_{OXA-181}$- and $bla_{OXA-232}$-carrying plasmids were nontypeable by PCR-based replicon typing (PBRT).

Structure of OXA-48-like-carrying plasmids

The complete sequences of all bla_{OXA-48}-like-carrying plasmids were determined. Illumina sequencing revealed three types of plasmid sequences (types A to C), with type A being the most prevalent including three subtypes (A0, A1, A2).

All OXA-48-carrying plasmids belonged to type A and were derivatives of the archetypal IncL bla_{OXA-48}-carrying plasmid pOXA-48 (Figure 1), originally described in the *K. pneumoniæ* 11978 isolate recovered in Turkey in 2001 and then reported worldwide.[50] Nineteen out of the 22 sequenced bla_{OXA-48}-carrying plasmid plasmids (type A0; Table 1) showed high degrees of similarity to each other and to pE71T (100% coverage, 99% identity), previously characterized from *K. pneumoniæ* E71T isolated in Ireland.[51] Plasmid pE71T differed from pOXA-48 by the insertion of two copies of IS*1R* element. The carbapenemase gene was part of the Tn*1999.2* transposon, which included IS*1R* integrated in IS*1999* located upstream of the bla_{OXA-48} gene.[2] The second IS*1R* was inserted into *orf25*. Plasmids pOXA-48_30715 and pOXA-48_30890 (type A1), both of which were isolated fromST11 *K. pneumoniæ* isolates, differed from pE71T by the insertion of a 1,911-bp fragment encoding a reverse transcriptase (RetA), upstream of *mucAB* operon. Plasmid pOXA-48_30891 (type A2) was a pE71T derivative carrying a novel variant of the Tn*1999.2* transposon (designated Tn*1999.5*), in which the *lysR* gene was truncated by the IS*Kpn19* element. Interestingly, plasmids pOXA-48_30715 and pOXA-48_30891 were characterized from two different *K. pneumoniæ* isolates recovered from the same patient (Table 1). Among all type Abla_{OXA-48}-like-carrying plasmids, no resistance genes other than bla_{OXA-48}were identified, as previously described for the pOXA-48 and its relatives.[37,50,51]

pOXA-181_29144 (type B) (Figure 1), encoding OXA-181, was an IncX3-type plasmid that was identical to pOXA181_EC14828 (100% coverage, 100% identity), which to date has been described only in China from an ST410 *E. coli* strain (WCHEC14828) isolated in 2014.[52] Similar to pOXA181_EC14828, the*qnrS1b* gene conferring low-level resistance to fluoroquinolones was identified in the sequence of pOXA-181_29144. Finally, plasmid pOXA-232_30929 (type C) appeared to be a derivative of pOXA-232 (Figure 1), a ColE2-type plasmid originally described from an ST2968 *E. coli* isolate and two ST14 *K. pneumoniæ* isolates recovered from patients who transferred from India to France in 2011.[49] Only one difference between the two plasmids was observed. A 5,981-bp segment consisting of the Tn*1000* transposon was present in pOXA-232_30929 and was found 477 bp upstream of the *repA* gene.

Finally, *de novo* assembly obtained a unique contig containing bla_{OXA-48}, for ST45 *K. pneumoniæ* and ST38 *E. coli*. Sequence analysis showed that these isolates harbored a 21.9-kb plasmid fragment containing bla_{OXA-48} flanked by IS*1R* elements integrated into their chromosomes. This plasmidic

fragment consisted of the IS*1R*-based composite transposon (Figure 1) Tn*6237*.[53] However, using the Illumina MiSeq platform, we were not able to identify the precise insertion site of Tn*6237*.

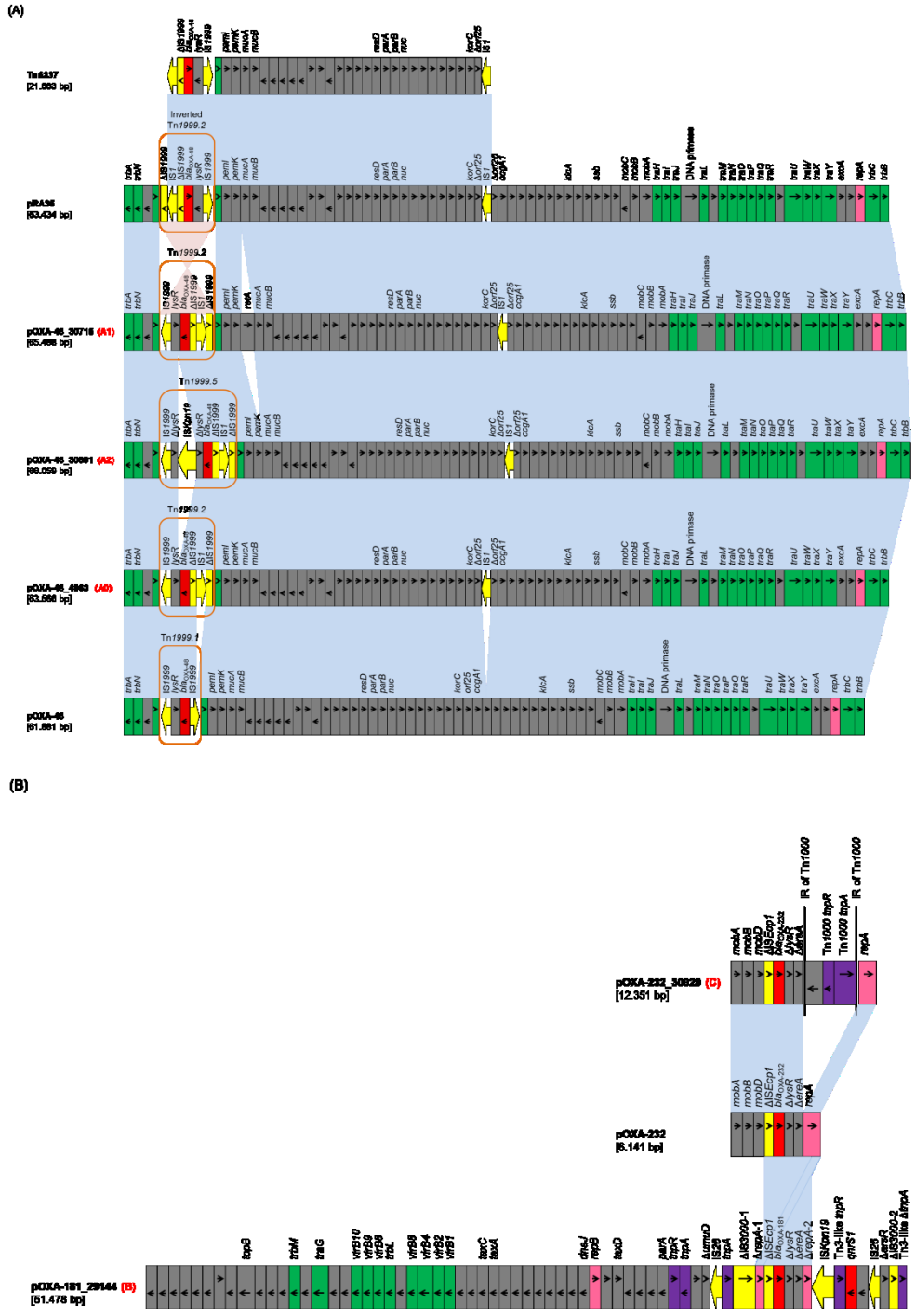

Figure 1. Linear maps of the *bla*$_{OXA-48}$-like-carrying plasmids. For each plasmid, the type of plasmid sequence is indicated in red next to the plasmid name. (A) Comparison of the IncL *bla*$_{OXA-48}$-like-carrying plasmids pOXA-48, pOXA-48_4963, pOXA-48_30715, pOXA-48_30891 and pRA35, and of the composite transposon Tn*6237*.[50,53] The boundaries of Tn*1999* like transposons are also shown. (B) Comparison of the *bla*$_{OXA-232}$-like-carrying plasmids pOXA-232 and pOXA-232_30929, and of the *bla*$_{OXA-181}$-like-carrying plasmids pOXA-181_29144.49 Open reading frames (ORFs) are shown as rectangles (arrows within rectangles indicate the direction of transcription). Intact insertion sequences (IS) are represented by arrows, while truncated IS elements appear as rectangles. Replicons of the plasmids are indicated as pink rectangles. Resistance genes, IS elements and transposases are shown in red, yellow, and purple, respectively. Green rectangles indicate genes responsible for the conjugative transfer of the plasmids. The remaining genes, including plasmid scaffold regions, are indicated as grey rectangles. Homologous segments (representing ≥99% sequence identity) are indicated by light blue shading, while pink shading shows inverted homologous segments.

Concluding remarks

In conclusion, the present study investigated the first cases and outbreaks of OXA-48-like-producing *Enterobacteriaceæ* isolates from the Czech Republic. Five of the patients had recently traveled abroad, with one of them being involved in the initiation of an outbreak (hospital D), while three OXA-48-like isolates (Kpn-82929, Kpn-29114 and Eco-32005) could be described as community-acquired since the patients had no history of previous hospitalization or travel abroad. The setting that was most affected was hospital B, in which an outbreak followed a long period with the sporadic occurrence of OXA-48 producers. In hospital B, the outbreak was associated with the spread of *K. pneumoniæ* isolates belonging to ST101. Most of the STs found in isolates of *K. pneumoniæ* (STs 11, 15, 45, 101, 395 and 461) and *E. coli* (ST38) have previously been associated with OXA-48-like-producing isolates from several geographical areas.[42–46]

In four of the patients, two or three different OXA-48 producers were identified during their hospitalization, supposing the *in vivo* horizontal transfer of the $bla_{\text{OXA-48}}$-carrying plasmid. Sequencing data showed the presence of the same $bla_{\text{OXA-48}}$-carrying plasmid in three of these isolates (Table 1), further confirming this hypothesis. In addition, the same $bla_{\text{OXA-48}}$-carrying plasmid (type A0) was identified in all isolates recovered from patients that were involved in the outbreak, which took place in hospital B.

Results from Illumina sequencing showed that pOXA-48-like plasmids played a major role in the dissemination of $bla_{\text{OXA-48}}$ gene in Czech hospitals. Among our isolates, a highly conserved $bla_{\text{OXA-48}}$-carrying plasmid, which was identical to the previously described pE71T, was observed in a polyclonal population of *K. pneumoniæ* isolates (of 5 different STs). Plasmid pE71T was also found in two *E. coli* isolates of different STs and one *E. cloacæ*.isolate.[51] Additionally, two novel pE71T derivatives (plasmids pOXA-48_30715 and pOXA-48_30891) were characterized from *K. pneumoniæ* isolates of STs 11 and 891, respectively. On the other hand, the OXA-181 and OXA-232 carbapenemases were encoded by different types of plasmids belonging to IncX3 and ColE2-like groups, respectively.

The data presented here contribute to the current knowledge of OXA-48-like-producing *Enterobacteriaceæ*. In agreement with the results of previous studies, our findings underline that OXA-48 producers pose an important public threat, mainly due to the difficulties with their detection and the rapid horizontal transfer of pOXA-48-like plasmids.

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4.2 Publication no. 2: Emergence of Sequence Type 252 *Enterobacter cloacæ* producing GES-5 carbapenemase in a Czech hospital

Note

Emergence of sequence type 252 *Enterobacter cloacae* producing GES-5 carbapenemase in a Czech hospital

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Abstract

ST252 *Enterobacter cloacæ*, producing GES-5 carbapenemase, was isolated in a Czech hospital. bla_{GES-5} was part of a novel class 1 integron, In1406, which also included a new allele of the *aadA15* gene cassette. In1406 was located on a ColE2-like plasmid, pEcl-35771cz (6,953 bp).

GES-5-producing *Enterobacter cloacæ* ST252

Acquired carbapenem-hydrolyzing β-lactamases are resistance determinants of increasing clinical importance in Gram-negative pathogens.[1] Of these, enzymes of the Ambler class A KPC type, class B type, including IMP-, VIM- and NDM-like metallo-β-lactamases, or the class D OXA-48 type have been mainly encountered in *Enterobacteriaceæ*.[2] However, sporadic studies have reported the emergence of *Enterobacteriaceæ* isolates producing the class A GES-5 β-lactamase, which possess weak but significant carbapenemase activity.[3-5] Here, we report the first case of a GES-5-producing *Enterobacter cloacæ* isolate (Ecl-35771cz) identified in the Czech Republic. We also describe the complete nucleotide sequence of the bla_{GES-5}-carrying plasmid harbored by Ecl-35771cz.

Ecl-35771cz was recovered from the leg-wounds of a diabetic patient, who was treated in a Czech hospital, in September of 2016. Ecl-35771cz was resistant to carbapenems, as determined by broth dilution method, and interpreted according to EUCAST criteria.[6,7] Of all the drugs tested, the isolate was susceptible to aminoglycosides, trimethoprim-sulfametoxazole, tigecycline and colistin (Table 1).

Carbapenemase production was hypothesized due to a positive result in the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) imipenem hydrolysis assay.[8] Ecl-35771cz tested negative by the EDTA-meropenem test.[9] While the respective boronic acid-meropenem-combined disc test appeared positive, indicating production of class A carbapenemase-type.[10] PCR screening for various carbapenemase-encoding genes followed by sequencing revealed the presence of the *bla*$_{GES-5}$ gene in Ecl-35771cz.[11]

The multilocus sequence typing (MLST) analysis classified the isolate into ST252.[12] *E. cloacæ* ST252 was originally identified among KPC-producing isolates from a healthcare institution in Pensylvania, USA.[13]

Attempts to transfer β-lactam resistance from Ecl-35771cz to rifampin-resistant *Escherichia coli* strain A15 by conjugation were unsuccessful. Plasmid DNA from Ecl-35771cz was extracted using a Qiagen Maxikit (Qiagen, Hilden, Germany) and used to transform *E. coli* DH5α cells. Transformants were selected on Luria-Bertani agar plates with ampicillin(50μg/ml), confirmed to be GES producers by PCR, and MALDI-TOF MS imipenem hydrolysis assay.[8,14] *bla*$_{GES-5}$-positive transformant exhibited resistance or decreased susceptibility to cefotaxime and cefuroxime, while it remained susceptible to the remaining antibiotics tested (Table 1). Plasmid analysis of Ecl-35771cz and its transformant, indicated transfer of a ~6-kb plasmid that hybridized strongly with a *bla*$_{GES}$-like probe.[15] This plasmid, designated pEcl-35771cz, was nontypeable by PCR-based replicon typing (PBRT).[16] Plasmid pEcl-35771cz was extracted from *E. coli* transformant, and was sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego,CA, USA). Sequencing, assembling of the reads, filling of sequence gaps, and analysis and annotation of the plasmid sequence were performed as described previously.[17]

Table 1. Antimicrobial susceptibility of *E. cloacae* and the *E. coli* DH5α harboring the GES-5-encoding plasmid pEcl-35771cz.

Isolate	MIC (μg/ml)[a]																		
	PIP	TZP	CTX	CZL	CRX	CAZ	FEP	ATM	MEM	ETP	GEN	AMK	TOB	CHL	TET	SXT	CST	TGC	CIP
***E .cloacæ* Ecl-35771cz**	>128	16	>8	>16	>64	>16	16	>16	>16	8	0.5	2	2	32	2	2	2	0.5	>8
***E. coli* DH5α (pEcl-35771cz)**	4	≤1	>8	8	8	1	≤0.12	≤0.12	≤0.12	≤0.12	≤0.25	1	0.12	≤1	≤1	≤0.12	≤0.25	0.12	≤0.12
***E. coli* DH5a (recipient)**	≤0.5	≤1	≤0.06	1	1	≤0.25	≤0.12	≤0.12	≤0.12	≤0.12	≤0.25	0.5	0.12	≤1	≤1	≤0.12	≤0.25	0.12	≤0.12

[a] PIP, piperacillin; TZP, piperacillin-tazobactam (inhibitor fixed at 4 μg/ml); CZL, cefazolin; CRX, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; MEM, meropenem; ETP, ertapenem; GEN, gentamicin; AMK, amikacin; TOB, tobramycin; CHL, chloramphenicol; TET, tetracycline; SXT, trimethoprim-sulfametoxazole; CST, colistin; TGC, tigecycline;CIP, ciprofloxacin.

Plasmid pEcl-35771cz is 6,953 bp in size. Analysis of the sequence revealed that *bla*$_{GES-5}$ is the first gene cassette of a novel class 1 integron structure, In1406 (Figure 1), with a hybrid 1 Pc promoter.[18] In1406 also included a novel allele of the *aadA15*, differing by G-to -T transversion (nt 3389 in GenBank accession no. MF370188) resulting in P176Q substitution compared with *aadA15* carried by In569 (GenBank accession no. HQ148722).The *aadA15* cassette (*aadA15Δ1*) was disrupted at the *attC* recombination site, and was followed by an unusual 3'conserved sequence (3'-CS) lacking the *qacEΔ1*and *sul1* gene cassettes. However, adjacent to *aadA15Δ1*, an 84-bp sequence exhibited similarity to the right end of In2 in Tn*21*.[19] The 5'-CS of In1406 was intact.

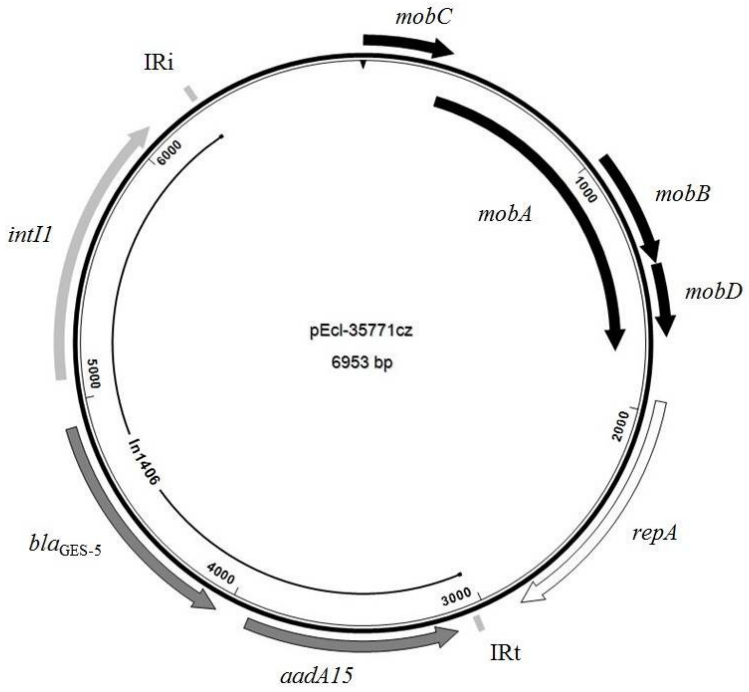

Figure 1. Circular genetic map of pEcl-35771cz. Arrows show directions of transcription of open reading frames. Sequences characteristic of the ColE2 like plasmid backbone are indicated by black arrows. White arrow indicates the repA gene. The *intI1* gene is shaded light gray, while dark gray arrows indicate the resistance genes. Position 1 is indicated by a vertical black arrow on strand ruler.

In1406 was inserted into a 3687-bp sequence (Figure 1; nt 1 to 3030 and 6296 to 6953) sharing common features with the backbone of the recently described plasmids pKP3-A, pOXA-232 and pOXA-232_30929, assigned to ColE2-like complex.[17,20,21] Interestingly, the latter plasmids have been associated with the dissemination of OXA-48-like β-lactamases, OXA-181 and OXA-232. A DNA comparison showed that *repA* gene (nt 1954 to 2871) of pEcl-35771cz exhibited the highest nucleotide similarity to replication regions of ColE2-like plasmids (78% coverage, 95% identity).[17,20,21] Additionally, pEcl-35771cz included four open reading frames (ORFs) encoding Mob proteins (MobC, MobA, MobB and MobD) forming a plasmid mobilization system.[20]

In conclusion, to our knowledge, this study presents the first GES-5-producing *E. cloacæ* strain in the Czech Republic. Notably, *E. coli* DH5a harboring pEcl-35771cz was susceptible to carbapenems. It might be due to weak carbapenemase activity of GES-5 enzyme, and lower expression driven by a hybrid 1 Pc promoter.[11,18]

The clinical data of the patient indicated that the GES-5 producer could be described as hospital acquired since the patient had no history of travelling abroad, while he had been hospitalized several times for the treatment of his diabetic wounds. In order to avoid further spread of CPE, the patient was isolated. Additionally, surveillance cultures from patients hospitalized, since September of 2016, were conducted, and no GES-producing isolates were identified indicating the success of isolations precautions. Sequencing data showed that bla_{GES-5} occurred in a novel class 1 integron, In1406, which was carried by a ColE2-like plasmid. These findings, in addition to previous reports documenting the role of

ColE2-like plasmids in the spread of OXA-48-like β-lactamases, confirm the significant role of this plasmid family in resistance dissemination.[17,20,21]

Nucleotide sequence accession numbers

The nucleotide sequence of the plasmid pEcl-35771cz has been assigned GenBank accession number MF370188.

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4.3 Publication no. 3: Characterization of NDM-encoding plasmids from *Enterobacteriaceæ* recovered from Czech hospitals

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Abstract

The aim of the present study was to characterize sporadic cases and an outbreak of NDM-like-producing *Enterobacteriaceæ* recovered from hospital settings, in Czechia. During 2016, 18 *Entrobacteriaceae* isolates including 9 *Enterobacter cloacæ* complex, 4 *Escherichia coli*, 1 *Enterobacter asburiæ*, 1 *Enterobacter intermedius*, 1 *Klebsiella pneumoniæ*, 1 *Klebsiella oxytoca*, and 1 *Raoultella ornithinolytica* that produced NDM-like carbapenemases were isolated from 15 patients. Three of the patients were colonized or infected by two different NDM-like producers. Moreover, an NDM-4-producing *Enterobacter*, isolated in 2012, was studied for comparative purposes. All *Enterobacter* isolates, recovered from the same hospital, were assigned to ST182. Additionally, two *E. coli* belonged to ST167, while the remaining isolates were not clonally related. Thirteen isolates carried $bla_{\text{NDM-4}}$, while six isolates carried $bla_{\text{NDM-1}}$ (n = 3) or $bla_{\text{NDM-5}}$ (n = 3). Almost all isolates carried bla_{NDM}-like-carrying plasmids being positive for the IncX3 allele, except ST58 *E. coli* and ST14 *K. pneumoniæ* isolates producing NDM-1. Analysis of plasmid sequences revealed that all IncX3 bla_{NDM}-like-carrying plasmids exhibited a high similarity to each other and to previously described plasmids, like pNDM-QD28, reported from worldwide. However, NDM-4-encoding plasmids differed from other IncX3 plasmids by the insertion of a Tn*3*-like transposon. On the other hand, the ST58 *E. coli* and ST14 *K. pneumoniæ* isolates carried two novel NDM-1-encoding plasmids, pKpn-35963cz and pEsco-36073cz. Plasmid pKpn-35963cz that was an IncFIB(K) molecule contained an acquired sequence, encoding NDM-1 metallo-β-lactamase (MBL), which exhibited high similarity to the mosaic region of pS-3002cz from an ST11 *K. pneumoniæ* from Czechia. Finally, pEsco-36073cz was a multireplicon A/C_2+R NDM-1-encoding plasmid. Similar to other type 1 A/C_2 plasmids, the $bla_{\text{NDM-1}}$ gene was located within the ARI-A resistance island. These findings underlined that IncX3 plasmids have played a major role in the dissemination of bla_{NDM}-like genes in Czech hospitals. In combination with further evolvement of NDM-like-encoding MDR plasmids through reshuffling, NDM-like producers pose an important public threat.

Introduction

Acquired carbapenem-hydrolyzing β-lactamases are resistance determinants of increasing clinical importance in Gram-negative pathogens. Of these, NDM-1 metallo-β-lactamase (MBL) was first described in *Klebsiella pneumoniæ* and *Escherichia coli* isolated in Sweden in 2008 from an Indian patient transferred from a New Delhi hospital.[1] Since then, NDM-1-producing bacteria, including clinical isolates of *Enterobacteriaceæ* and *Acinetobacter baumannii*, have been reported from the Indian subcontinent but also worldwide.[2]

In Czechia, the occurrence of NDM-producing bacteria was rare, with only three sporadic cases being detected during 2011-2013. These cases included an NDM-1-producing *A. baumanni* isolated from a patient repatriated from Egypt, an NDM-4-producing strain of an *Enterobacter* species from a patient previously hospitalized in Sri Lanka and a ST11 *K. pneumoniæ* isolate carrying two NDM-1-encoding plasmids, from Slovakia.[3-5] However, an increase in the isolation frequency of NDM-like-producing *Enterobacteriaceæ* from Czech hospitals was observed, during 2016.

Thus, the aim of the present study was to characterize the NDM-like producers detected in Czech hospitals, during 2016. Also, we describe the complete nucleotide sequences of representative bla_{NDM}-like-carrying plasmids harbored by the studied isolates.

Materials and methods

Bacterial isolates and confirmation of carbapenemase production

In 2016, Czech hospitals referred a total of 410 *Enterobacteriaceæ* isolates with a meropenem MIC of >0.125 µg/ml to the National Reference Laboratory for Antibiotics.[6] Species identification was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). All isolates were tested for carbapenemase production by the MALDI-TOF MS meropenem hydrolysis assay.[7] Isolates that were positive by the MALDI-TOF MS meropenem hydrolysis assay were subjected to metallo-β-lactamase, KPC, and OXA-48 detection using the double-disc synergy test with EDTA, the phenylboronic acid disc test, and the temocillin disc test, respectively.[8-10] Additionally, carbapenemase genes (bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48}-like) were detected by PCR amplification.[1,11-13] PCR products were sequenced as described below. Isolates positive for bla_{NDM}-like genes were further studied. Moreover, the NDM-4-producing *Enterobacter* isolate, recovered at the University Hospital Pilsen (Pilsen, Czechia) during 2012, was included in this study for comparative purposes.[4]

Susceptibility testing

The MICs of piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, meropenem, ertapenem, gentamicin, amikacin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, colistin, and tigecycline were determined by the broth dilution method.[14] Data were interpreted according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org).

Typing

All isolates were typed by multilocus sequence typing (MLST).[15–18] The databases at:

- https://pubmlst.org/ecloacæ/,
- http://mlst.warwick.ac.uk/mlst/dbs/Ecoli,
- http://bigsdb.web.pasteur.fr/klebsiella and https://pubmlst.org/koxytoca/

were used to assign STs.

Detection of β-lactamases

The β-lactamase content of all *bla*$_{NDM}$-like-positive isolates was determined by isoelectric focusing (IEF). Bacterial extracts were obtained by sonication of bacterial cells suspended in 1% glycine buffer and clarified by centrifugation. Sonicated cell extracts were analyzed by IEF in polyacrylamide gels containing ampholytes (pH 3.5 to 9.5; AP Biotech, Piscataway, NJ). The separated β-lactamases were visualized by covering the gel with the chromogenic cephalosporin nitrocefin (0.2 mg/ml; Oxoid Ltd., Basingstoke, United Kingdom).[19]

On the basis of the IEF data, PCR detection of various *bla* genes was performed by the use of primers specific for *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{CMY}$, as reported previously.[20–23] Both strands of the PCR products were sequenced using an ABI 377 sequencer (Applied Biosystems, Foster City, CA).

Transfer of *bla*$_{NDM}$-like genes

Conjugal transfer of *bla*$_{NDM}$-like genes from the clinical strains was carried out in mixed broth cultures, using the rifampin-resistant *E. coli* A15 laboratory strain as a recipient.[24] Transconjugants were selected on MacConkey agar plates supplemented with rifampin (150 mg/l) and ampicillin (50 mg/l). Plasmid DNA from clinical isolates, which failed to transfer *bla*$_{NDM}$-like by conjugation, was extracted using a Qiagen Maxi kit (Qiagen, Hilden, Germany) and used to transform *E. coli* DH5α cells. The preparation and transformation of competent *E. coli* cells were done using calcium chloride.[25] Transformants were selected on Luria-Bertani agar plates with ampicillin (50 mg/l). Transconjugants or transformants were confirmed to be NDM-like producers by PCR and the MALDI-TOF MS meropenem hydrolysis assay.[1,7]

Plasmid analysis

To define the genetic units of the *bla*$_{NDM}$-like genes, the plasmid contents of all NDM-producing clinical and recombinant strains were analyzed by pulsed-field gel electrophoresis (PFGE) of total DNA digested with S1 nuclease (Promega, Madison, WI, USA).[26] Following PFGE, the DNA was transferred to a BrightStar-Plus positively charged nylon membrane (Applied Biosystems, Foster City, CA) and hybridized with digoxigenin-labeled *bla*$_{NDM}$-like probe.

Plasmid incompatibility (Inc) groups were determined by the PCR-based replicon typing (PBRT) method, using total DNA from transconjugants or transformants.[27,28] Additionally, the IncR replicon was detected as described previously.[29]

Detection of characteristic regions

Based on the results from Illumina sequencing (see below), six PCRs targeting characteristic regions of NDM-4-ncoding IncX3 plasmids and ST182 *Enterobacter* genomes sequenced during this study were designed. The selected regions included: (i) a Tn3-like transposon found in NDM-4-encoding IncX3 plasmids, and (ii) four insertions identified in the genome of Encl-922 (see section: Comparative analysis of *Enterobacter* isolates). All NDM-producing clinical or recombinant strains were screened for the presence of the regions described above by the use of specific primers (Table S1).

Plasmid and chromosome sequencing

Ten plasmids were selected for complete sequencing. These plasmids were selected as representatives of different origins, plasmid sizes and hospitals. Additionally, *E. cloacæ* isolates Encl-922 and Encl-44578 were also selected for whole genome sequencing. These two isolates were selected as representatives of different isolation periods.

Plasmid DNAs from transconjugants or transformants were extracted using a Qiagen Large-Construct kit (Qiagen, Hilden, Germany). Additionally, the genomic DNAs of *Enterobacter* isolates were extracted using a DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy). Multiplexed DNA libraries were prepared, using the Nextera XT Library Preparation kit, and 300-bp paired-end sequencing was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) using the MiSeq v3 600-cycle Reagent kit. Initial paired-end reads were quality trimmed using the Trimmomatic tool v0.33 with the sliding window size of 4 bp, required average base quality ≥17 and minimum read length of 48 bases Genomic DNA reads of *E. cloacæ* were consequently assembled using the de Bruijn graph-based *de novo* assembler SPAdes v3.9.1, using k-mer sizes 21, 33, 55, 77, 99 and 127.[30,31] For assembly of the plasmids, reads were mapped to the reference *E. coli* K-12 substrain MG 1655 genome (GenBank accession no. U00096) using the BWA-MEM algorithm, in order to filter out the chromosomal DNA.[32] Then, all the unmapped reads were assembled in the same way as described above. The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (www-is.biotoul.fr/), and the open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using the Mauve v2.3.1 program.[33]

Antibiotic resistance genes were identified using the ResFinder 2.1 tool (https://cge.cbs.dtu.dk/services/ResFinder/) with an identity threshold of >90%.[34]

Comparative analysis of *E. cloacæ* clinical isolates

Comparative genomic analysis of *Enterobacter* clinical strains was based on statistics calculated by QUAST v4.5 and VarScan v2.3.9 tools.[35,36] All quality trimmed Illumina reads of Encl-922 were mapped to contigs of Encl-44578, employing BWA-MEM algorithm v0.7.12 and SAMtools v1.3, for the format conversions and analysis of the results.[32,37] Then, single nucleotide polymorphisms (SNPs) and indels were detected employing VarScan with parameters set as follows: minimum read depth at a position =6, minimum base quality at a position =20 and minimum variant allele frequency threshold of 0.45.

Moreover, SNPs and indels located in a region within 127 bp from any edge of a contig, as well as SNPs and indels harbored by contigs smaller than 2 kb were excluded from further analysis. Remaining SNPs and indels were also manually checked and refined by visualization of mapped data via Tablet v1.14.04.10.[38] Differences in assembly of *E. cloacæ* genomes were inspected using QUAST's Icarus viewer.[39] In order to examine whether SNPs and indels were located in intergenic or coding regions, as well as to find out what are the differences in genetic information between studied isolates, contigs of clinical strains were annotated using Prokka v1.10.[40] Genes harboring SNPs were compared against NCBI's conserved domain database via CD-Search to identify conserved domain hits.[41,42] Finally, sequencing data of clinical strains were examined for the presence of prophage sequences using PHAST web server.[43]

Nucleotide sequence accession numbers

The nucleotide sequences of the pEsco-5256cz, pEncl-922cz, pRor-30818cz, pKpn-35963cz, pEsco-36073cz, pEncl-44578cz, pEnas-80654cz, pEnin-51781cz, pEsco-4382cz and pKlox-45574cz plasmids have been deposited in GenBank under accession numbers MG252891, MG252892, MG252893, MG252894, MG252895, MG833402, MG833403, MG833404, MG833405 and MG833406, respectively. Whole genome assemblies of *Enterobacter* isolates were deposited in NCBI under accession number PRJNA432167.

Results

Carbapenemase-producing *Enterobacteriaceæ*

A total of 40 *Enterobacteriaceæ* isolates showing carbapenemase activity on MALDI-TOF MS meropenem hydrolysis assay were recovered from Czech hospitals during 2016. PCR screening showed that 18 of the isolates were positive for bla_{NDM}, 14 isolates were positive for $bla_{\text{OXA-48}}$, while the remaining 8 isolates were positive for bla_{KPC}.

NDM-like-producing isolates

Altogether, 18 nonrepetitive isolates producing NDM-like carbapenemases were isolated from 15 patients in 2016. Among them, 9 were identified to be *E. cloacæ* complex, 4 were identified to be *E. coli*, while the remaining isolates belonged to unique species (*Enterobacter asburiæ*, *Enterobacter intermedius*, *K. pneumoniæ*, *Klebsiella oxytoca*, and *Raoultella ornithinolytica*). Three of the patients were colonized or infected by two different NDM-like producers (Table 1).

NDM-like producers were collected from five Czech hospitals located in three different Czech cities. In hospital B1, an outbreak that included ten patients diagnosed with NDM-like-producing *Enterobacteriaceæ* lasted the studied period. Additionally, two patients colonized or infected with NDM-like producers were reported in hospital B2. The three remaining cases were identified in three different hospitals. None of the patients, treated in hospital B1, had recently traveled abroad or had been previously hospitalized. The patient treated in hospitals was directly repatriated from a hospital in China, while clinical data weren't available for the remaining patients.

Additionally, the NDM-4-producing *Enterobacter* isolate identified in 2012, was studied.[4]

Table 1. Characteristics of NDM-like-producing *Enterobacteriaceae*

Isolate[a]	Isolation mn/yr (hospital)	Material (infection/ colonization)	ST	β-Lactamase content	Size of NDM-encoding plasmid (kb)[b]	Replicon of NDM-encoding plasmid	Additional resistance markers
E. xiangfangensis							
Encl-922	09/2012 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55 (53.683)**	IncX3	
Encl-66918	04/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-89040	06/2016 (B1)	Bile (infection)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-44578	07/2016 (B1)	Venous catheter (infection)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55 (53.683)**	IncX3	
Encl-89485○	07/2016 (B1)	Bile (infection)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-91221	09/2016 (B1)	Throat swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-93141	10/2016 (B1)	Peritoneal catheter (infection)	ST182	NDM-4, CTX-M-15, OXA-1	**~55**	IncX3	
Encl-98042	11/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1	**~55**	IncX3	
Encl-98047■	11/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
Encl-98546	12/2016 (B1)	Rectal swab (colonization)	ST182	NDM-4, CTX-M-15, OXA-1, TEM-1	**~55**	IncX3	
E. asburiæ							
Enas-80654○	07/2016 (B1)	Bile (infection)	NA	NDM-4, CTX-M-15	**~55 (53.683)**	IncX3	
E. intermedius							
Enin-51781	10/2016 (B1)	Rectal swab (colonization)	NA	NDM-4, CTX-M-15, OXA-1	**~55 (53.683)**	IncX3	
E. coli							
Esco-14290	06/2016 (B2)	Nasal swab (colonization)	ST167	NDM-5, CTX-M-15, TEM-1	~45	IncX3	
Esco-5256▲	07/2016 (B2)	Bronchoalveolar lavage (infection)	ST167	NDM-5, CTX-M-15, TEM-1	~45 (46.161)	IncX3	
Esco-36073	09/2016 (A1)	Urine (infection)	ST58	NDM-1, CMY-16, OXA-10, CTX-M-15, TEM-1	**~300 (300.958)**	IncR, IncA/C_2	*floR*, *tet*(A), *strAB*, *sul2*, *aacA4*, *aphA7*, *dfrA14*, *arr-2*, *cmlA1*, *aadA1*, *aphA6*, *sul1*
Esco-4382■	12/2016 (B1)	Rectal swab (colonization)	ST69	NDM-4, CTX-M-15, TEM-1	**~55 (53.683)**	IncX3	
K. oxytoca							
Klox-45574▲	07/2016 (B2)	Rectal swab (colonization)	ST2	NDM-5	~45 (46.161)	IncX3	
K. pneumoniæ							
Kpn-35963	09/2016 (A2)	Urine catheter (infection)	ST14	NDM-1, SHV-12, CTX-M-15, OXA-1	~150 (161.324)	IncFIB	*aacA4*, *dfrA14*, *mph*(A)
Raoultella ornithinolytica							
Ror-30818	09/2016 (C)	Rectal swab (colonization)	NA	NDM-1, SHV-12, CTX-M-15, OXA-1, TEM-1	~55 (53.051)	IncX3	

[NA] Not applicable.
[a] White circles, black squares, and black triangles each indicate the NDM-like-producing isolates recovered from the same patient.
[b] Data for plasmids found in transconjugants are shown in bold; data for plasmids observed in transformants are underlined.

All 19 NDM-like producers exhibited resistance to piperacillin, piperacillin-tazobactam, cephalosporins and ertapenem (Table S2), while the observed variations in the MICs of aztreonam might reflect the presence of additional resistance mechanisms in some of the isolates. Seventeen of the NDM-like producers also exhibited resistance to ciprofloxacin; 15 were resistant to gentamicin, 13 were resistant to trimethoprim-sulfamethoxazole, 1 was resistant to amikacin and 1 was resistant to colistin, whereas all isolates were susceptible to tigecycline.

The population structure of NDM-like-producing isolates studied by MLST is shown in Table 1. All *Enterobacter* isolates, which were recovered from hospital B1, belonged to ST182. Of note was that the NDM-4-producing *Enterobacter* that was isolated, in 2012, from the patient previously hospitalized in Sri Lanka was also assigned to ST182.[4] ST182 *Enterobacter* isolates were previously identified in Mexico and were associated with the production of NDM-1 enzyme.[44,45] Two of *E. coli*, both of which were from hospital B2, belonged to ST167. *E. coli* ST167 was recently found among NDM-5-producing isolates from

different healthcare institutions in China.[46,47] The two remaining *E. coli* isolates were not clonally related and belonged to different STs (ST58 and ST69). The *K. pneumoniæ* isolate was assigned to the high risk clone ST14, while the *K. oxytoca* isolate was classified into ST2 that belongs to a growing international clonal complex (CC2).[48,49]

Sequencing of the PCR products revealed three bla_{NDM}-type genes encoding the NDM-1, NDM-4 and NDM-5 enzymes (Table 1).[1,50,51] NDM-5 is an NDM-1-related MBL variant that differs from NDM-1 by two amino-acid substitutions, Val88Leu and Met154Leu, the former one being its only change with NDM-4. Thirteen of the isolates, all of which were from hospital B1, were found to produce the NDM-4 MBL (Table 1). The three isolates from hospital B2 produced the NDM-5 enzyme, while the three remaining isolates that were recovered from sporadic cases in three different hospitals expressed NDM-1 carbapenemase. Additionally, most of bla_{NDM}-like-positive isolates were confirmed to coproduce the extended-spectrum β-lactamase CTX-M-15 (n = 18) either alone or along with TEM-1 (n = 13) and/or OXA-1 (n = 13), whereas the *K. pneumoniæ* and *R. ornithinolytica* isolates also expressed the SHV-12 enzyme. The ST58 NDM-1-producing *E. coli* isolate coproduced CMY-16, CTX-M-15, OXA-10 and TEM-1 β-lactamases.

bla_{NDM}-like-carrying plasmids

The bla_{NDM}-like genes from all clinical strains were transferred by conjugation (n = 14) or transformation (n = 5) (Table 5). All bla_{NDM}-like-positive recombinants exhibited resistance to piperacillin, piperacillin-tazobactam, cephalosporins and ertapenem, while they remained susceptible to meropenem (Table S2). The three NDM-1-producing recombinants also exhibited resistance to aztreonam. Additionally, most of bla_{NDM}-like-positive recombinants (n = 18) were susceptible to non-β-lactam antibiotics.

Plasmid analysis of NDM-4-producing donor and transconjugant strains revealed the transfer of plasmids, all of which were ~55 kb in size (Table 1). The three NDM-5-producing transformants harbored plasmids of ~45 kb, whereas the three remaining recombinants carried bla_{NDM-1}-positive plasmids of different sizes (~55 kb, ~150 kb and ~300 kb). Replicon typing showed seventeen of the plasmids, including those sizing ~45 kb, and ~55 kb, were positive for the IncX3 allele. The bla_{NDM-1}-positive plasmid of ~300 kb was positive for replicons R and A/C, whereas the one remaining bla_{NDM-1}-carrying plasmid was nontypeable by the PBRT method.[27,28]

Structure of bla_{NDM}-like-carrying plasmids

The complete sequence of bla_{NDM}-like-carrying plasmids representative of different plasmid sizes, replicons, and resistance genes (n = 10) was determined (Table 1). Sequence analysis revealed that all IncX3 bla_{NDM}-like-carrying plasmids exhibited a high similarity to each other and to previously described NDM-like-encoding plasmids, belonging to IncX3 group, reported from worldwide.[52-54] The bla_{NDM-5}-positive plasmids, pEsco-5256cz and pKlox-45574cz, were almost identical to NDM-5-encoding plasmid pNDM-QD28 (100% coverage, 99% identity) (GenBank accession no. KU167608) that was characterized from a ST167 *E. coli* in China.[53] Differences among these plasmids consisted in few SNPs

(n = 5), almost all located in mobile elements. Similar to pNDM-QD28, no other resistance genes were detected in these plasmids. Compared to other IncX3 NDM-encoding plasmids, all bla_{NDM-4}-encoding plasmids differed by the insertion of a Tn3-like transposon (nt 7108-14624 in pEncl-44578cz) downstream *topB* gene (Figure 1). The Tn3-like sequence was composed by the 38-bp inverted repeats (IR) of the transposon, *tnpA*, *tnpR* and two ORFs encoding hypothetical proteins. Target site duplications of 5 bp (GTACC) at the boundaries of the Tn3-like element indicated insertion by transposition. Of note was that the sequence of pEncl-922cz, isolated in 2012, was identical to the respective sequences of NDM-4-encoding plasmids recovered in the same hospital, during 2016.[4] PCR screening confirmed the presence of the Tn3-like transposon in all NDM-4-encoding IncX3 plasmids, isolated in hospital B1, while Tn3-like wasn't detected in the remaining bla_{NDM}-like-positive plasmids that belonged to IncX3 group. Furthermore, the bla_{NDM-1}-positive plasmid, pRor-30818cz, harbored an additional 7875-bp sequence (nt 40617-48491 in pRor-30818cz) encoding the extended-spectrum β-lactamase SHV-12 (Figure 1). A similar SHV-12-encoding region was found in the IncX3 bla_{NDM-1}-positive plasmid pKP04NDM (100% coverage, 99% identity) (GenBank accession no. KU314941) described from a *K. pneumoniæ* isolate in China.

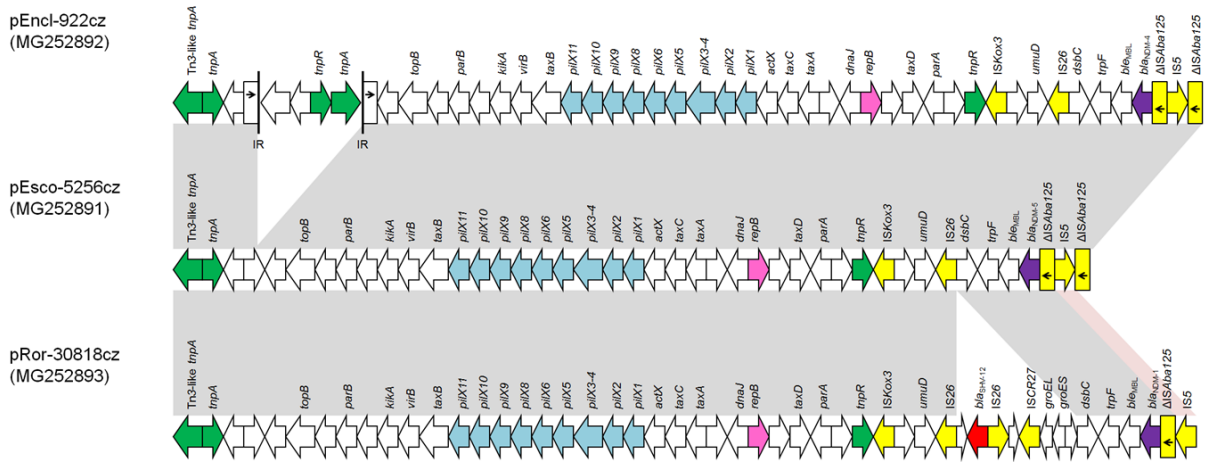

Figure 1. Comparison of linear maps of the NDM-like-encoding IncX3 plasmids pEncl-922cz, pEsco-5256cz, and pRor-30818cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Replicons of the plasmids are shown in pink. bla_{NDM}-like genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. Light blue arrows indicate genes responsible for the conjugative transfer of the plasmids. The remaining genes, including plasmid scaffold regions, are shown in white. Homologous segments (representing ≥99% sequence identity) are indicated by light gray shading, while pink shading shows inverted homologous segments.

The NDM1-encoding plasmid pKpn-35963cz that was nontypeable by the PBRT method was 161324 bp in size.[27] Plasmid pKpn-35963cz was composed of two distinct parts: a contiguous plasmid backbone of 115998 bp (nt 1-58655 and 103982-161324) and an acquired sequence of 45326 bp (nt 58656-103981). The plasmid backbone, which shared similarities with the respective regions of plasmid p1605752FIB (GenBank accession no. CP022125) recovered from a pan-resistant isolate of *K. pneumoniæ* from the United States, harbored regions responsible for replication [*repB* gene; IncFIB(K) replicon], conjugative transfer (*tra* and *trb* genes) and plasmid maintenance (*vagCD*, *psiAB*, *umuCD* and *parAB* operons, and

ssb gene) (Figure 2). The acquired sequence of pKpn-35963cz contained a 17836-bp segment (nt 77360-95195) encoding NDM-1, which was similar to the mosaic region of pS-3002cz (99% identity). pS-3002cs was characterized from an ST11 *K. pneumoniæ* isolate identified in Czechia.[5] The acquired sequence of pKpn-35963cz contained two additional segments that have also been described in pS-3002cz. The first segment (nt 65518-72935) included genes encoding an EcoRII methylase and EcoRII endonuclease, and the class 1 integron In191 carrying the *dfrA14* resistance gene. The second segment (nt 101342-103981) contained fragments of transposons Tn*1000* (ΔTn*1000*) and Tn*1331* (ΔTn*1331*). ΔTn*1331* comprised *tnpR* and *aacA4* resistance gene. Furthermore the acquired sequence of pKpn-35963cz carried a macrolide resistance operon [*mph*(A)], and regions encoding OXA-1 and CTX-M-15 β-lactamases (Figure 2). In the acquired sequence of pKpn-35963cz, intact and truncated copies of several mobile elements that may have been implicated in the formation of this region were found.

The plasmid pEsco-36073cz, which encoded the NDM-1 carbapenemase, is 300,958 bp in size. The plasmid showed a complex structure, being composed of sequences of diverse origin (Figure 3). A 170314-bp sequence (nt 232204-300958 and 1-101559) resembled the type 1 A/C_2 plasmid pRH-1238 (94% coverage, 99% identity) (Figure 3), characterized from a *Salmonella enterica* serovar Corvallis strain isolated from a migratory wild bird in Germany.[55] Analysis of A/C_2-associated sequence by the core gene PMLST (cgPMLST) scheme indicated that it belonged to cgST3.4.[56] The A/C_2 backbone was composed of regions responsible for replication (*repA* gene), conjugative transfer (Tra1 and Tra2 regions), and plasmid maintenance (*higBA* and *parAB* operons and *xerD*- and *kfrA*-like genes). Apart from the backbone, pEsco-36073cz carried the bla_{CMY-2}-like-containing region, and the ARI-B and ARI-A resistance islands, as previously described in other type 1 A/C_2 MDR plasmids.[57,58] The bla_{NDM-1} gene was located within ARI-A, in a genetic environment similar to those previously identified in pRH-1238.[55] However, unlike in pRH-1238, the ARI-A of pEsco-36073cz lacked the macrolide resistance determinant *mphA-mel-repAciN*. Furthermore, a class 1 integron with *aacA4* and *aphA1* gene cassettes was located between *resI* and *resII* sites of the Tn*1696* module. The ARI-A of pEsco-36073cz also carried a new integron, In1459, whose variable region comprised the *dfrA14*, *arr-2*, *cmlA1*, bla_{OXA-10}, *aadA1* cassettes. Additionally, pEsco-36073cz included fragments resembling the backbone of the recently described IncR plasmid pKP1780, and sequences previously found in the plasmid pPSP-a3e and in the chromosomes of several Gram-negative rods.[59,60] Genes encoding for resistance to arsenate, cooper and mercury were identified in the three remaining acquired regions of pEsco-36073cz.

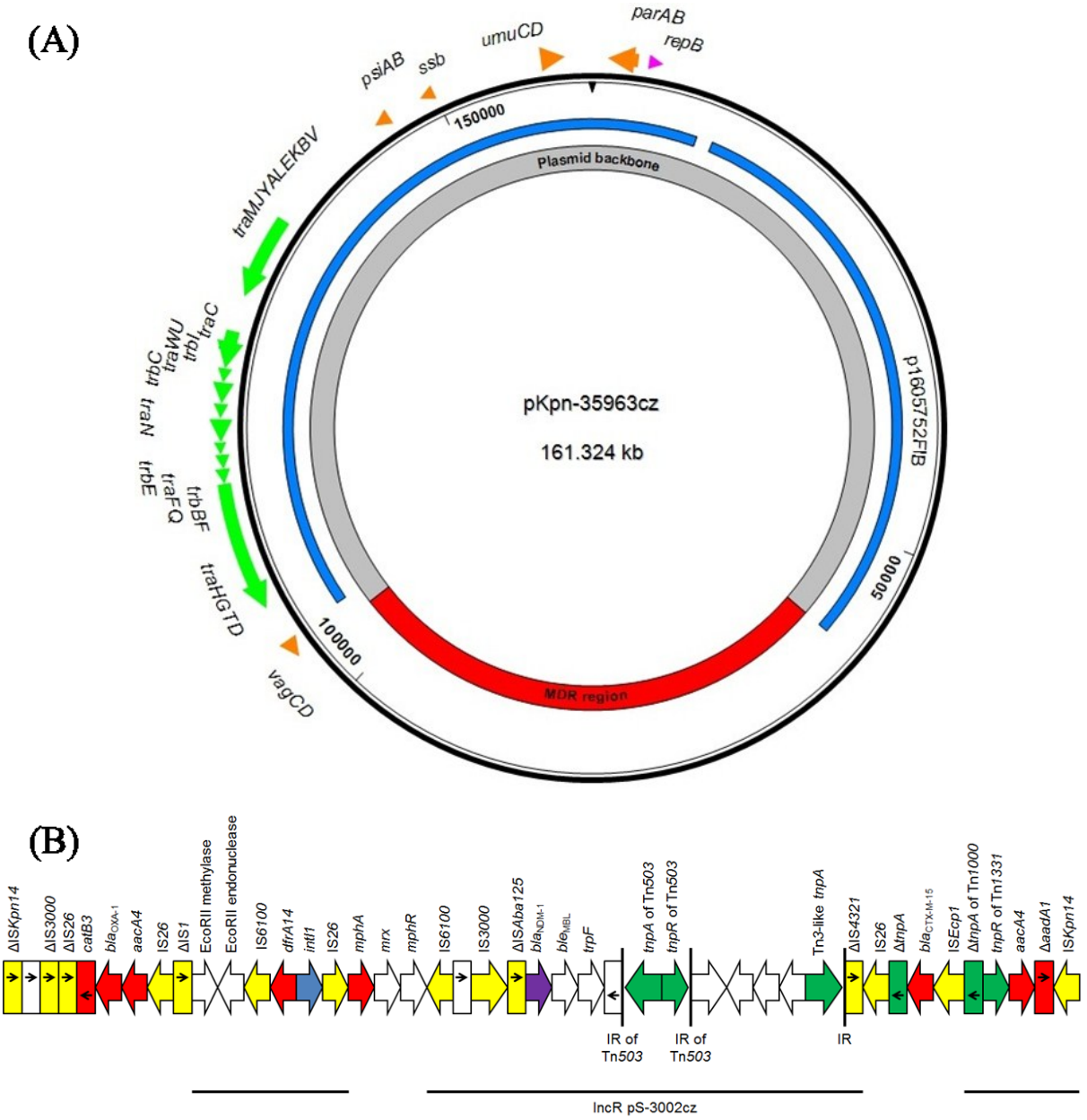

Figure 2. (A) Overview of the plasmid pKpn-35963cz. The innermost circles show the main regions of the plasmids. Similarities with other plasmids are shown in the next circle; each color represents a unique plasmid. In the outer circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in green and orange, respectively. (B) Linear map of the multidrug resistance region (MDR) of the plasmid pKpn-35963cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). *bla*$_{NDM}$-like genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. *intI1* genes are shaded blue. The remaining genes are shown in white. Thin lines below the map correspond to highly similar sequences from other plasmids.

Comparative analysis of *Enterobacter* isolates

'*In silico*' *hsp60* typing of the genome sequences showed that both isolates belonged to the recently recognized *E. xiangfangensis* species.[61,62]

Since all *Enterobacter* isolates belonged to the same ST and carried the same IncX3 *bla*$_{NDM-4}$-carrying plasmid, the WGS data of clinical strains Encl-922 and Encl-44578 were compared, using QUAST and

VarScan tools, in order to examine the phylogenetic relationship of the isolates recovered in 2012 and 2016.

Comparative analysis of *Enterobacter* clinical isolates revealed that the genome of Encl-922 exhibited extensive similarity (99.87% identity) to the genome of Encl-44578. Sixteen SNPs were identified in the genome of Encl-922, compared to that of Encl-44578, five of which were located within prophage regions (Table 2). Interestingly, Encl-922 harbored three large insertions of 8,933 bp (nt 439392-448324 in node 2), of 17,903 bp (nt 17786-35688 in node 32) and of 13,165 bp (nt 1-13165 in node 27; prophage sequence PHAGE_Salmon_SPN3UB_NC_019545). Additionally, Encl-922 harbored an insertion of 33-bp sequence (AACCCTCTCCCCAAAGGGGAGAGGGGACGATTA) located in an intergenic region. Moreover, Encl-922 showed a single nucleotide (G) deletion leading to CDS annotation change of general stress protein 39 to putative oxidoreductase YghA. Analysis of whole genome sequencing (WGS) data by PHAST web server found five intact prophage sequences (PHAGE_Haemop_HP2_NC_003315, PHAGE_Salmon_SPN3UB_NC_019545, PHAGE_Entero_mEp390_NC_019721, PHAGE_Pseudo_PPpW_3_NC_023006, and PHAGE_Salmon_SP_004_NC_021774) and one questionable prophage region (PHAGE_Entero_SfI_NC_027339), in both Enterobacter isolates. However, Encl-922 included one additional incomplete prophage region (PHAGE_Salmon_SPN3UB_NC_019545), which was absent from the Encl-44578 genome.

Screening by PCR and sequencing identified that all Enterobacter isolates, recovered during 2016, didn't harbor any of the four mentioned insertions. Thus, this finding indicated that Enterobacter isolates from 2016 differed from Encl-922.

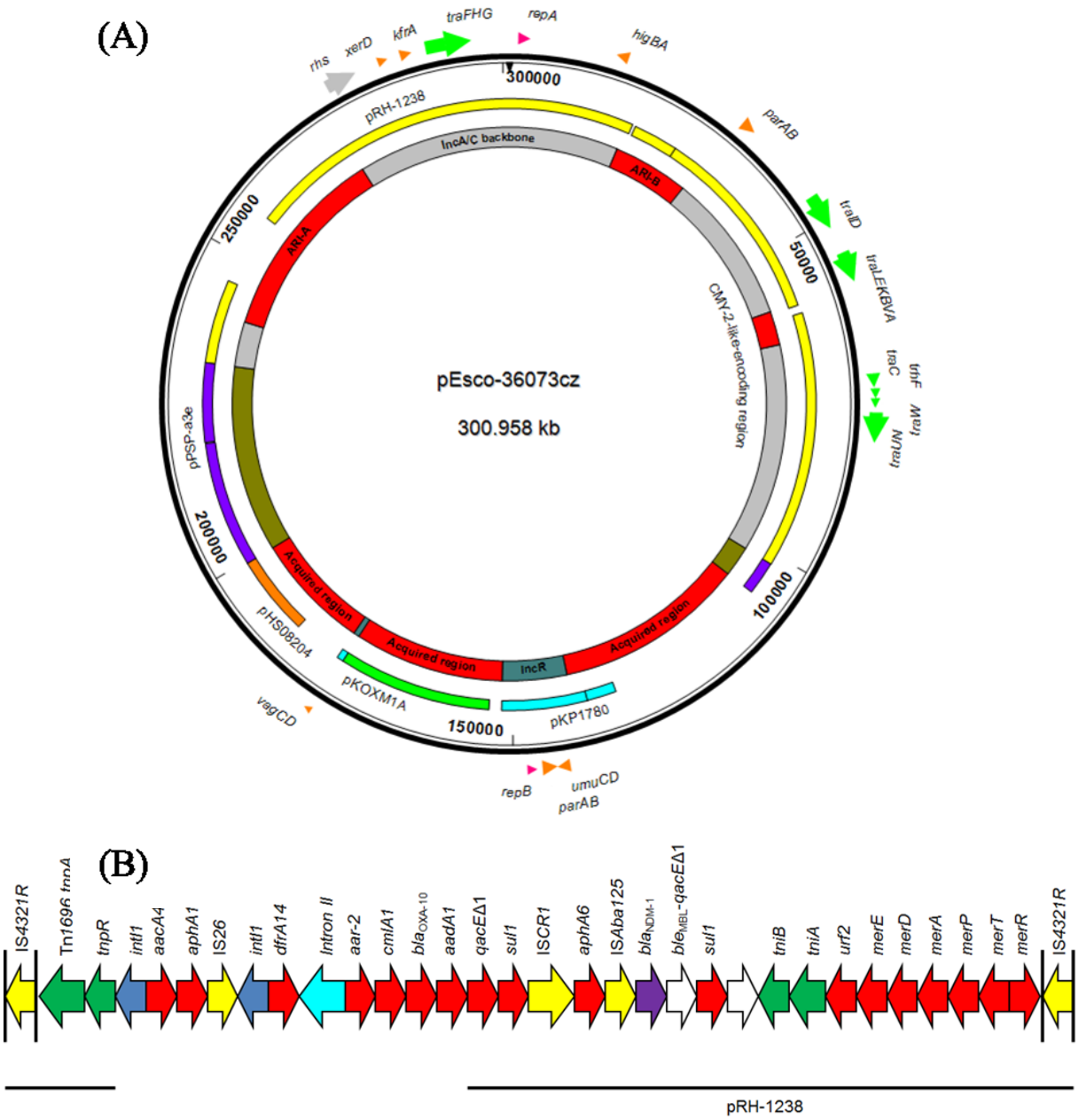

Figure 3. (A) Overview of the plasmid pEsco-36073cz. The innermost circles show the main regions of the plasmids. Similarities with other plasmids are shown in the next circle; each color represents a unique plasmid. In the outer circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in green and orange, respectively. (B) Linear map of the ARI-A resistance island of the plasmid pEsco-36073cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). *bla*$_{NDM}$-like genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. intI1 genes are shaded blue; teal blue arrow indicates the group II intron. The remaining genes are shown in white. Thin lines below the map correspond to highly similar sequences from other plasmids.

Table 2. Summary table of sixteen SNPs found between the genomes of *Enterobacter* isolates Encl-44578 (reference) and Encl-922 (query).

PROKKA name	Conserved domain classification	Enzyme Commision number	Contig	SNP	Gene length (aa)	aa substitution
_[a]	–	–	2	T64623G	-	-
_[a]	–	–	7	T88097G	-	-
Methyl viologen resistance protein SmvA	MFS transporter	–	8	T51220C	496	M293T
D-amino acid dehydrogenase small subunit	D-amino acid dehydrogenase	1.4.99.1	23	A46893G	432	S395S
NADP-dependent malic enzyme	NADP-dependent malic enzyme	1.1.1.40	2	A296564G	759	N584N
Glyoxylate/hydroxypyruvate reductase A	Glyoxylate/hydroxypyruvate reductase A	1.1.1.79	4	G113111A	312	R267H
Ribonuclease E	Ribonuclease E	3.1.26.12	4	T156395C	1035	H685R
Hypothetical protein	–	–	38	C784A	369	T239N
Hypothetical protein	Similar to protein YjaG	–	39	A24170G	196	I61V
Low-affinity gluconate transporter	Low-affinity gluconate transporter	–	6	T100479C	421	S277P
Arabinose operon regulatory protein	DNA-binding transcriptional regulator	–	12	A66284G	281	N193S
Anaerobic dimethyl sulfoxide reductase chain B	DMSO_dmsB family protein	–	35	T1976G	205	K120Q
Tail length tape measure protein	COG5281 and Phage_HK97_TLTM domain-containing protein	–	5	G24809A	1154	L824L
Tail length tape measure protein	COG5281 and Phage_HK97_TLTM domain-containing protein	–	5	T24845C	1154	A836A
Tail length tape measure protein	COG5281 and Phage_HK97_TLTM domain-containing protein	–	5	C24893A	1154	G852G
Terminase-like family protein	P family protein	–	26	G7615T	589	R485L

[a] The first two SNPs are located in intergenic regions.

Discussion

The present study investigated sporadic cases and an outbreak of NDM-like-producing *Enterobacteriaceæ* recovered from Czech hospitals, during 2016. Specifically, 12 NDM-4-producing isolates, which belonged to *E. xiangfangensis* (n = 9), *E. asburiæ* (n = 1), *E. intermedius* (n = 1) and *E. coli* species, 3 NDM-5 producers of *E. coli* (n = 2) and *K. oxytoca* (n = 1) species, and one *E. coli*, one *K. pneumoniæ* and one *R. ornithinolytica* producing NDM-1 MBL were characterized.

The setting that was most affected was hospital B1, in which an outbreak of NDM-4-producing ST182 *E. xiangfangensis* isolates took place. Of note was that the *Enterobacter*, isolated in 2012 from a patient who had been previously hospitalized in Sri Lanka, also belonged to ST182 and harbored an IncX3 $bla_{\text{NDM-4}}$-positive plasmid being identical to respective plasmids characterized from *Enterobacter* isolates recovered from patients treated in hospital B1 (Table 1), during 2016.[4] However, comparative genome analysis revealed the presence of four insertions in the genome of *Enterobacter* Encl-922 isolate. These insertions were not found in the genomic DNA of *Enterobacter* isolates from 2016, suggesting a second insertion event of NDM-4-producing *Enterobacter* isolates in Czech hospitals.

In three of the patients, two different NDM-like producers were identified during their hospitalization, supposing the *in vivo* horizontal transfer of bla_{NDM}-like-carrying plasmids. Sequencing and PCR screening data revealed the presence of the same $bla_{\text{NDM-4}}$- or $bla_{\text{NDM-5}}$-carrying plasmid in these isolates (Table 1). These results confirmed the hypothesis of the *in vivo* horizontal transfer of bla_{NDM}-like-carrying plasmids.

Results from Illumina sequencing showed that IncX3 plasmids have played a major role in the dissemination of *bla*$_{NDM}$-like genes in Czech hospitals, which is in agreement with the findings from previous studies from worldwide.[52-54] In the current study, three *bla*$_{NDM}$-type genes, encoding the NDM-1, NDM-4, and NDM-5 enzymes, were associated with IncX3 plasmids exhibiting high similarity to each other. Considering also the fact that NDM-1, NDM-4 and NDM-5 differ by one or two amino-acid substitutions may indicate the possibility that *bla*$_{NDM}$-like genes encoding NDM-1-related variants have evolved in the same plasmid type. Additionally, Illumina data showed the presence of a unique sequence, a Tn*3*-like transposon, in sequenced *bla*$_{NDM-4}$-carrying plasmids. PCR confirmed the presence of the Tn*3*-like sequence in all transconjugants, carrying *bla*$_{NDM-4}$-positive plasmids. Thus, the PCR targeting the Tn*3*-like sequence was able to distinguish *bla*$_{NDM-4}$-positive plasmids from other IncX3 plasmids carrying *bla*$_{NDM-1}$ or *bla*$_{NDM-5}$. On the other hand, two of the sporadic isolates carried novel NDM-1-encoding plasmids. Plasmid pKpn-35963cz that was an IncFIB(K) molecule contained an acquired sequence, encoding NDM-1 MBL, which exhibited high similarity to the mosaic region of pS-3002cz from an ST11 *K. pneumoniæ* from Czechia.[5] Whereas plasmid pEsco-36073cz was a multireplicon A/C$_2$+R NDM-1-encoding plasmid, being a fusion derivative of sequences of diverse origin. Similar to other type 1 A/C$_2$ plasmids (Villa et al., 2015; Harmer and Hall, 2015), the *bla*$_{NDM-1}$ gene was located within the ARI-A resistance island.[55,57]

In conclusion, the data presented here contribute to the current knowledge of NDM-like-producing *Enterobacteriaceæ*. In agreement with previous studies, our findings punctuate that NDM-like producers constitute an important public threat, mainly due to the rapid horizontal transfer of IncX3 *bla*$_{NDM}$-carrying plasmids but, also, due to further evolvement of NDM-like-encoding MDR plasmids via reshuffling.

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Table S1. Oligonucleotide primers.

Name	Sequence (5′–3′)	Usage	Reference
pNDM-F	CGTGGCTCTTGTCATGCTGA	Tn3-like segment mapping with pNDM4-R	This study
pNDM4-R	TAACGACAAAGATCAGGAGCA	Tn3-like segment mapping with pNDM-F	This study
pNDM4-F	TGCAGGTTCGCTGAAGCTG	Tn3-like segment mapping with pNDM-R	This study
pNDM-R	AGGGAAGTAGTCTCTGATATCT	Tn3-like segment mapping with pNDM4-F	This study
922.9k-F	GATCGGCAGTAGAGGTGGA	9-kb segment mapping with 922.9k-R	This study
922.9k-R	TACCAAAGCAACAGCTGACG	9-kb segment mapping with 922.9k-F	This study
922.13k-F	GCTCTACAGCAGCGTTCCAG	13-kb segment mapping with 922.13k-R	This study
922.13k-R	AGTGGGAAAGCGTTGCAGATC	13-kb segment mapping with 922.13k-F	This study
922.18k-F	TGATACATGAACAAGGCAGATG	18-kb segment mapping with 922.18k-R	This study
922.18k-R	GTGCGATCAATGGTTAACTCA	18-kb segment mapping with 922.18k-F	This study
922.33b-F	ATGGCGAAACTGCCCTCGA	33-bb insertion mapping with 922.33b-R	This study
922.33b-R	TCTGTCTGAACGTGCTGGCT	33-bb insertion mapping with 922.33b-R	This study

Table S2. Antimicrobial susceptibility of NDM-like-producing clinical and recombinant strains.

Isolate	MIC (mg/l) of:[a]															
	Pip	Tzp	Ctx	Caz	Fep	Atm	Mem	Etp	Gen	Amk	Cmp	Tet	Sxt	Cip	Col	Tgc
Encl-922	>128	>128	>8	>16	>16	>16	4	>2	16	2	16	>32	>4	>8	0.25	1
Trc Encl-922	128	128	>8	>16	16	≤0.12	1	2	≤0.5	≤0.25	4	0.5	≤0.03	≤0.06	0.25	0.25
Encl-66918	>128	>128	>8	>16	>16	>16	4	>2	16	2	16	>32	>4	>8	0.25	1
Trc Encl-66918	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-89040	>128	>128	>8	>16	>16	>16	2	>2	16	4	16	>32	>4	>8	0.25	1
Trc Encl-89040	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-44578	>128	>128	>8	>16	>16	>16	2	>2	16	1	16	>32	>4	>8	0.25	1
Trc Encl-44578	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-89485	>128	>128	>8	>16	>16	>16	4	>2	16	1	16	>32	>4	>8	0.25	1
Trc Encl-89485	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-91221	>128	>128	>8	>16	>16	>16	4	>2	>32	1	16	>32	>4	>8	0.25	1
Trc Encl-91221	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-93141	>128	>128	>8	>16	>16	>16	4	>2	16	1	16	>32	0.5	>8	≤0.12	1
Trc Encl-93141	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-98042	>128	>128	>8	>16	>16	>16	2	>2	32	1	16	>32	1	>8	0.25	1
Trc Encl-98042	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-98047	>128	>128	>8	>16	>16	>16	2	>2	16	1	16	>32	>4	>8	0.25	1
Trc Encl-98047	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Encl-98546	>128	>128	>8	>16	>16	>16	16	>2	16	1	16	>32	0.25	0.12	0.25	1
Trc Encl-98546	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Enas-80654	>128	>128	>8	>16	>16	0.5	16	>2	≤0.25	≤0.5	16	8	0.12	8	>16	1
Trc Enas-80654	128	128	>8	>16	8	≤0.12	1	2	≤0.5	≤0.25	4	1	≤0.03	≤0.06	0.25	0.25
Enin-51781	>128	128	>8	>16	>16	>16	2	>2	16	1	16	>32	1	1	0.25	1
Trc Enin-51781	128	128	>8	>16	16	≤0.12	1	2	≤0.5	≤0.25	4	0.5	≤0.03	≤0.06	0.25	0.25
Esco-36073	>128	>128	>8	>16	>16	16	2	>2	8	4	>32	>32	>4	≤0.06	0.25	0.25
Trc Esco-36073	>128	>128	>8	>16	>16	16	2	>2	2	2	>32	32	>4	≤0.06	0.25	0.25
Esco-4382	>128	>128	>8	>16	>16	≤0.12	1	>2	32	1	4	>32	>4	>8	0.25	0.25
Trc Esco-4382	128	128	>8	>16	16	≤0.12	1	2	≤0.5	≤0.25	4	0.5	≤0.03	≤0.06	0.25	0.25
Esco-5256	>128	>128	>8	>16	>16	1	8	>2	≤0.25	≤0.5	4	>32	>4	>8	≤0.12	0.12
Trf Esco-5256	128	128	>8	>16	>16	≤0.12	2	>2	≤0.5	≤0.25	2	0.5	≤0.03	≤0.06	0.25	0.25
Esco-14290	>128	>128	>8	>16	>16	>16	2	2	16	1	16	>32	>4	>8	0.25	0.12
Trf Esco-14290	128	128	>8	>16	>16	≤0.12	2	>2	≤0.5	≤0.25	2	0.5	≤0.03	≤0.06	0.25	0.25
Klox-45574	>128	>128	>8	>16	>16	>16	4	>2	≤0.25	≤0.5	>32	>32	>4	2	≤0.12	0.5
Trf Klox-45574	128	128	>8	>16	>16	≤0.12	2	>2	≤0.5	≤0.25	2	0.5	≤0.03	≤0.06	0.25	0.25
Kpn-35963	>128	>128	>8	>16	>16	>16	2	>2	0.5	4	16	>32	1	>8	0.25	0.5
Trf Kpn-35963	>128	64	>8	>16	8	>16	0.5	2	0.5	4	1	16	0.25	≤0.06	0.25	0.25
Ror-30818	>128	>128	>8	>16	>16	>16	8	>2	>32	>64	>32	4	>4	>8	0.25	1
Trf Ror-30818	>128	>128	>8	>16	16	>16	1	>2	0.5	4	1	16	0.25	≤0.06	0.25	0.25

[a] Pip, piperacillin; Tzp, piperacillin-tazobactam (inhibitor fixed at 4 mg/l); Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Cmp, chloramphenicol; Tet, tetracycline; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin; Col, colistin; Tgc, tigecycline.

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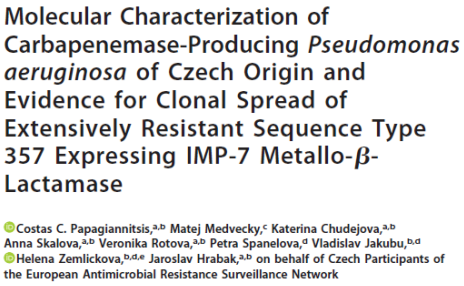
4.4 Publication no. 4: Molecular characterization of carbapenemase-producing *Pseudomonas æruginosa* of Czech origin and evidence for clonal spread of extensively resistant sequence type 357 expressing IMP-7 metallo-β-lactamase



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Abstract

The objective of this study was to perform molecular surveillance for assessing the spread of carbapenemase-producing *Pseudomonas. æruginosa* in Czech hospitals. One hundred thirty-six carbapenemase-producing isolates were recovered from 22 hospitals located throughout the country. Sequence type 357 (ST357) dominated (n = 120) among carbapenemase producers. One hundred seventeen isolates produced IMP-type (IMP-7 [n = 116] and IMP-1 [n = 1]) metallo-β-lactamases (MBLs), 15 produced the VIM-2 MBL, and the remaining isolates expressed the GES-5 enzyme. The bla_{IMP}-like genes were located in three main integron types, with In-p110like being the most prevalent (n = 115). The two other IMP-encoding integrons (In1392 and In1393) have not been described previously. bla_{VIM-2}-carrying integrons included In59-like, In56, and a novel element (In1391). bla_{GES-5} was carried by In717. Sequencing data showed that In-p110-like was associated with a Tn*4380*-like transposon inserted in genomic island LESGI-3, in *P. æruginosa* chromosome. The other integrons were also integrated into

the *P. æruginosa* chromosome. These findings indicated the clonal spread of ST357 *P. æruginosa*, carrying the IMP-7-encoding integron In-p110, in Czech hospitals. Additionally, the sporadic emergence of *P. æruginosa* producing different carbapenemase types, associated with divergent or novel integrons, punctuated the ongoing evolution of these bacteria.

Introduction

Pseuodomonas æruginosa is one of the most clinically important opportunistic pathogens, characterized by intrinsic resistance to a wide variety of antimicrobials.[1,2] However, in recent years, this species has turned resistant to all β-lactams, including carbapenems.[3] Although in *P. æruginosa* carbapenem resistance mostly arises from mutations that lead to the loss or inactivation of the porin OprD or upregulation of efflux pumps, production of carbapenemases is also increasingly reported.[4] Serine carbapenemases of the KPC, GES and OXA types have been encountered sporadically in this pathogen, with a limited geographical dissemination.[5-7] The emergence of *P. æruginosa* isolates producing metallo-β-lactamases (MBLs), mainly of VIM and IMP types, has been widely reported, and the spread of these bacteria has caused a public health crisis of global dimensions.[8,9]

MBLs are zinc-dependent enzymes commonly characterized by the ability to hydrolyze all β-lactams (with the exception of monobactams), including carbapenems. Their activity is not affected by the currently available β-lactamase inhibitors (i.e., clavulanic acid, tazobactam, sulbactam, or avibactam). Contrary to bla_{NDM} genes, bla_{VIM} and bla_{IMP} occur as gene cassettes in class 1 integrons or, more rarely, integrons of class 2 or 3.[9-11] These integrons often also contain gene cassettes conferring resistance to other antibiotics, like aminoglycosides and trimethoprim. Integrons cannot mobilize themselves but often reside within transposon structures, which, in turn, may be found in plasmids or in chromosomes. Frequently, class 1 integrons found in chromosomes are associated with genomic islands (GIs) of pathogenic bacteria, like the *Salmonella* genomic island 1 (SGI1).[12-14] These islands may also contain other resistance- or virulence-associated genes.[15,16]

Furthermore, in *P. æruginosa*, the production of MBLs commonly has been associated with multiresistant high-risk clones belonging to sequence types (STs) 111, 175, and 235.[17] A recent study has reported the spread of extensively drug-resistant ST235 *P. æruginosa* throughout Russia and into Belarus and Kazakhstan via clonal dissemination, underlining the importance of this ST.[18]

In the Czech Republic, carbapenem-resistant *P. æruginosa* strains are currently a critical problem in the management of health care-associated infections. The first MBL-producing *P. æruginosa* isolates were identified in 2008. These isolates that produced the IMP-7 enzyme were assigned to ST357, which was previously reported in IMP-1-producing *P. æruginosa* from Japan.[19-21] In another study, carbapenemase-producing *P. æruginosa* isolated from a hospital in Brno, during the period 2009 to 2011, belonged to STs 111 and 357, and carried VIM-2-encoding integrons In-p385 (*aacA29a*-bla_{VIM-2}) and In56 (bla_{VIM-2}) or the IMP-7-encoding integron In-p110(*aacA4*-*orf105*/*orfD*-bla_{IMP-7}-*aacA4*-bla_{OXA-2}-*orfE*-

-like).[22,23] However, the previous data are the outcome of reports describing the sporadic emergence of MBL-producing *P. æruginosa* in Czech hospitals at the beginning of the spread of these bacteria.

Accordingly, during this study, we organized surveillance for assessing the spread of carbapenemase-producing *P. æruginosa* in Czech hospitals, and characterizing them.

Results and Discussion

Carbapenemase-producing *P. æruginosa*

In 2015, a total of 194 nonrepetitive *P. æruginosa* isolates that were nonsusceptible to meropenem were referred to the National Reference Laboratory for Antibiotics (NRL) from 43 hospitals, of which 16 were located in Prague's metropolitan area (Central Bohemian Region). Isolates were derived from blood (n = 75, 38.7%), urine (n = 47, 24.2%), respiratory secretions (n = 33, 17.0%) and other material (34, 17.5%). Clinical material was not reported for the remaining five isolates. Additionally, 94 representative meropenem-susceptible (MER-S) *P. æruginosa* isolates, collected during 2015, were also studied.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) imipenem hydrolysis assay indicated carbapenemase production in 136 out of the 194 meropenem-nonsusceptible isolates.[24] All MER-S isolates were negative in the MALDI-TOF MS imipenem hydrolysis assay. In 132 of the isolates, the EDTA-meropenem test appeared to be positive, indicating MBL production.[25] The remaining four isolates tested positive by the boronic acid-meropenem-combined disc test suggesting carbapenemase production of the Ambler class A type.[26] PCR screening showed that 117 of the isolates were positive for the presence of bla_{IMP} and 15 isolates were positive for bla_{VIM} genes. The four MBL-negative isolates were positive for the presence of bla_{GES} genes. Nineteen of the carbapenemase producers, carrying bla_{IMP} genes, were recovered from positive blood cultures. Carbapenemase producers were collected from 22 hospitals, located throughout the country (Figure 1).

Susceptibilities, in terms of MIC ranges, are presented in Table 1. MIC_{50} and MIC_{90} values for the group of 136 carbapenemase-producing *P. æruginosa* (CPP) isolates were >64 μg/ml for ceftazidime and cefepime, and >32 μg/ml for meropenem. The majority of CPP isolates was resistant to ceftazidime (n = 136, 100%), cefepime (n = 134, 98.5%), meropenem (n = 134, 98.5%), piperacillin (n = 134, 98.5%) and piperacillin-tazobactam (n = 127, 93.8%). One hundred thirty-four (98.5%) CPP isolates also exhibited resistance to ciprofloxacin, 131 (96.3%) were resistant to tobramycin, 117 (86.0%) were resistant to gentamicin, and 63 (46.3%) were resistant to amikacin, while only 5 (3.7%) isolates were resistant to colistin. The 58 non-carbapenemase-producing *P. æruginosa* (N-CPP) isolates exhibited lower MICs of ceftazidime, cefepime and meropenem than those observed for CPP isolates. Additionally, MICs of non-β-lactam antibiotics, except colistin, were higher for the groups of CPP and N-CPP isolates than for MER-S isolates. The majority of MER-S isolates were also susceptible to cefepime (n = 89, 94.7%), ceftazidime (n = 88, 93.6%), piperacillin-tazobactam (n = 82, 87.2%) and piperacillin (n = 81, 86.2%).

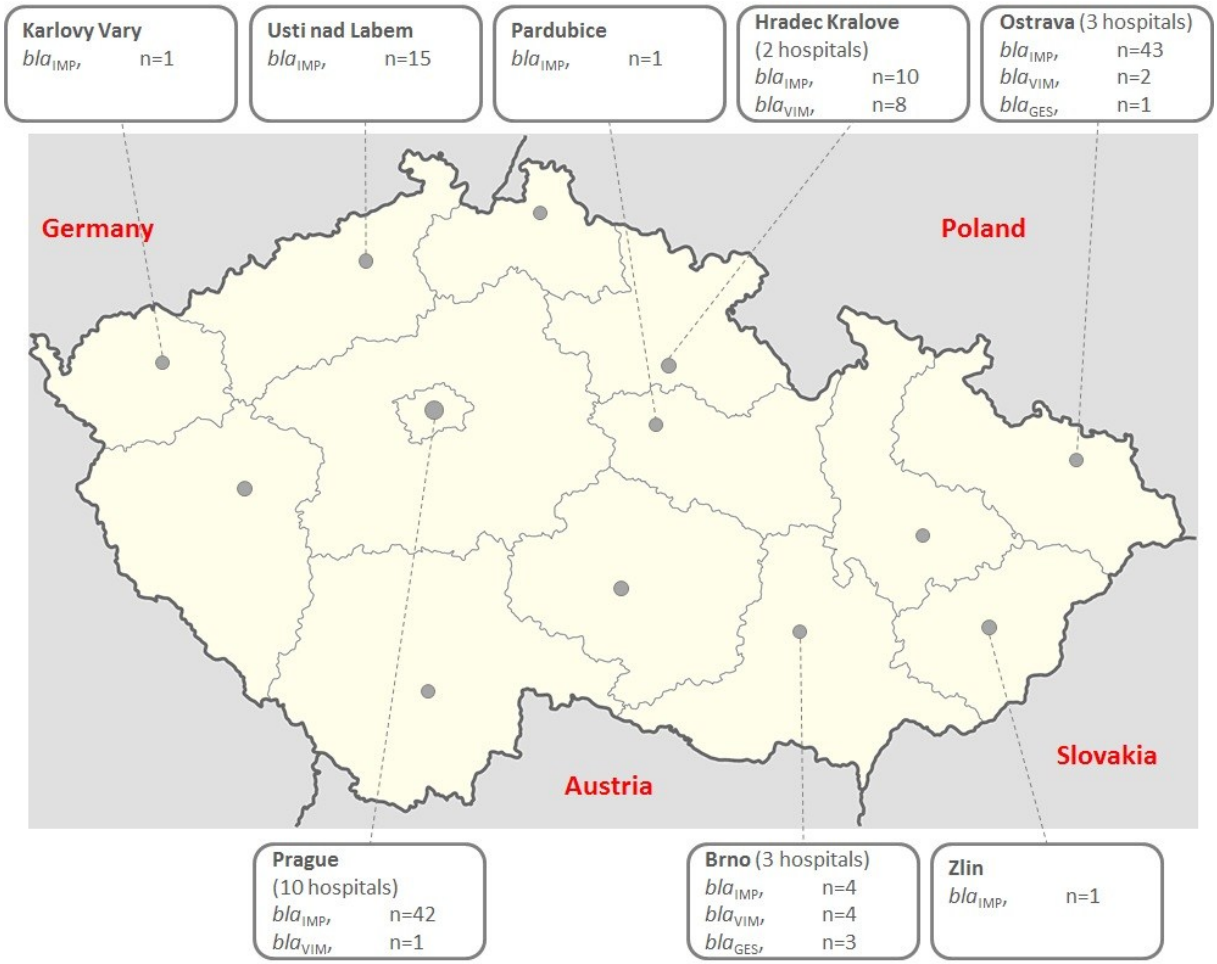

Figure 1. Geographic map showing the locations of the hospitals as well as the number of the carbapenemase-producing *P. æruginosa* isolates (CPP) collected during the study.

The population structure of *P. æruginosa* isolates studied by MLST is shown in Figure 2. The CPP isolates comprised 7 STs. ST357 was the most prevalent accounting for 120 CPP isolates (Table 1). The majority of ST357 isolates were associated with production of IMP-type MBLs (n = 115), while only 5 out of the 15 isolates that produced VIM type MBLs belonged to ST357. Thirteen of the CPP isolates were distributed in STs 111 (n = 9) and 235 (n = 4), which have been considered as high-risk clones.[17] The three remaining CPP isolates belonged to distinct STs. However, significant genetic diversity was found in the group N-CPP and MER-S isolates (Table S1). The group of 58 N-CPP isolates comprised 29 clones, with STs 175 (n = 10) and 235 (n = 12) accounting for 22 of the isolates. On the other hand, 58 different STs were identified among the group of 93 MER-S isolates. Most of the observed STs have been reported previously from *P. æruginosa* isolates of Czech origin.[27] However, five N-CPP and six MER-S isolates were assigned to novel STs (ST2297, ST2304, ST2305, ST2350, ST2351, ST2351, ST2352, ST2353, ST2354, ST2355 and ST2356). Of note was that high risk clones, ST235 and ST357, were not found among the MER-S isolates.

Furthermore, all *P. æruginosa* isolates were examined for the presence of the ExoS, ExoT, ExoU and ExoY toxin-encoding genes. The *exoT* (n = 287, 100%) and *exoY* (n = 281, 97.9%) genes were present in

the majority of the isolates (Table S1), while *exoU* was found in only 184 (64.1%) of the isolates. The *exoS* (n = 110, 38.3%) gene was identified in less than half of the isolates. These results are in agreement with previous studies reporting that even if *exoY* and *exoT* are present in nearly all clinical isolates, a significant number lack either *exoS* or *exoU*.[28,29] Additionally, our results confirmed that the high-risk clones belonging to STs 111 and 175 were associated with the copresence of *exoS*, *exoT* and *exoY* genes, while those belonging to STs 235 and 357 were associated with the copresence of *exoT*, *exoU* and *exoY*. Previous studies have demonstrated that the presence of ExoS, ExoT, or ExoU secretion correlates with a higher risk of mortality.[30] In particular, ExoU correlates with acute cytotoxicity and lung damage.[29]

Table 1. Susceptibility data of *P. æruginosa* isolates[a]

Carbapenemase content	ST	No. of isolates	MIC range (μg/ml)[b] for:									
			Pip	Tzp	Caz	Fep	Mer	Gen	Tob	Amk	Cip	Col
IMP-1	446	1	8	8	>64	>64	>32	>32	32	64	0.25	≤0.25
IMP-7	233	1	128	128	>64	>64	16	4	1	16	>8	≤0.25
IMP-7	357	115	32 to >128 (64/128)	16 to >128 (64/128)	32 to >64 (>64/>64)	16 to >64 (>64/64)	8 to >32 (>32/>32)	1 to >32 (32/>32)	4 to >32 (>32/>32)	2 to >64 (16/32)	8 to >8 (>8/>8)	≤0.25 to >32 (≤0.25/0.5)
VIM-2	111	9	16 to >128	16 to >128	16 to >64	8 to 64	8 to >32	2 to 16	32 to >32	16 to >64	>8	≤0.25 to 8
VIM-2	253	1	128	128	>64	64	32	8	4	4	0.125	≤0.25
VIM-2	357	5	32 to 64	8 to 64	32 to 64	16 to 32	>32	1 to >32	0.5 to >32	1 to >64	>8	≤0.25
GES-5	235	4	64	64	16	8 to 16	>32	>32	>32	64	>8	≤0.25 to 0.5
Carbapenemase producers		**136**	**8 to >12 (64/128)**	**8 to >128 (64/128)**	**16 to >64 (>64/>64)**	**8 to >64 (>64/>64)**	**8 to >32 (>32/>32)**	**1 to >32 (32/>32)**	**0.5 to >32 (>32/>32)**	**1 to >64 (16/64)**	**0.125 to >8 (>8/>8)**	**≤0.25 to >32 (≤0.25/0.5)**
Non-carbpenemase producers		**58**	**4 to >128 (32/>128)**	**4 to >128 (32/>128)**	**2 to 64 (8/32)**	**2 to 32 (8/16)**	**4 to >32 (8/32)**	**0.5 to >32 (8/>32)**	**0.5 to >32 (4/>32)**	**≤0.5 to 64 (8/64)**	**0.125 to >8 (>8/>8)**	**≤0.25 to 16 (≤0.25/0.5)**
Meropenem-susceptible isolates		**93**	**≤1 to >128 (8/64)**	**≤1 to >128 (4/32)**	**≤0.5 to 64 (2/8)**	**0.5 to 32 (2/8)**	**0.125 to 2 (0.5/2)**	**≤0.25 to >32 (2/4)**	**≤0.25 to >32 (0.5/2)**	**≤0.5 to 16 (4/8)**	**<0.06 to >8 (0.125/4)**	**≤0.25 to 0.5 (≤0.25/≤0.25)**

[a] Rows representing the total number of isolates per category are in bold.
[b] MIC_{50} and MIC_{90} values are also presented (in parentheses) for several groups of isolates. Pip, piperacillin; Tzp, piperacillin-tazobactam (inhibitor fixed at 4 μg/ml); Caz, ceftazidime; Fep, cefepime; Mer, meropenem; Gen, gentamicin; Tob, tobramycin; Amk, amikacin; Cip, ciprofloxacin; Col, colistin.

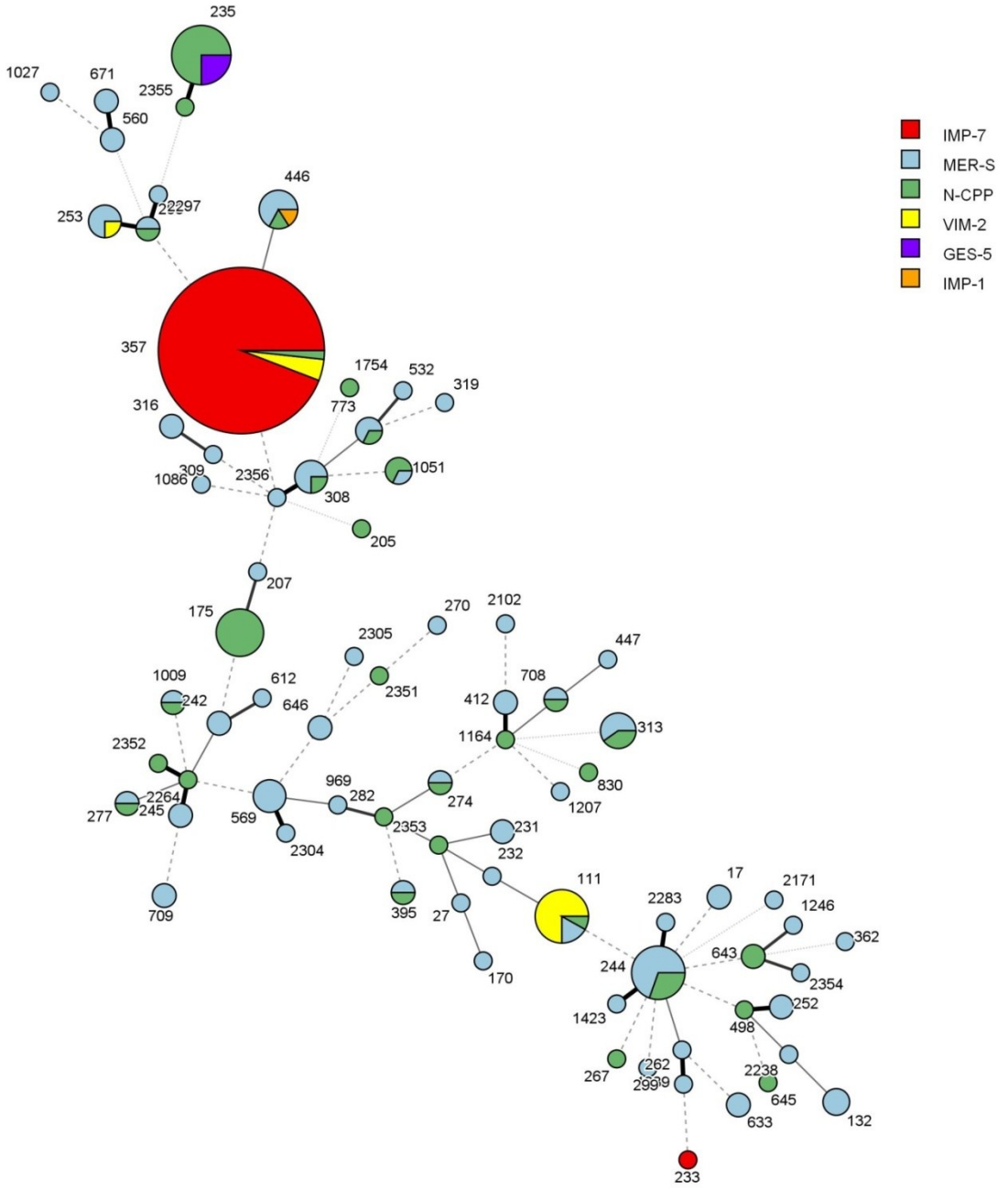

Figure 2. Minimal spanning tree of 287 *P. æruginosa* isolates, recovered from Czech hospitals during 2015, showing sequence types (STs) versus carbapenemase content. Each circle corresponds to an ST. The area of each circle is proportional to the number of isolates. The style of the connecting lines between STs correspond to the number of allelic differences: up to 3 differences, solid lines; above 3 allelic differences, dashed lines.

Carbapenemase-encoding integrons

Characterization of the regions, flanking the carbapenemase-encoding genes, by PCR mapping and sequencing showed that bla_{IMP}-like genes were located in three main types of class 1 integrons (Table 2). The most prevalent were In-p110-like integrons, identified in 115 ST357 CPP isolates. The canonical In-p110 occurred in 94 ST357 isolates.[22] However, the 21 remaining ST357 IMP-7 producers carried an In-p110 derivative with absent the *orf105/D* cassette. Among ST233 and ST446 IMP-producing isolates

were identified new integron types, designated In1393 and In1392, respectively. On the other hand, the most common (n = 9) *bla*$_{VIM}$-carrying integron was an In59 derivative, differing from In59 by having a second copy of *aacA29a* instead of *aacA29b*.[31] In59-like integrons were found in ST111 CPP isolates. Among ST357 VIM producers, the class 1 integron In56 was identified.[23] The ST253 VIM-producing isolate carried a new integron, In1391. Finally, the class 1 integron In717 with *bla*$_{GES-5}$ was identified in the ST235 CPP isolates.[13]

Chromosomal location of carbapenemase-encoding integrons

Repeated attempts to transfer carbapenemase-encoding genes from *P. æruginosa* isolates, representative of different STs and integron types (n = 22), into *Escherichia coli* DH5a were unsuccessful. This finding suggested the chromosomal location of the carbapenemase-encoding integrons.

Thus, the complete sequence of these isolates (Table 3) was determined. Illumina sequencing revealed that, in all cases, carbapenemase-encoding integrons were inserted in the chromosomes of *P. æruginosa* isolates.

Table 2. Integrons with carbapenemase-encoding genes identified in *P. æruginosa* isolates from Czech hospitals.

Integron types	Integron variants	ST	No. of isolates	Geographical area (No. of hospitals)	Gene cassette array	GenBank entry	Reference
In-p110-like	In-p110	357	94	Brno (1) Hradec Kralove (2) Karlovy Vary (1) Ostrava (3) Pardubice (1) Prague area (8) Usti nad Labem (1)	5'CS-*aacA4-orf105/D-bla*$_{IMP-7}$-*aacA4-bla*$_{OXA-2}$-*orfE*-3'CS	JX982232	(22)
	In-p110-like 1	357	21	Brno (2) Ostrava (1) Prague area (3) Ustinad Labem (1)	5'CS-*aacA4-bla*$_{IMP-7}$-*aacA4-bla*$_{OXA-2}$-*orfE*-3'CS	KY860567	This study
In1393		233	1	Prague area	5'CS-*bla*$_{IMP-7}$-*cmlA8-bla*$_{OXA-246}$-3'CS	KY860568	This study
In1392		446	1	Brno	5'CS-*aadB-bla*$_{IMP-1}$-*aadA1a*-3'CS	KY860569	This study
In59-like	In59[a]				5'CS-*aacA29a-bla*$_{VIM-2}$-*aacA29b*-3'CS	AF263519	(46)
	In59-like 1	111	9	Brno (1) Hradec Kralove (1)	5'CS-*aacA29a-bla*$_{VIM-2}$-*aacA29a*-3'CS	KY860571	This study
In56		357	5	Brno (1) Ostrava (1) Prague area (1)	5'CS-*bla*$_{VIM-2}$-3'CS	AF191564	(23)
In1391		253	1	Prague area	5'CS-*bla*$_{VIM-2}$-*aacA8-gcuD*-3'CS	KY860572	This study
In717		235	4	Brno (1) Ostrava (1)	5'CS-*bla*$_{GES-5}$-*aacA4-gcuE15-aphA15*-IS*Pa21e*-3'CS	JF826499	(13)

[a] In59 was not identified in this study and is shown here only for comparison reasons.

Table 3. Characteristics of 25 *P. æruginosa* isolates sequenced by Illumina platform.

Isolate	Geographical area (Hospital)	ST	Carbapenemase content	Carbapenemase-encoding integron				Additional resistance genes	GenBank entry
				Integron variant	In-associated transposon	In-associated GI or ICE	Chromosomal location		
Pae-31448cz	Brno (BB)	ST111	VIM-2	In59-like 1	Tn5060-like	ICE1	PA4541.1	–	KY860571
Pae-31929cz	Hradec Kralove (NA)	ST111	VIM-2	In59-like 1	Tn5060-like	ICE1	PA4541.1	–	–
Pae-29327cz	Prague area (A0)	ST175	–	–	–	–	–	*aadB*, *aadA13*, *sul1*	–
Pae-32301cz	Prague area (A63)	ST233	IMP-7	In1393	–	–	PA5101	bla_{OXA-4}, *aadA2*, *tetA*(G), *cmlA9*	KY860568
Pae-30094cz	Brno (ZN1)	ST235	GES-5	In717	Tn4380-like	LESGI-3	PAO2583	*aadA6*, *tetA*(G)	KY860573
Pae-30653cz	Prague area (TN)	ST235	GES-5	In717	Tn4380-like	LESGI-3	PAO2583	*aadA6*, *tetA*(G)	–
Pae-29931cz	Prague area (A2)	ST235	–	–	–	–	–	bla_{OXA-2}, *aacA31*, *sul1*	–
Pae-29785cz	Prague area (A41)	ST253	VIM-2	In1391	Tn5563-like	PACS171b GI	*endA*	*aadA6*, *aacA31*, *strAB*	KY860572
Pae-28606cz	Ostrava (OV1)	ST357	VIM-2	In56	Tn3-like	PAGI-56	PA0069	-	-
Pae-29652cz	Brno (BB)	ST357	VIM-2	In56	Tn3-like	PAGI-56	Pa0069	bla_{LCR-1}, *aadB*, *aacA4*, *aphA7*, *strAB*	KY860570
Pae-29480cz	Zlin (ZL)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-29533cz	Karlovy Vary (KV2)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-30039cz	Prague area (A31)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-30351cz	Prague area (A0)	ST357	IMP-7	In-p110-like 1	Tn4380-like	LESGI-3	PAO2583	*strAB*	KY860567
Pae-30418cz	Hradec Kralove (HK)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-30652cz	Ostrava (TN)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-31092cz	Brno (BB)	ST357	IMP-7	In-p110-like 1	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-31360cz	Ostrava (OV1)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	KY860566
Pae-31897cz	Prague area (KL)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-31912cz	Pardubice (PA2)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-31927cz	Prague area (PB)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*cmx*, *strAB*	-
Pae-31975cz	Usti nad Labem (UL)	ST357	IMP-7	In-p110	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-32048cz	Prague area (A0)	ST357	IMP-7	In-p110-like 1	Tn4380-like	LESGI-3	PAO2583	*strAB*	-
Pae-31025cz	Hradec Kralove (HK)	ST357	-	-	-	-	-	bla_{OXA-2}, *aacA4*, *sul1*, *strAB*	-
Pae-32183cz	Brno (BB)	ST446	IMP-1	In1392	Tn4380-like	LESGI-3	PAO2583	*aphA6*, *strAB*	KY860569

In-p110-like, In1392 and In717 integrons

In-p110-like, In1392 and In717 integrons were in LESGI-3, located in the chromosome (Figure 3). LESGI-3 was previously reported in *P. æruginosa* LESB58, an epidemic strain from the United Kingdom.[32] All three integrons were embedded in the mercury resistance transposon Tn*4380*. The IRi of the integrons

were located downstream *resI* site of the Tn*4380* module in precisely the same position as In717 in Tn*6163*.[13] Similar to Tn*6163* in *P. æruginosa* C79, an IS*1071* element was inserted into the *tnpA* gene of the transposon.

The 3'conserved segment (3'CS) of In-p110 was truncated 526 bp after the start codon of *sul1*. Downstream of Δ*sul1*, a 2,875-bp fragment of a Tn*3*-like transposon, consisting of the IRtnp of the transposon and the 3' end of *tnpA*, was found. The *tnpA* gene was probably deleted due to insertion of an IS*6100* element.

Unlike Tn*6163*in *P. æruginosa* C79, a sequence composed of a gene encoding a resolvase, an *aphA6* resistance gene, and a Tn*5393* transposon was found, next to IS*1071*, in the In1392-carrying Tn*4380*-like transposon.[13] Similar to In717 in Tn*6163*, the 3'CS of In1392 was disrupted, after the start codon of *orf5*, by an IS*6100* element.

Unlike in *P. æruginosa* C79, the 3'CS of In717 was truncated after the start codon of *sul1* in ST235 *P. æruginosa* Pae-30094cz and Pae-30653cz isolates. Downstream of Δ*sul1*, there was a *cmlA9-tetR*(G)-*tetA*(G)-IS*CR3-groEL/intI1-sul1-orf5* sequence. This sequence appeared as part of the bla_{VIM-2}-containing Tn*501*-like transposon from *P. æruginosa* isolates from northeast Ohio.[33] *orf5* was disrupted by an IS*6100* element.

In all three cases, the Tn*4380*-like *mer* module was found next to IS*6100*.

In1393

Sequencing data showed that In1393 was also located in *P. æruginosa* chromosome (Figure 3). Both the 5'CS and 3'CS of the integron were intact. The IRi of In1393 was adjacent to a 9,685-bp sequence (nucleotide [nt] 9349 to 19033 in GenBank accession no. KY860568) exhibiting no identity with already characterized transposition modules. However, the putative products of this sequence showed high amino acid sequence similarity (from 95% to 99%) with TniQ-, TnsD-, TnsA, TnsB- and TnsC-like transposition proteins from *Pseudomonas* sp. strain BAY1663 (GenBank accession no. AZSV01000008). This sequence was found at the boundary of *P. æruginosa* chromosome. An IS*6100* element, adjoining the 3'CS of In1393, was identified next to the other boundary of *P. æruginosa* chromosome.

In56

In both ST357 VIM-2 producers, sequenced during this study, the integron In56 was associated with a Tn*3*-like transposon that was inserted in a novel genomic island (Figure 4), designated PAGI-56. PAGI-56 included open reading frames encoding proteins of various functions (e.g., metabolic activities, DNA recombination, and regulation of gene expression) and hypothetical proteins of unknown function. GIs closely related to PAGI-56 have been reported previously in *P. æruginosa* isolates IOMTU 133 (77% coverage; 100% identity) (GenBank accession no. AP017302), PA7 (77% coverage; 99% identity), and W36662 (55% coverage; 99% identity) (GenBank accession no. CP008870).[34] PAGI-56 was inserted in *P. æruginosa* chromosome, into a gene encoding for a DNA repair photolyase (PA0069 in GenBank accession no.AE004091), in the same position as that reported in the aforementioned isolates. PAGI-56

differed from the GI identified in *P. æruginosa* IOMTU 133 by the presence of an additional 8,002-bp sequence (nt 28607 to 36608 in GenBank accession no. KY860570) including a mer operon and coding sequences for proteins with unknown function. Additionally, a 19,505-bp segment (nt 115171 to 134675 in GenBank accession no. AP017302) was probably deleted due to insertion of the In56-carrying Tn3-like transposon. The IRi of In56 was located within the *urf-2* gene of the transposon, while an IS6100 element was found downstream of *orf5* of integron 3'CS. IS6100 was found at the boundary of PAGI-56.

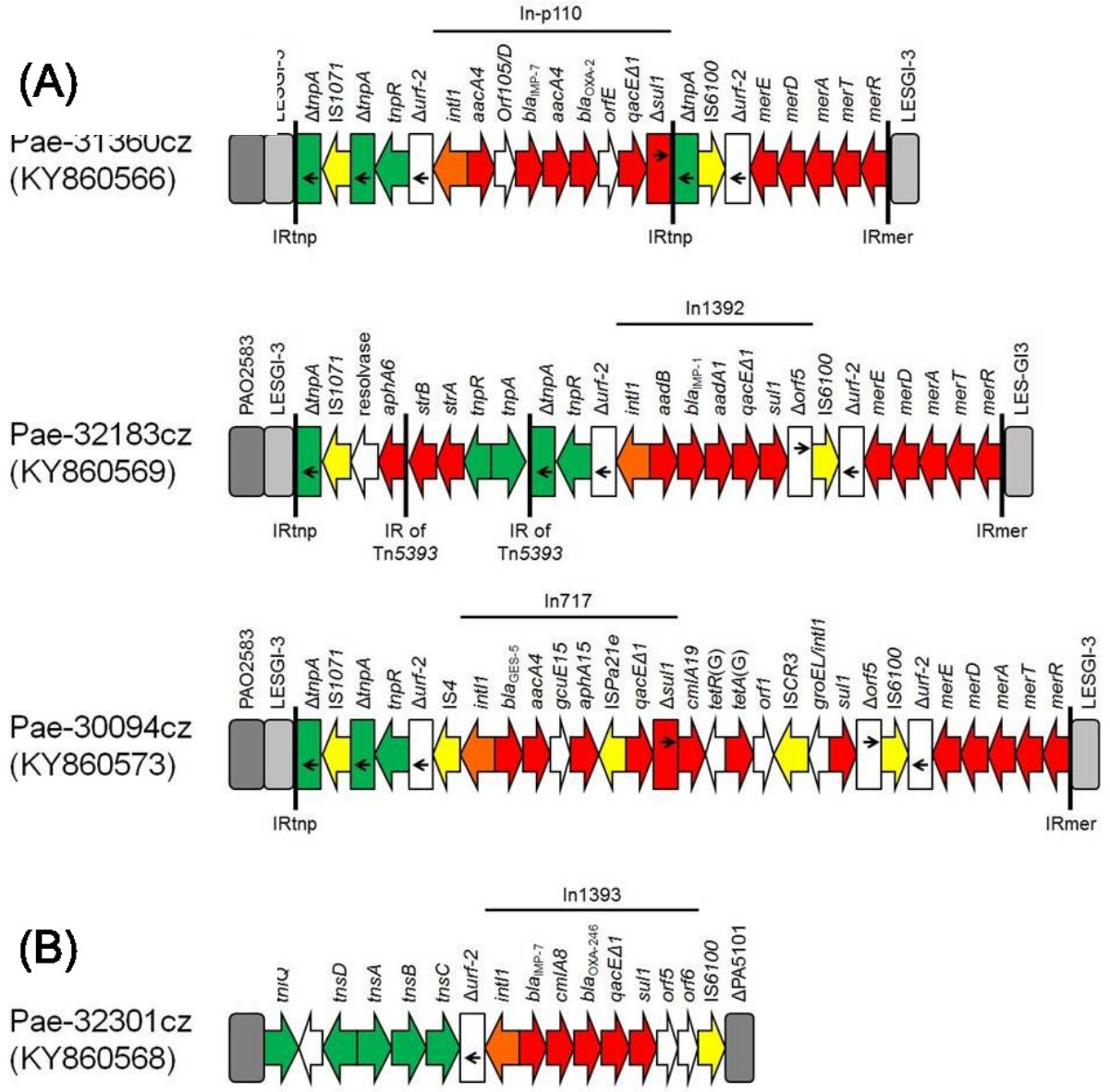

Figure 3. Linear maps of the genetic context of carbapenemase-encoding class 1 integrons In-p110, In1392 and In717 inserted in LESGI-3 (A), and In1393 integrated into *P. æruginosa* chromosome (B). Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements and transposases are shown in red, yellow and green, respectively. *intI1* genes are shaded orange. Sequences associated with GIs are shaded light gray; dark gray rectangles indicate *P. æruginosa* chromosome. The remaining genes are shown in white.

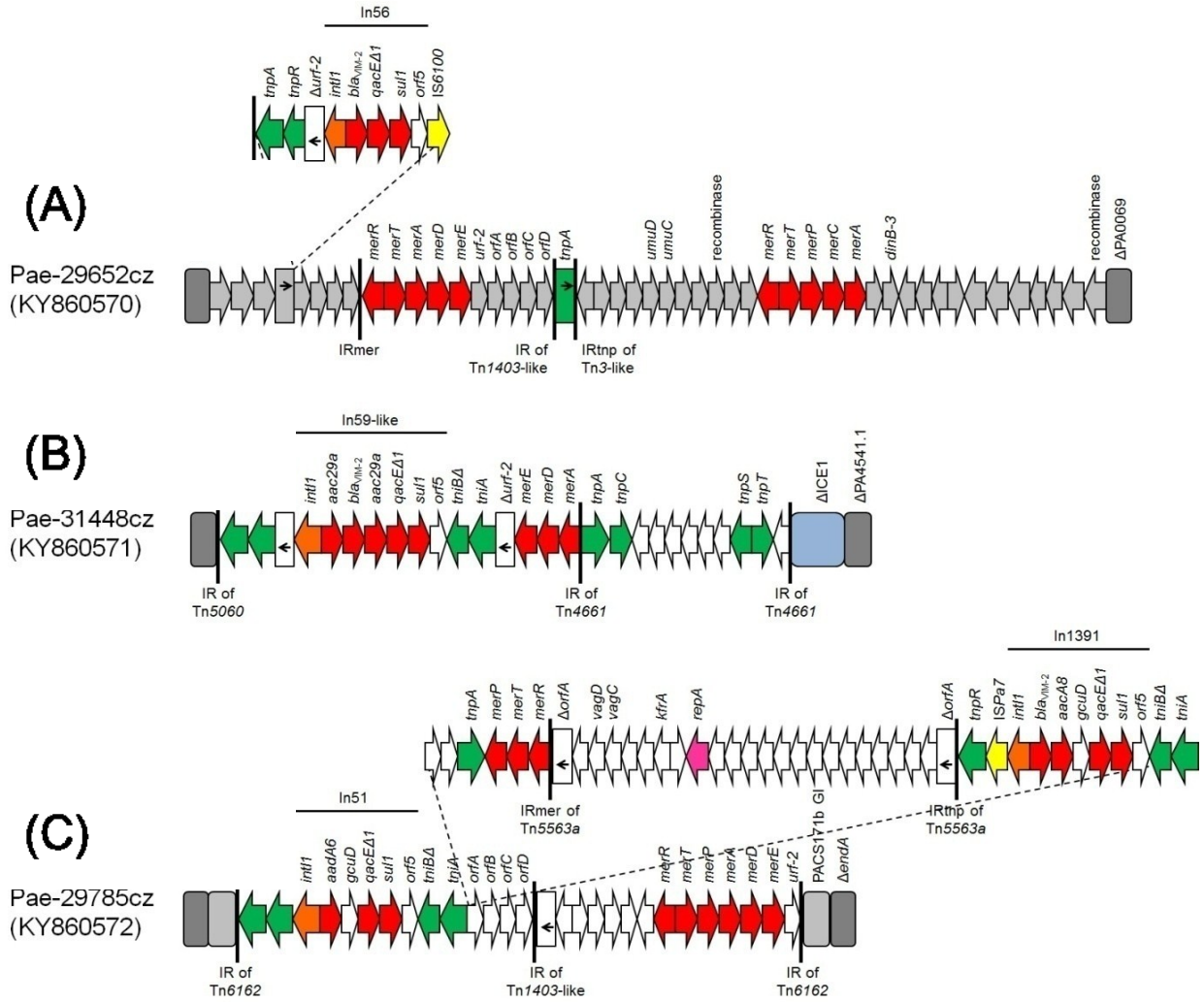

Figure 4. Linear maps of the genetic context of carbapenemase-encoding class 1 integrons In56, located in the novel PAGI-56 (A), In59-like associated with ICE1 (B), and In1391, inserted in PACS171b GI (C). Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements and transposases are shown in red, yellow and green, respectively. *intI1* genes are shaded orange, while the *repA* gene of pAMBL2 is shown in pink. Sequences associated with GIs are shaded light gray; dark gray rectangles indicate the *P. æruginosa* chromosome; ICE1 is shown in blue. The remaining genes are shown in white.

In59-like 1

In both ST111 isolates, sequenced by Illumina platform, the In59-like integron was inserted in a Tn*5060*-like transposon located in the *P. æruginosa* chromosome (Figure 4). The mercury resistance Tn*5060*, which is most closely related to the ancestor of Tn*21* prior to integron acquisition, has been reported previously from *Pseudomonas* sp. strain A19-1 isolated from Siberian permafrost.[35] The IRi of In59-like was located within the *urf-2* gene of the transposon, while a defective Tn*402*-like *tni* module (ΔTn*402*-like) composed of *tniB*Δ4 and *tniA* was found downstream of *orf5* and next to the *mer* operon of Tn*5060*. However, the Tn*5060 mer* module lacked a 1,847-bp fragment including the *merC*, *merP*, *merT* and *merR* genes, and the IRmer of the transposon (Δ*mer*). The Tn*3*-like transposon Tn*4661*, which carried two divergently transcribed *tnpS* and *tnpT* genes for cointegrate resolution, was identified adjacent to Δ*mer* and to a partially deleted ICE1-like integrative conjugative element (ΔICE1).[36]

Interestingly, a previous study showed that ICE1 was present in 67% of ST111 *P. æruginosa* isolates.[37] ΔICE1 was integrated into $tRNA^{Lys}$ gene (PA4541.1 in GenBank accession no. AE004091), a site similar to that previously reported for most analyzed *P. æruginosa* isolates. It is likely that insertion of the Tn*4661* transposon deleted the remaining parts of Δ*mer* and ΔICE1.

In1391

In1391 was in PACS171b GI (Figure 4) that includes open reading frames encoding proteins responsible for tellurite resistance, metabolic activities, DNA recombination and gene regulation.[38] PACS171b GI was inserted into the chromosomal *endA* gene. In1391 was embedded in a mercury resistance Tn*6162*-like element, which is a Tn*1403* related transposon.[39] Similar to Tn*6162* in *P. æruginosa* C79, 5-bp direct repeats of the target (GTCAT) were identified at the boundaries of the transposon, and the class 1 integron In51 containing *aadA6* and *gcuD* gene cassettes was located in the Tn*6162* module.[13] The 3'CS of In51 was followed by a partially deleted Tn*402*-like *tni* module, carrying *tniB*Δ4 and *tniA*. Next to ΔTn*402*-like, a 20,045-bp (nt 44098 to 64142) sequence that comprised two fragments of Tn*5563a* transposon flanking a central sequence, which exhibited extensive similarity with the VIM-1-encoding plasmid pAMBL2 from *P. æruginosa* PAO1 (GenBank accession no. KP873171), was found.[40] The IRi of In1391 was located between *resII* and *resI* sites of the Tn*5563a* module. However, an IS*Pa7* element was present between the 3'end of *intI1* and IRi. Similar to In51 in Tn*6162*, a ΔTn*402*-like *tni* module followed by the *orfDCBA* and *mer* operons was identified adjacent to the 3'CS of In1391.[13] However, the region intervening between *orfDCBA* and *mer* operons was in an inverted orientation. The described transposon may have resulted by integration of a pAMBL2-like plasmid, carrying a Tn*5563*-like with In1391, into Tn*6162*.

Further analysis of WGS data

Analysis of whole-genome sequencing (WGS) data by the ResFinder 2.1 tool revealed that the majority of the sequenced isolates included additional genes for resistance to aminoglycosides, tetracyclines, trimethoprim and chloramphenicol (Table 3). Fifteen out of the sixteen ST357 isolates carried the *strA* and *strB* resistance genes. Interestingly, one IMP-7-producing ST357 isolate included the recently described *cmx* gene (GenBank accession no. U85507) conferring resistance to chloramphenicol, while one ST357 VIM-2 producer harbored the bla_{LCR-1}oxacillinase gene.[41] Examination of quinolone resistance-determining regions of *gyrA*, *gyrB*, *parC* and *parE*, and of the *mexR*, *nfxB* and *mexT* genes, which regulate the MexAB-OprM, MexCD-OprJ, and MexEF-OprN multidrug efflux systems, showed the presence of several amino acid substitutions (Table S2).[4] The majority of these substitutions have been reported previously from both ciprofloxacin-susceptible and ciprofloxacin-resistant isolates. However, all ciprofloxacin-resistant isolates presented the T83I amino acid substitution in GyrA, which previously has been associated with increased quinolone resistance.[42] Finally, in 21 of the isolates, the sequence of *oprD* showed point mutations predicted to result in early termination of translation (Table S2), which is consistent with increased carbapenem resistance, even in non-carbapenemase-producing isolates.[43]

Phylogenetic analysis

Bayesian analysis of the 24 core genomes resulted in well-defined clusters (Figure 5), which corresponded to different STs. The largest cluster was composed of isolates, which belonged to ST357. Isolates of STs 111 and 235 were grouped in monophyletic subgroups. The tree topology was greatly supported by 100% posterior probabilities for all clades representing different STs. Additionally, the results of Bayesian analysis indicated that IMP-7-producing ST357 *P. æruginosa* isolates, recovered from different geographical locations and hospitals, were closely related. This finding further supported the clonal spread of extensively resistant ST357 *P. æruginosa*, expressing IMP-7 MBL, in Czech hospitals.

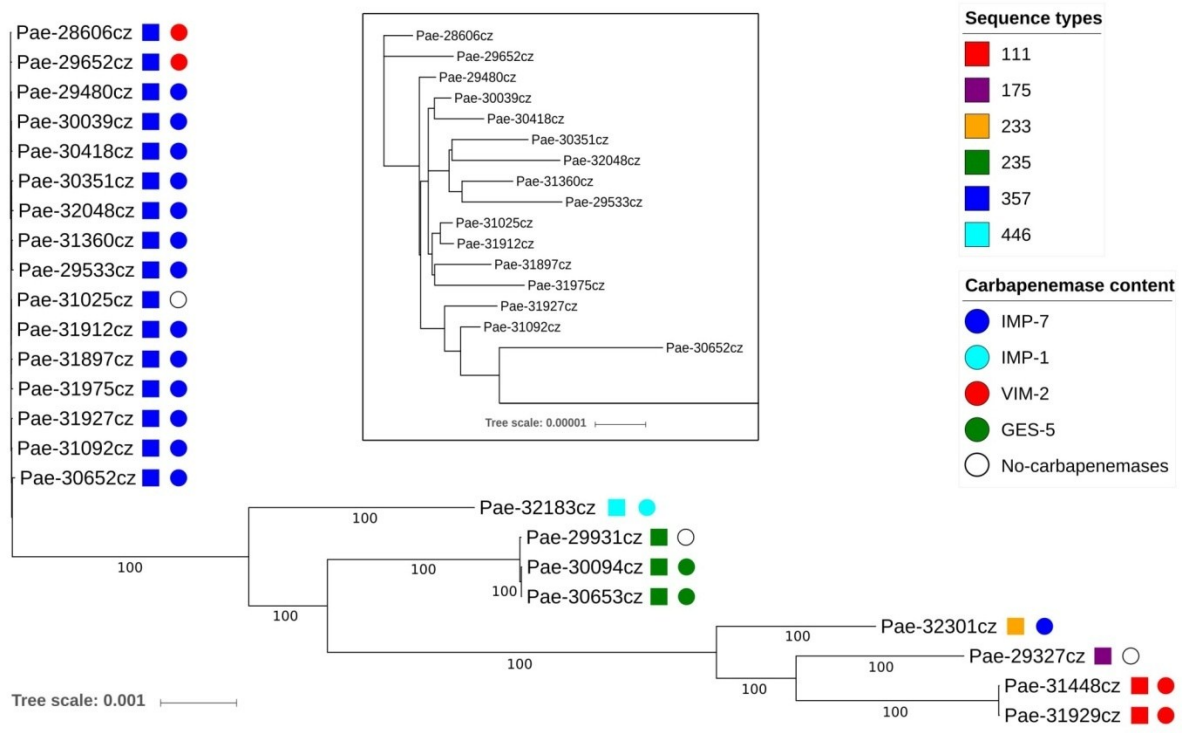

Figure 5. Phylogenetic tree of *P. æruginosa* isolates, which were sequenced using the Illumina platform, based on Bayesian statistics constructed from core genome alignment. The boxed portion shows the clade of ST357 isolates. Branch lengths indicate the number of base changes per site. STs and carbapenemase content are indicated as squares and circles, respectively, of different colors. Numbers at the branched represent their posterior probabilities.

Concluding remarks

To our knowledge, this is the first nationwide surveillance study on carbapenemase-producing *P. æruginosa* isolates from the Czech Republic using a deep molecular-genetic typing procedure. One hundred thirty-two CPP isolates were collected from 22 hospitals, located throughout the country. The majority of CPP isolates belonged to ST357 and carried the IMP-7-encoding integron In-p110, which indicates the clonal spread of these isolates in Czech hospitals. WGS data showed that, in all sequenced ST357 isolates, In-p110-like integrons were associated with a Tn*4380*-like transposon inserted in LESGI-3, which was located in the same position of *P. æruginosa* chromosome. Additionally, phylogenetic analysis showed that all ST357 isolates were clustered in a monophyletic group (Figure 5), indicating

that they were close relatives. These findings further supported the hypothesis regarding the clonal spread of ST357 IMP-7-producing isolates in Czech hospitals. Analysis of WGS data revealed the presence of additional resistance genes, of T83I amino acid substitution in GyrA and of premature stop codons in *oprD* gene that can be implicated in the development of extensively multidrug-resistant bacteria, limiting therapeutic options.[4,18,42,43] Additionally, all ST357 IMP-7 producers carried the virulence genes *exoT*, *exoU* and *exoY*, which previously have been associated with increased pathogenicity and mortality of the bacterium.[29,30] These data, which are in agreement with the results of previous studies, highlighted the important role of high-risk clones, such as STs 111, 175, 235 and 357, in the successful dissemination of clinically important resistance determinants.[17,18]

Furthermore, the sporadic emergence of *P. æruginosa* isolates producing different carbapenemase types (VIM-2, GES-5 and IMP-1), which were associated with divergent or novel integron structures, underlined the ongoing evolution of these bacteria. This evolution will probably further aggravate the situation. Therefore, there is a need of utmost importance to limit this public health problem. Thus, infection control practices in Czech hospitals should be improved by: (i) performance of surveillance cultures for detection of carbapenemase-producing bacteria upon admission of patients, (ii) periodical reinforcement of hygiene practices, (iii) control of frequent transfer of patients between different hospitals, and (iv) isolation of colonized or infected patients.

Materials and methods

Bacterial isolates and confirmation of carbapenemase production

In 2015, 43 microbiological laboratories, covering all of the Czech Republic, collected nonrepetitive *P. æruginosa* isolates that were nonsusceptible to meropenem (MIC >2 μg/ml). All meropenem-nonsusceptible isolates were sent to the National Reference Laboratory (NRL) for Antibiotics for further analysis. Species identification was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). All isolates were tested for carbapenemase production by MALDI-TOF MS imipenem hydrolysis assay.[24] Isolates that were positive by MALDI-TOF MS imipenem hydrolysis assay were subjected to phenotypic detection of carbapenemases using double-disc synergy test (DDST) with EDTA, and the phenylboronic acid disc test, respectively.[25,26] Carbapenemase genes (bla_{KPC}, bla_{GES}, bla_{VIM}, bla_{IMP}, bla_{NDM} and bla_{OXA-48}-like) were detected by PCR amplification.[44-48] Additionally, 94 meropenem-susceptible *P. æruginosa* isolates (representative of all different hospitals and susceptibility profiles; that are part of the European Antimicrobial Resistance Surveillance Network (EARS-Net) recovered from positive blood cultures of patients treated in Czech hospitals during 2015 were included in this study for comparative purposes.

Susceptibility testing

MICs of piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, meropenem, tobramycin, gentamicin, amikacin, colistin and ciprofloxacin were determined by the broth dilution method.[49] Data

were interpreted according to the criteria (version 7.1) of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org).

Typing

All *P. æruginosa* isolates were typed by multilocus sequence typing (MLST).[50] The database at https://pubmlst.org/pæruginosa was used for assigning STs. Based on allelic profiles, a graphical tool-based minimal spanning tree build in Bionumerics 7.6 (Applied-Maths, Austin, TX) was constructed.

Detection of virulence-associated genes

All isolates were PCR screened for the presence of genes encoding ExoS, ExoT, ExoU and ExoY toxins of the *P. æruginosa* type III secretion system.[51] Primers and conditions for PCR amplification were used as described previously.[52]

Integron analysis

Variable regions of class 1 integrons with bla_{IMP}-, bla_{VIM}- and bla_{GES}-like genes were amplified in two parts, from the 5' conserved segment (5'CS) to carbapenemase-encoding cassette, and from carbapenemase-encoding cassette to the 3'CS.[22] Whole-gene arrays were sequenced using an ABI 3500 sequencer (Applied Biosystems, Foster City, CA). The Integrall integron database (http://integrall.bio.ua.pt) was used to analyze and assign integron sequences.[53]

Transfer of carbapenemase-encoding genes

Twenty-two carbapenemase-producing *P. æruginosa* (CPP) isolates were selected, in order to define the genetic units carrying the detected carbapenemase-encoding genes. These isolates were selected as representatives of all different STs, integron-types and hospitals.

Plasmid extractions from *P. æruginosa* isolates were carried out using a Qiagen Maxi Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Transformations were attempted by electroporation into *E. coli* DH5a cells. Transformants were selected on Luria Bertani agar plates with ampicillin (50 μg/ml).

Whole genome sequencing

The 22 CPP isolates, used in transformation experiments, were selected for whole-genome sequencing. Additionally, three non-carbapenemase-producing *P. æruginosa* were selected for comparison purposes.

The genomic DNAs of *P. æruginosa* were extracted using the DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy), and were sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Initial paired-end reads were quality trimmed using Trimmomatic tool v0.32 and assembled via de Bruijn graph-based de novo assembler SPAdes v3.6.0.[54,55] The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISFinder database (www-is.biotoul.fr/), and the open reading frame

(ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using Mauve 2.3.1.[56]

Antibiotic resistance genes were identified using the ResFinder 2.1 tool (https://cge.cbs.dtu.dk/services/ResFinder/) with an identity threshold >90%.[57]

Bayesian analysis

Additionally, Illumina data were used for phylogenetic analysis. Briefly, core-genomes were extracted employing NUCmer v3.1 for detection of shared genome content among isolates, and a home-made script for extraction of core sequences.[58] The resulting core genome sequences were 5.468 Mbp (range, 5,467,960-bp to 5,468,214-bp). They then were aligned by MAFFT v7.215 using default gap penalties, memsave parameter and progressive FFT-NS-2 strategy.[59] For the purposes of our analysis, we applied Bayesian statistics via MrBayes v3.2.6 using the following parameters: mixed model of nucleotide substitution, gamma-distributed rates among sites, four Monte Carlo Markov chains for 1,000,000 cycles, chains were sampled every 1,000th generation, and first 25% of the samples discarded as burn-in.[60] The obtained summary statistics of Bayesian analysis were the following: average standard deviation of split frequencies 0.0035, maximum standard deviation of split frequencies 0.0301, average potential scale reduction factor 1.000; and maximum potential scale reduction factor, 1.010. The final tree topology was generated using 50% majority-rule consensus and was visualized via iTOL v3.5.2 and edited by Inkscape v0.91 (www.inkscape.org).[61]

Nucleotide sequence accession numbers

One nucleotide sequence representing each integron-type was submitted to the GenBank, under accession numbers KY860566, KY860567, KY860568, KY860569, KY860570, KY860571, KY860572 and KY860573.

Table S1. STs and virulence-associated genes identified among *P. æruginosa* isolates collected from Czech hospitals, during 2015.

ST	No. of isolates			Virulence-associated genes
	Carba-penemase producers	Non-carba-penemase producers	Meropenem-susceptible isolates	
17	–	–	2	*exoS, exoT, exoY*
27	–	–	1	*exoS, exoT, exoY*
111	9	1	2	*exoS, exoT, exoY*
132	–	–	3	*exoS, exoT, exoY*
170	–	–	1	*exoS, exoT, exoY*
175	–	10	–	*exoS, exoT, exoY*
205	–	1	–	*exoT, exoU*
207	–	–	1	*exoT, exoU, exoY*
231	–	–	2	*exoS, exoT, exoY*
232	–	–	1	*exoS, exoT*
233	1	–	–	*exoS, exoT, exoY*
235	4	12	–	*exoT, exoU, exoY*
242	–	–	2	*exoS, exoT, exoY*
244	–	4	9	*exoS, exoT, exoY*
245	–	–	2	*exoS, exoT, exoY*
252	–	–	2	*exoS, exoT, exoY*
253	1	1	4	*exoT, exoU, exoY*
262	–	–	1	*exoS, exoT, exoY*
267	–	1	–	*exoS, exoT, exoY*
270	–	–	1	*exoS, exoT, exoY*
274	–	1	1	*exoS, exoT, exoY*
277	–	–	1	*exoS, exoT, exoY*
282	–	1	–	*exoS, exoT, exoY*
299	–	–	1	*exoS, exoT, exoY*
308	–	1	3	*exoT, exoU, exoY*
309	–	–	1	*exoT, exoU*
313	–	2	3	*exoS, exoT, exoU*
316	–	–	2	*exoT, exoU, exoY*
319	–	–	1	*exoT, exoU, exoY*
357	120	2	–	*exoT, exoU, exoY*
362	–	–	1	*exoS, exoT, exoY*
395	–	1	1	*exoS, exoT, exoY*
412	–	–	2	*exoS, exoT, exoY*
446	1	1	4	*exoT, exoU, exoY*
447	–	–	1	*exoS, exoT, exoY*
498	–	1	–	*exoS, exoT, exoY*
532	–	–	1	*exoT, exoU, exoY*
560	–	–	2	*exoT, exoU, exoY*
569	–	–	4	*exoS, exoT, exoY*

ST	No. of isolates			Virulence-associated genes
	Carba-penemase producers	Non-carba-penemase producers	Meropenem-susceptible isolates	
612	–	–	1	*exoS, exoT, exoY*
633	–	–	2	*exoS, exoT, exoY*
643	–	2	–	*exoS, exoT, exoY*
645	–	1	–	*exoS, exoT, exoY*
646	–	–	2	*exoS, exoT, exoY*
671	–	–	2	*exoT, exoU, exoY*
708	–	1	1	*exoS, exoT, exoY*
709	–	–	2	*exoS, exoT, exoY*
773	–	1	2	*exoT, exoU, exoY*
830	–	1	–	*exoT, exoU*
969	–	–	1	*exoS, exoT, exoY*
1009	–	1	1	*exoS, exoT*
1027	–	–	1	*exoT, exoU, exoY*
1051	–	2	1	*exoT, exoU, exoY*
1086	–	–	1	*exoT, exoU, exoY*
1164	–	1	–	*exoS, exoT, exoY*
1207	–	–	1	*exoS, exoT, exoY*
1239	–	–	1	*exoS, exoT, exoY*
1246	–	–	1	*exoS, exoT, exoY*
1423	–	–	1	*exoS, exoT, exoY*
1754	–	1	–	*exoS, exoT, exoY*
2102	–	–	1	*exoS, exoT, exoY*
2171	–	–	1	*exoS, exoT*
2211	–	–	1	*exoT, exoU, exoY*
2238	–	–	1	*exoT*
2264	–	1	–	*exoS, exoT*
2283	–	–	1	*exoS, exoT, exoY*
2297	–	–	1	*exoT, exoU, exoY*
2304	–	–	1	*exoS, exoT, exoY*
2305	–	–	1	*exoS, exoT, exoY*
2350	–	–	1	*exoS, exoT, exoU, exoY*
2351	–	1	–	*exoS, exoT, exoY*
2352	–	1	–	*exoS, exoT*
2353	–	1	–	*exoS, exoT, exoY*
2354	–	–	1	*exoS, exoT, exoY*
2355	–	1	–	*exoT, exoU*
2356	–	–	1	*exoT, exoU, exoY*

Table S2. Susceptibility levels of meropenem and ciprofloxatin, and amino acid alterations in the *gyrA*, *gyrB*, *parC*, *parE*, *mexR*, *nfxB*, *mexT* and *oprD* genes of 25 *P. æruginosa* isolates sequenced by Illumina platform.

Isolate	ST	C.c.[a]	MIC (μg/ml)[b]		Amino acid changes[c]							OprD[f]
			Mem	Cip	GyrA[d]	GyrB[e]	ParC	ParE	MexR	NfxB	MexT	
Pae–31448cz	ST111	VIM-2	8	>8	T83I, V671I, G860S, D892E, A900G, S903A, +S912, +E913	SB1	S87L, F254V, A346Q	T89I, I91T	V126E	E124A	L26V	Non-SC
Pae-31929cz	ST111	VIM-2	32	>8	T83I, V671I, G860S, D892E, A900G, S903A, +S912, +E913	SB1	S87L, F254V, A346Q	T89I, I91T	V126E	E124A	L26V	*94
Pae-29327cz	ST175	–	16	>8	T83I	SB1	S87W, L168Q, F254V, A346Q, P752T	T89I, I91T	–	E124A	L26V	*142
Pae-32301cz	ST233	IMP-7	16	>8	T83I, D652Y	SB1	S87L, F254V, A346Q	T89I, I91T	–	E124A	L26V	Non-SC
Pae-30094cz	ST235	GES-5	>32	>8	T83I	SB1	S87L, F254V, A346Q	T89I, I91T, D533E	V126E	E124A	L26V	*238
Pae-30653cz	ST235	GES-5	>32	>8	T83I	SB1	S87L, F254V, A346Q	T89I, I91T, D533E	V126E	E124A	L26V	*238
Pae-29931cz	ST235	–	8	>8	T83I	SB1	S87L, F254V, A346Q	T89I, I91T, D533E	K44M, V126E	E124A	L26V	*264
Pae-29785cz	ST253	VIM-2	32	0.125	+E909, +S910	SB1	F254V, A346Q	T89I, I91T, D533E	V126E	R21H, D56G, E124A	L26V	Non-SC
Pae-28606cz	ST357	VIM-2	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*142
Pae-29652cz	ST357	VIM-2	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*142
Pae-29480cz	ST357	IMP-7	>32	>8	T83I	SB1, E469D	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-29533cz	ST357	IMP-7	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-30039cz	ST357	IMP-7	>32	8	T83I	SB1, E469D	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-30351cz	ST357	IMP-7	>32	8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-30418cz	ST357	IMP-7	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-30652cz	ST357	IMP-7	16	>8	T83I	SB1, E469D	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	Non-SC
Pae-31092cz	ST357	IMP-7	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-31360cz	ST357	IMP-7	>32	>8	T83I	SB1, E469D	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-31897cz	ST357	IMP-7	16	8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-31912cz	ST357	IMP-7	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-31927cz	ST357	IMP-7	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-31975cz	ST357	IMP-7	>32	>8	T83I	SB1,E469 D	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-32048cz	ST357	IMP-7	>32	>8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-31025cz	ST357	–	>32	8	T83I	SB1	S87L, F254V, A346Q, P752T	T89I, I91T, D533E	V126E	E124A	L26V	*301
Pae-32183cz	ST446	IMP-1	>32	0.25	+E909, +S910	SB1	F254V, A346Q, A587T	T89I, I91T, D533E	V126E	E124A	L26V	*166

[a] C.c.,carbapenemase content.
[b] Mem, meropenem; Cip, ciprofloxacin.
[c] Sequences were compared to those under GenBank accession no. L29417 for *gyrA*, AB005881 for *gyrB*, AB003428 for *parC*, AB003429 for *parE*, U23763 for *mexR*, X65646 for *nfxB*, AJ007825 for *mexT*, and AE004091 for *oprD*.
[d] Symbol + indicate the duplication of the specific amino acids.
[e] SB1 indicate substitution of amino acids G151-S152-A153-V154-P155-T156-A157-R158-S159-G160-R161-R162 to V151-P152-Q153-F154-P155-L156-R157-E158-V159-G160-E161.
[f] The coding sequences for OprD proteins were examined for the presence of stop codods. Non-SC indicates the absence of a stop codon in the deduced protein sequences. Symbol * indicates the stop codon position in the deduced protein sequences.

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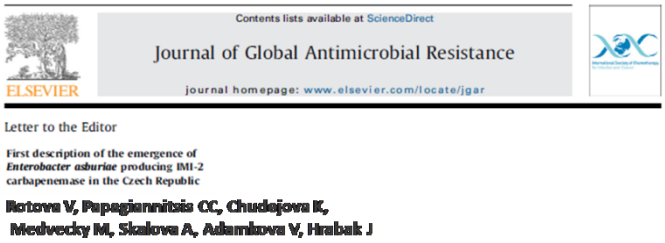
4.5 Publication no. 5: First description in Czech Republic of emergence of an *Enterobacter asburiæ* producing an IMI-2 carbapenemase



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IMI-2-producing *Enterobacter asburiæ*

The acquired class A carbapenemase IMI-1, was originally described in an *Enterobacter cloacæ* isolated in a Californian hospital in1984.[1] Since their first description, IMI-type carbabenemases have occasionally been detected in *Enterobacteriaceæ* from the USA, Europe, the Far East, and South Africa. Here we report a case of an IMI-2-producing *Enterobacter asburiæ* identified in the Czech Republic.

In 2016, *E. asburiæ* Easb-36567cz was recovered from a patient admitted to a Czech hospital. Easb-36567cz was isolated from a rectal swab during routine screening for carbapenemase-producing *Enterobacteriaceæ* (CPE). Easb-36567cz was resistant to aminopenicillins, aminopenicillin-sulbactam combinations, second-generation cephalosporins, aztreonam, carbapenems and colistin but susceptible to piperacillin-tazobactam, cefotaxime, ceftazidime, and various non-β-lactam antibiotics (Table 1). Carbapenemase production was hypothesized by a positive result in the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) imipenem hydrolysis assay.[2] Easb-36567cz tested negative by the ethylene diamine tetra-acetic acid (EDTA)-meropenem test, whilst the respective boronic acid-meropenem combined-disc test appeared positive indicating production of a class A carbapenemase. PCR and sequencing showed that Easb-36567cz carried $bla_{\text{IMI-2}}$.[3]

Conjugal transfer of $bla_{\text{IMI-2}}$ to rifampin-resistant *Escherichia coli* strain A15 was achieved by mating experiments in mixed-broth cultures, using rifampin (150 mg/L) and ampicillin (50 mg/L) as selective agents. The β-lactam resistance phenotype of Easb-36567cz was transferred (Table 1) at a frequency of

ca. 10^{-3} per donor cell. Transconjugants were confirmed to be IMI producers by PCR and MALDI-TOF MS imipenem hydrolysis assay.[2,3] bla_{IMI-2}-positive transconjugant exhibited resistance to amino-penicillins, aminopenicillin-sulbactam combinations, and imipenem, whilst it remained susceptible to the remaining antibiotics tested (Table 1). The plasmid location of the bla_{IMI-2} gene was demonstrated by S1 nuclease analysis of Easb-36567cz and its transconjugant, followed by hybridization with a digoxigenin-labelled bla_{IMI} probe. Plasmid analysis indicated transfer of a single plasmid (pEasb-36567cz) of ca. 80 kb that hybridized strongly with the bla_{IMI} probe (data not shown). Plasmid pEasb-36567cz was positive for the FII allele using the replicon typing method.

Plasmid DNA from the IMI-producing transconjugant was extracted using a Qiagen Large-construct kit (Qiagen, Hilden, Germany), and was sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA).Sequencing, assembling of the reads, filling of sequence gaps, and analysis of the plasmid sequence were performed as described previously.[4]

Table 1. Antimicrobial susceptibility of *E. asburiae* and the *E. coli* A15 transconjugant harboring the IMI-2-encoding plasmid pEasb-36567cz.

Isolate	MIC (mg/L) of[a]:																			
	Amp	Ams	Pip	Tzp	Cfz	Cxm	Ctx	Caz	Fep	Atm	Imp	Mem	Etp	Gen	Amk	Tet	Sxt	Cip	Col	Fos
E. asburiæ Eae-36567cz	>128	64	16	2	>16	64	0.25	0.5	1	4	>32	>16	>2	1	1	1	0.5	0.12	8	12
E. coli A15 pEasb-36567cz	128	16	8	≤1	>16	4	0.12	0.25	0.25	0.5	>32	1	0.25	0.5	1	1	0.5	≤0.06	0.25	2
E. coli A15 (recipient)	≤1	2	≤1	≤1	2	2	≤0.06	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.03	≤0.25	≤0.5	1	≤0.03	≤0.06	0.25	2

[a] Amp, ampicillin; Ams, ampicillin-sulbactam (inhibitor fixed at 4 mg/L); Pip, piperacillin; Tzp, piperacillin-tazobactam (inhibitor fixed at 4 mg/L); Cfz, cefazolin; Cxm, cefuroxime; Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Imp, imipenem; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Tet, tetracycline; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin; Col, colistin; Fos, fosfomycin.

Sequencing data showed that plasmid pEasb-36567cz is 77,824 bp in size. pEasb-36567cz showed a high degree of similarity to pJF-787 (99% coverage; 99% identity), previously characterized from *Klebsiella variicola* strain H152460787 (GenBank accession no. KX868552). The plasmid backbone was composed of regions responsible for replication (*repA* gene), conjugative transfer (*tra* and *trb* genes), and maintenance (*parAB*and *psiAB* operons, and *klcA* gene) of the plasmid (Figure S1).The LysR-type regulator gene (*imiR-2*) was found upstream ofbla_{IMI-2}. In their genetic environment, IS*Ec36* was detected upstream of*imiR-2*, whilst remnants of mobile elements IS*1*, Tn*3*-like and IS*Eae1* as well as an intact insertion sequence exhibiting 86% identity to IS*Sgsp1* (GenBank accession no. HE578057) were found downstream of bla_{IMI-2} (Figure S1). pEasb-36567cz carried no additional resistance genes.

To our knowledge, this is the first description of an IMI carbapenemase-producing *Enterobacteriaceæ* from the Czech Republic. Of note was that IMI-2-producing *E asburiæ* Easb-36567cz was isolated from a patient with no history of travelling abroad or previous hospitalization. This finding indicates the spread potential of carbapenemase genes via routes that remain largely unknown. The patient was isolated to avoid further spread of CPE and was successfully treated for his main disease.

IMI carbapenemases, together with closely related NMC-A β-lactamase, are mainly found in *Enterobacter* spp. and have remained overall uncommon in the clinical setting, unlike other class A

carbapenemase such as the KPC-type enzymes. Similarly to previous reports, the *bla*$_{IMI-2}$ gene was located in a conjugative plasmid and was linked to mobile elements.[3,5] These features may be involved in the future spread of this emerging resistance mechanism between different members of *Enterobacteriaceæ*. Therefore, accurate detection of IMI-producing *Enterobacteriaceæ*, which exhibit unusual antimicrobial resistance profiles, is of utmost importance since such bacteria can act as hidden sources of clinically important resistance determinants.

Funding

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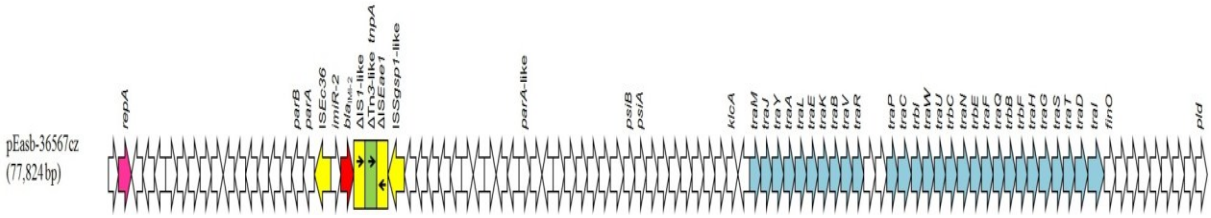

Figure S1. Linear map of the *bla*$_{IMI-2}$-carrying plasmid pEasb-36567cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements and transposases are shown in red, yellow and green, respectively. The *repA* genes is shaded pink, while *tra* and *trb* genes are shown in light blue. The remaining genes are shown in white.

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5 Discussion

Systematic surveillance and molecular-genetic analysis of carbapenemase-producing isolates are of particular worldwide importance. The works summarized in this dissertation thesis are focusing on molecular-epidemiological characterization of carbapenemase-positive clinical isolates detected in hospitals in the Czech Republic. Special attention is paid to variants of carbapenemases which are being rapidly disseminated throughout Czechia and in last years significantly contributed to the huge increase of incidence of carbapenemase-producing isolates in our country. Moreover, analysis of less frequent carbapenem-hydrolyzing enzymes, detected for the first time in the Czech Republic, may be a warning sign indicating further possible dissemination.

5.1 The dissemination of OXA-48-like-producing *Enterobacteriaceæ* in the Czech Republic

The OXA-48-like enzymes have recently rapidly spread throughout the world. The considerable increase of carbapenemase incidence in Czechia since 2015 is significantly associated with the spread of OXA-48 enzymes. Molecular analysis of all OXA-48-like-positive isolates obtained till the end of 2015 in Czech hospitals was performed.

Altogether, 26 non-repetitive isolates producing OXA-48-like carbapenemases were isolated from 20 patients, collected in 7 Czech hospitals located throughout the Czech Republic until 2015. Among them, 22 of the isolates were identified to be *K. pneumoniæ*, 3 *Escherichia coli*, and 1 *Enterobacter cloacæ*. Four of the patients were colonized or infected by two or three different species or strains of OXA-48-like producers. Five of the patients had recently travelled abroad, with one of them being involved in the initiation of an outbreak, which highlights the need of routine screening of patients repatriated from foreign countries. Three OXA-48-like isolates could be described as community acquired since the patients had no history of previous hospitalization or travel abroad. This fact is of a great concern, since it refers the possibility of dissemination in community with potentially devastating consequences on spread of the antibiotic resistance. In most affected hospital, an outbreak followed a long period with the sporadic occurrence of OXA-48 producers.

The sequence typing showed, that most of the sequence types (STs) found in isolates of *K. pneumoniæ* and *E. coli* have previously been associated with OXA-48-like-producing isolates from several geographical areas.[1,2,3,4,5] Additionally, most of $bla_{\text{OXA-48}}$-like-positive isolates were confirmed to coproduce the extended spectrum β-lactamase either alone, or along with another β-lactamases. Combination of resistance mechanisms creates isolates resistant to wide spectrum of antibiotics, which can have a serious clinical repercussion.

Three $bla_{\text{OXA-48}}$-type genes encoding the OXA-48, OXA-181, and OXA-232 enzymes were found. Twenty-four of the isolates were found to produce the OXA-48-β-lactamase, while one *K. pneumoniæ* isolate produced the OXA-181 enzyme and one remaining *K. pneumoniæ* isolate coproduced the OXA-232 and NDM-1 metallo-β-lactamase. Twenty four isolates were diagnosed to have the blaOXA-48-like genes located on plasmid, whereas one isolate of K. pneumonia and one isolate of E. coli harbored a plasmid fragment containing $bla_{\text{OXA-48}}$ flanked by IS*1R* elements integrated into their chromosomes.

Most of diagnosed *bla*$_{OXA-48}$-carrying plasmids were derivatives of the archetypal IncL *bla*$_{OXA-48}$-carrying plasmid pOXA-48, originally described in the *K. pneumoniæ* 11978 isolate recovered in Turkey in 2001 and then reported worldwide.[6] Nineteen out of the 22 sequenced *bla*$_{OXA-48}$-carrying plasmids showed high degrees of similarity to each other and to pE71T (100% coverage, 99% identity), previously characterized from *K. pneumoniæ* E71T isolated in Ireland.[7] Plasmid pE71T differed from pOXA-48 by the insertion of two copies of the IS*1R* element (the carbapenemase gene was part of the Tn*1999.2* transposon). Additionally, two novel pE71T derivatives were characterized from *K. pneumoniæ* isolates. The first one differed from pE71T by the insertion of a 1,911-bp fragment encoding a reverse transcriptase, the second one was a pE71T derivative carrying a novel variant of the Tn*1999.2* transposon (designated Tn*1999.5*).

On the other hand, the OXA-181 and OXA-232 carbapenemases were encoded by different types of plasmids belonging to IncX3, which to date has been described only in China and ColE2-type plasmid originally recovered from patients who was transferred from India to France in 2011.[8,9]

In four of the patients, two or three different OXA-48 producers were identified during their hospitalization, supposing the *in vivo* horizontal transfer of the *bla*$_{OXA-48}$-carrying plasmid. Sequencing data showed the presence of the same *bla*$_{OXA-48}$-carrying plasmid in three of these isolates, further confirming this hypothesis.

Results from whole genome sequencing showed that pOXA-48-like plasmids played a major role in the dissemination of the *bla*$_{OXA-48}$ gene in Czech hospitals, which is in agreement with the findings from previous studies reported worldwide.[6,7,10]

5.2 The first detection of GES-5-producing *Enterobacter cloacæ* in the Czech Republic

The GES enzymes, quite rare until now, are currently increasingly reported from distinct geographical areas. Especially, GES-5 variant is frequently reported from many European countries.[11,12]

The first case of GES-5-producing *E. cloacæ* identified in the Czech Republic in 2016 was described. The ST252 *E. cloacæ* was obtained from a chronically ill patient with anamnesis of repeated hospitalizations, but with no history of traveling abroad. Mentioned sequence type was previously described in association with KPC-producing *E. cloacæ* nosocomial strains in the United States.[13] The *bla*$_{GES-5}$ gene was the first gene cassette of a novel class 1 integron, In1406, located on a plasmid of approximately 7kb in size (pEcl-35771cz). Moreover, a novel allele of the *aadA15* gene cassette was identified. Interestingly, In1406 was found to be inserted in a sequence (3687kb) sharing common genetic features with the backbone of several recently described plasmids, assigned to ColE-type complex, associated with the dissemination of OXA-48-like genes (OXA-181, OXA-232).[14-16] The finding of GES-enzymes located on ColE-type plasmids is of particular interest, since there exists a possible threat of their rapid dissemination, as was exemplified in case of OXA-48-like enzymes.

5.3 The dissemination of NDM-like producing *Enterobacteriaceæ* in the Czech Republic

An alarming increase of NDM-carbapenemases was detected in the Czech Republic from 2016. The rapid spread of NDM-producers is largely involved in the overall continuous increase of carbapenemase-producing strains in the Czech Republic, observed since 2015.

Eighteen non-repetitive isolates producing NDM-like carbapenemases were isolated from 15 patients, collected in 5 Czech hospitals located in 3 Czech cities, in 2016. Among them, 10 of the isolates were identified to belong to *E. cloacæ* complex, 4 isolates were determined as *E. coli*, while the remaining isolates were identified as *Kluyvera intermedia*, *K. pneumoniæ*, *K. oxytoca*, and *Raoultella ornithinelytica*. Moreover, the NDM-4-producing isolate of *E. cloace* complex, obtained in the Czech Republic in 2012 from a patient repatriated from Sri Lanka, was added for comparative reasons. Notably, most of the NDM-like-positive isolates coproduce ESBL enzymes.

An outbreak which took place in hospital B1, counting 10 affected patients, was included in the study. None of these patients had recently traveled abroad. Additionally, two patients carrying NDM-like-producers were reported in hospital B2. Remaining isolates were obtained from three different hospitals. One patient with a sporadic NDM-like-producing isolate was directly repatriated from China. Three patients were colonized or infected by two different NDM-like producers.

Twelve of the isolates (ten of *E. cloacæ* complex, one *K. intermedia*, one *E. coli*), all of which were recovered from the outbreak in hospital B1, were identified as NDM-4-positive. The vast majority of the NDM-4-producing *E. cloacæ* complex isolates (except one *E. asburiæ* strain) belonged to ST182. Additionally, the isolate of *E. cloacæ* complex obtained in 2012 also belong to ST182 and harbored $bla_{\text{NDM-4}}$ gene. Interestingly, isolates of NDM-1-producing *E. cloacæ* complex ST182 were previously reported from Mexico.[17] Plasmids harboring $bla_{\text{NDM-4}}$ were positive for the IncX3 allele, as well as the *E. cloacæ* complex isolate, from 2012.

The three isolates (two *E. coli*, one *K. oxytoca*), all of them recovered from hospital B2, were found to produce NDM-5 enzyme. The $bla_{\text{NDM-5}}$ genes were localized on IncX3 plasmids. Two *E. coli* isolates, obtained in hospital B2, belonged to ST167. Notably, ST167 *E. coli* have been repeatedly reported in association with NDM-5-producing isolates from China.[18,19] The *K. oxytoca* isolate was classified as ST2, belonging to a growing international clonal complex.[20]

The three remaining isolates (*E. coli*, *K. pneumoniæ*, *R. ornithinolytica*), identified as sporadic cases in three different hospitals, expressed NDM-1 carbapenemase. NDM-1-positive isolates harbored plasmids of different sizes (approximately 55kb, 150kb, and 300kb). The $bla_{\text{NDM-1}}$ gene of *R. ornithinolytica* isolate was localized on IncX3 plasmid of approximately 55kb. Moreover, the plasmid harbored an additional sequence encoding SHV-12 enzyme, similar to SHV-12-encoding region previously described in *K. pneumoniæ* isolate from China (GeneBank accession no. KU314941). The $bla_{\text{NDM-1}}$-positive plasmid of approximately 300kb in size, detected in *E. coli* isolate, was identified as a multireplicon A/C_2+R, constitute a fusion derivative of sequences of different origin. Plasmid pKpn-35963cz of approximately 150kb, recovered from *K. pneumoniæ* isolate which was not typable by the PCR-based replicon typing

(PBRT) scheme, was identified as IncFIB(K) molecule containing an acquired sequence.[21] Plasmid pKpn-35963cz composed of two distinct parts: a contiguous plasmid backbone (of approximately 115kb) and an acquired sequence (of approximately 45kb). The plasmid backbone showed similarities with the respective regions of plasmid p1605752FIB reported from a pan-resistant *K. pneumoniæ* strain from the United States (GenBank accession no. CP022125). The acquired sequence contained a segment encoding the NDM-1 enzyme which was highly similar to the mosaic region of pS-3002cz previously described from a clinical isolate of NDM-1-positive *K. pneumoniæ* reported in the Czech Republic.[22]

All IncX3 bla_{NDM}-like-carrying plasmids exhibited a high similarity to each other and to previously described NDM-like encoding IncX3 plasmids reported worldwide.[19,23] Two plasmids harboring bla_{NDM-5} genes recovered from hospital B2 were almost identical (differing in few SNPs) to previously described NDM-5-carrying plasmid recovered from Chinese isolate *E. coli* ST167.[19] Compared to other IncX3 NDM-encoding plasmids, all isolates expressing the bla_{NDM-4} gene differed by the insertion of a Tn*3*-like transposon. Notably, the plasmid of *E. cloacæ* complex isolate obtained in hospital B1 in 2012 was identical to bla_{NDM-4}-carrying plasmids recovered during 2016. Nevertheless, comparative genome analysis identified the presence of four insertion sequences in the genome of *E. cloacæ* complex isolate from 2012, while these insertions were not detected in isolates obtained during 2016, suggesting a second insertion event of NDM-4-producing *E. cloacæ* complex isolates in the Czech Republic.

Three of the patients were identified to possess two different NDM-producing strains, indicating a horizontal gene transfer of the bla_{NDM}-like genes. Sequencing and PCR data showed the presence of the same bla_{NDM-4}- and bla_{NDM-5}-carrying plasmid in these isolates, further confirming this hypothesis.

The study confirms that IncX3 plasmids play a major role in the spread of NDM-like enzymes in the Czech Republic. The increasing incidence of NDM-positive isolates in Czechia, which is in concordance with the worldwide situation, is of particular interest since the dissemination via horizontal gene transfer seems to be extremely successful.

5.4 The first nationwide surveillance of carbapenemase-producing *Pseudomonas æruginosa* isolates in the Czech Republic

Carbapenemase-positive *P. æruginosa* isolates are frequently reported among carbapenamse-producing Gram-negative bacteria in our geographical area. First nationwide surveillance of carbapenemase-positive *P. æruginosa* isolates detected during 2015 throughout the hospitals in the Czech Republic was performed, including deep molecular genetic typing. Altogether, 136 carbapenem-positive isolates were reported from 22 Czech hospitals. Moreover, 58 carbapenem-resistant *P. æruginosa* isolates without carbapenemase activity and 93 representative meropenem-susceptible *P. æruginosa* isolates were included in the study for comparative reasons.

Overall, 132 *P. æruginosa* MBL-producers were identified during 2015 in Czech hospitals. The bla_{IMP} genes were found in 117 isolates, including 116 IMP-7- and 1 IMP-1-producers, whereas 15 isolates harbored the bla_{VIM-2} gene. Moreover, 4 remaining isolates were found to be GES-producers. Data from molecular sequence typing showed that ST357 was the most prevalent sequence type, accounting for 120

MBL-producers. The majority of ST357 isolates was connected with IMP-7 production (n = 115), whereas only 5 VIM-positive isolates belonged to ST357. Thirteen of the isolates were distributed in ST111, including 9 VIM-2 producers, and ST235 including all GES-5-positive isolates. The three remaining isolates belonged to different STs. Nevertheless, significant genetic diversity was found in all the isolates without carbapenemase activity. The group of 58 carbapenem-resistant isolates without carbapenemase-production comprised 29 clones, with ST175 and ST235 accounting for 22 isolates. In the group of 93 meropenem-susceptible isolates 58 different STs were identified. Interestingly, 11 isolates without carbapenemase activity was assigned to novel STs.

Notably, regional spread of *P. æruginosa* ST357 producing IMP-7 MBL in Central Europe was reported by Hrabák et al. in 2011.[24] Study focusing on characterization of carbapenemase-producing isolates of *P. æruginosa* from 2009 to 2011 isolated in Brno (Czech Republic) classified the VIM-2- and IMP-7-producing isolates as ST111 and ST357.[25] Interestingly, ST357 IMP-1-producing *P. æruginosa* isolates were previously reported from Japan.[26] And moreover, study performed by Woodford et al. focusing on widespread clonal spread multiresistant Gram-negative bacteria classified ST235 between high-risk clones.[27] The high-risk clones (STs 357 and 235) were not found among meropenem-susceptible isolates included in this study.

The bla_{IMP}-like genes were found in three main types of class 1 integron. The most prevalent were In-p110-like integrons, identified in 115 ST357 carbapenemase-positive isolates. The In-p110 integron, previously described by Papagiannitsis et al., was detected in 94 ST357 isolates, whereas 21 remaining ST357 IMP-7 producers harbored an In-p110 derivative.[25] Moreover, two new integron types, designated In1393 and In1392, were reported among IMP-7 producers of ST233 and ST446. The most common bla_{VIM}-carrying integron was a derivative of an I59 integron, previously characterized from French isolates, which was found among all ST111 VIM-2-producing isolates.[28] Among ST357 VIM-producers, the class 1 integron In56, previously described in clinical isolates from France, was identified.[29] In ST253 VIM-2 producers, a new integron In1391 was described. The ST235 GES-positive isolates were identified to harbor In717 integron, previously described by authors from Australia.[30]

The whole genome sequencing data confirmed a chromosomal location of carbapenemase-encoding integrons of *P. æruginosa* isolates. In all sequenced ST357 isolates, the In-p110-like integrons were associated with a Tn*4380*-like tansposon inserted in LESGI-3 genomic island, located in the same position of *P. æruginosa* chromosome. The LESGI-3 was previously described from an epidemic strain of *P. æruginosa* detected in the UK.[31] Moreover, the association with Tn*4380*-like transposon was identified for In1392 and In717 transposons. The In1393 showed amino acid sequence similarities with transposition proteins previously found in *P. æruginosa* strain described by Hungarian authors (GeneBank accession no. AZSV01000008). In both ST357 VIM-2-producing strains, the integron In*56* was associated with Tn3-like transposon located in a novel genomic island, designated PAGI-56. Genomic islands closely related to PAGI-56 have been previously reported in *P. æruginosa* isolates from the USA and Japan (GenBank accession no. AP017302, CP008870). The In59-like integron identified in two ST111 VIM-2-producing

isolates was inserted in a Tn*5060*-like transposon. Interestingly, the Tn*5060* transposon has been previously reported from *P. æruginosa* strain isolated from Siberian permafrost.[32] The In*1391* integron was embedded in Tn*6162*-like element, which is a Tn*1403*-related transposon.[33]

A particularly important fact was showed by the phylogenetic analysis, indicating that IMP-7-producing ST357 *P. æruginosa* isolates, recovered from different hospitals throughout the Czech Republic, were closely related. These finding further supported the hypothesis regarding clonal spread of ST357 IMP-producing high-risk clone among Czech hospitals. Moreover, analysis of whole-genome sequencing data revealed the presence of additional resistance genes in majority of the isolates. Additionally, all *P. æruginosa* isolates were examined for the presence of ExoS, ExoT, ExoU, and ExoY toxin-encoding genes. Notable, all ST357 IMP-7-producers possess the virulence genes *exoT*, *exoU*, and *exoY* which have been previously found in association with a higher risk of mortality among patients infected by isolates expressing these type III secretory proteins.[34] Moreover, *exoU* gene expression was found to correlates with acute cytotoxicity and epithelial injury.[35]

The study highlighted the importance of dissemination of high-risk clones in the Czech Republic such as ST111, 175, 235, and 357. Furthermore, the sporadic emergence of *P. æruginosa* isolates expressing VIM-2, GES-5, and IMP-1 carbapenemases associated with divergent or novel integron structures emphasize the ongoing evolution of such bacterial isolates, further aggravating the situation.

5.5 The first detection of IMI-2-producing *Enterobacter asburiæ* in the Czech Reublic

IMI carbapenemases, are predominantly found in *Enterobacter* sp. and have remained overall uncommon in clinical settings. However, the first detected cases have already occurred in our country.

The first case of IMI-2-producing *E. asburiæ* identified in the Czech Republic in 2016 was described. The isolate was obtained from a patient with no history of traveling abroad or previous hospitalization. Plasmid analysis identified an IncFII plasmid of an approximately 80kb harboring $bla_{\text{IMI-2}}$ gene. Sequencing data found a high similarity to pJF-787 plasmid, previously characterized from *Klebsiella variicola* strain by authors from the UK (GenBank accession no. KX868552). The LysR-type regulatory gene (*imiR-2*) was localized upstream of the $bla_{\text{IMI-2}}$ gene. In their genetic environment, IS*Ec36* was found upstream of the LysR-type gene, while remaining mobile elements IS*1*, Tn-3-like transposase, IS*Eae1*, and an intact IS highly similar (86%) to IS*Sgsp1* previously described from IncX3 plasmid of *Shigella sp.* in Denmark (GeneBank accession no. HE578057) were detected downstream of the $bla_{\text{IMI-2}}$ gene.

No possible source of IMI-2-positive acquisition was identified, indicating a possible partial silent spread of carbapenemase genes via unknown routes, constituting an alarming public threat. Even though the occurrence of IMI-2 enzymes remain rare, the finding of the $bla_{\text{IMI-2}}$ gene on conjugative plasmid linked to mobile genetic elements is highlighting the emergence of possible future spread.

6 Conclusion

The spread of carbapenem-resistant Gram-negative bacteria in the hospital settings is nowdays one of the most serious health-care problems. Large expansion of carbapenemase-encoding genes throughout the world can be, to a large extent, explained by a frequent association with mobile genetic elements. Nevertheless, the clonal spread of high-risk clones also plays a fundamental role in ongoing worldwide dissemination. Both of these ways have been proven to participate in the successful spread of carbapenemase-producers in the Czech Republic. Moreover, many carbapenemase-positive isolates possess other unrelated mechanisms of antibiotic resistance, as was clearly exemplified in presented studies. Therefore, selection pressure can be caused not only by β-lactams but even structurally unrelated antimicrobial agents may contribute to their dissemination.

Of particular interest are the cases without possible ascertainable links of infection/colonization by carbapenemase-producers, which may represent community or autochtonous acquisition. Although the spread of carbapenem-resistant Gram-negative bacteria mainly occurs among hospitalized patients, isolates obtained from community settings (especially OXA-48- and NDM-producers) are increasingly reported from different European countries.[36,37] The collection of carbapenemase-producers included in this dissertation thesis comprises several isolates, obtained from patients without previous hospitalization or traveling abroad, lacking the traceable source of origin which indicates a dissemination via unrecognized ways.

Early identification of carbapenemase-producing bacteria in clinical infections or ideally even in the carriage stage, is mandatory to prevent the development of nosocomial outbreaks. Notably, similar strategy has been successfully implemented in northern European countries for hospital-acquired methicillin-resistant *Staphylococcus aureus*.[38]

Multidrug-resistant bacterial strains constitute a huge clinical and economic burden enormously affecting the outcome of infected patients.[39] Subsequently, there is an urgent need for new antimicrobial agents. Novel approaches in pharmaceutical research such as overcoming the intrinsic resistance of Gram-negative bacteria by designing glycopeptides analogues which can permeate the outer membrane can leads to interesting therapeutic options.[40] Notably, World Health Organization (WHO) has recently published a *Global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics*, classifying carbapenem-resistant *Enterobacteriaceæ*, *Pseudomonas æruginosa* and *Acinetobacter baumannii* on the first position.[41] Worryingly, a national survey of infectious disease specialists in 2011, performed by Infectious disease Society of America (IDSA) Emerging Infections Network, showed that more than 60% of participants had reported a pan-resistant isolate within a prior year.[42]

A well-concerted worldwide effort is crucial to prevent the further dissemination of carbapenemase-producing Gram-negative bacteria and thus to avoid an uncontrollable situation as is now observed for ESBL producers.

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7 Attachements

7.1 Curriculum vitæ

Name Maiden name	Anna Šrámková, MUDr. (M.D.) Skálová
Date & Place of Birth	7th April, 1989, Stod, Czech Republic
Address	Studentská 2089/69, 323 00, Plzeň
Sex	Woman
Present Position	Since 2014 Ph.D. student – Department of Clinical Microbiology, Biomedical Center, Faculty of Medicine in Pilsen, Charles University Since 2015 Doctor of medicine – Department of Clinical Microbiology, University Hospital in Pilsen
Work Address and Contacts	Alej Svobody 80, 323 00, Plzeň, Czech Republic
Education	2008–2014 Faculty of Medicine in Pilsen, Charles University; specialization: general medicine 2000–2008 grammar school, Gymnázium Mikulášské náměstí 23, Plzeň
Language Skills	Czech – mother tongue English – advanced German – basic
Internship	Department of Clinical and Experimental Medicine, Careggi University Hospital, Florence, Italy (2016, 1 month)
Technical Skills	Microbiology: Experienced in Microbiology techniques. Isolation, identification and characterization of bacteria (mainly *Enterobacteriaceæ*, *Pseudomonas* spp.); experienced in Clinical microbiology, especially in bacteriology and molecular-genetic diagnostics, molecular epidemiology. Molecular Biology: Experienced in molecular biology techniques, e.g., real time quantitative PCR, RT-PCR and related molecular biology works based on DNA amplification, DNA sequencing, southern blotting, hybridization, transformation, conjugation, PFGE, MLST. Protein Analysis: Isoelectric focusing, MALDI-TOF mass spectrometry techniques.
H-Index	3 (9.6.2018, Scopus)
Sum of Times Cited without Self-Citations	24 (9.6.2018, Scopus)
Number of Publications in Journals with IF	10
Publications	See list of publications

Professional experiences	Posters (Abstracts in Proceedings): European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2018 • Poster – Characterization of NDM-like-producing *Enterobacteriaceæ* isolated in Czech hospitals (Rotova V., Bitar I., Medvecky M., Skalova A., Chudejova K., Jakubu V., Bergerova T., Zemlickova H., Papagiannitsis CC, Hrabak J.) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2017 • ePoster – Evaluation and validation of HRC assay for detection and confirmation of carbapenemases in *Enterobacteriaceæ* (Rotova V., Skalova A., Chudejova K., Papagiannitsis C. C., Hrabak J.) • Poster – Molecular characterization of MBL-producing *Pseudomonas æruginosa* isolates in Czech hospitals (Papagiannitsis C. C., Chudejova K., Medvecky M., Skalova A., Rotova V., Jakubu V., Zemlickova H., Hrabak J.) • Poster – Automatic deposition of bacteria and yeast on MALDI target using MALDI Colonyst robot (Hrabak J., Chudejova K., Rotova V., Papagiannitsis C. C., Bohac M., Skalova A., Bergerova T.) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2016 • ePoster – Molecular epidemiological analysis of OXA-48 producing *Enterobacteriaceæ* in the Czech Republic with an evidence of horizontal gene transfer (Skalova A., Chudejova K., Rotova V., Bergerova T., Jakubu V., Zemlickova H., Papagiannitsis C. C., Hrabak J.) • poster – Complete nucleotide sequences of three IncA/C2-type plasmids carrying In416-like integrons with bla_{VIM} genes from *Enterobacteriaceæ* isolates of Greek origin (Papagiannitsis C. C., Dolejska M., Izdebski R., Giakkoupi P., Skalova A., Chudejova K., Dobiasova H., Vatopoulos A., Derde L. P. G., Bonten M. J., Gniadkowski M., Hrabak J.)

7.2 List of publications

Publications	Impact factor (Web of science)	Citations (Web of Science, 9.6.2018)	Citations (Scopus, 9.6.2018)
Papagiannitsis CC, Dolejska M, Izdebski R, Giakkoupi P, Skalova A, Chudejova K, Dobiasova H, Vatopoulos AC, Derde LP, Bonten MJ, Gniadkowski M. Characterisation of IncA/C2 plasmids carrying an In416-like integron with the blaVIM-19 gene from Klebsiella pneumoniæ ST383 of Greek origin. International Journal of Antimicrobial Agents. 2016 Feb 1;47(2):158-62.	4.307	6	6
Skalova A, Chudejova K, Rotova V, Medvecky M, Studentova V, Chudackova E, Lavicka P, Bergerova T, Jakubu V, Zemlickova H, Papagiannitsis CC, Hrabak J. Molecular characterization of OXA-48-like-producing Enterobacteriaceæ in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids. Antimicrobial Agents and Chemotherapy. 2017 Feb 1;61(2):e01889-16.	4.302	7	11
Rotova V, Papagiannitsis CC, Skalova A, Chudejova K, Hrabak J. Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity. Journal of Microbiological Methods. 2017 Jun 1;137:30-3.	1.79	6	7
Chudejova K, Bohac M, Skalova A, Rotova V, Papagiannitsis CC, Hanzlickova J, Bergerova T, Hrabak J. Validation of a novel automatic deposition of bacteria and yeasts on MALDI target for MALDI-TOF MS-based identification using MALDI Colonyst robot. PloS one. 2017 Dec 29;12(12):e0190038.	2.806	0	0
Papagiannitsis CC, Medvecky M, Chudejova K, Skalova A, Rotova V, Spanelova P, Jakubu V, Zemlickova H, Hrabak J. Molecular Characterization of Carbapenemase-Producing Pseudomonas æruginosa of Czech Origin and Evidence for Clonal Spread of Extensively Resistant Sequence Type 357 Expressing IMP-7 Metallo-β-Lactamase. Antimicrobial Agents and Chemotherapy. 2017 Dec 1;61(12):e01811-17.	4.302	0	0
Rotova V, Papagiannitsis CC, Chudejova K, Medvecky M, Skalova A, Adamkova V, Hrabak J. First description of the emergence of Enterobacter asburiæ producing IMI-2 carbapenemase in the Czech Republic. Journal of Global Antimicrobial Resistance. 2017 Oct 10;11:98.	1.276	0	0
Chudejova K, Rotova V, Skalova A, Medvecky M, Adamkova V, Papagiannitsis CC, Hrabak J. Emergence of sequence type 252 Enterobacter cloacæ producing GES-5 carbapenemase in a Czech hospital. Diagnostic Microbiology and Infectious Disease. 2018 Feb 1;90(2):148-50.	2.401	0	0
Chalupova M, Skalova A, Hajek T, Geigerova L, Kralova D, Liska P, Hecova H, Molacek J, Hrabak J. Bacterial DNA detected on pathologically changed heart valves using 16S rRNA gene amplification. Folia Microbiologica. 2018 May 22:1-5.	1.521	–	0
Jamborova I , Johnston B, Papousek I, Kachlikova K, Micenkova L, Clabots C, Skalova A, Chudejova K, Dolejska M, Literak I, Johnson JR. Extensive genetic commonality among wildlife, wastewater, community, and nosocomial isolates of *Escherichia coli* sequence type 131 (*H*30R1 and *H*30Rx Subclones) that carry $bla_{\text{CTX-M-27}}$ or $bla_{\text{CTX-M-15}}$. Antimicrobial Agents and Chemotherapy. 2018; Article in press.	4.302	–	–
Paskova V, Medvecky M, Skalova A, Chudejova K, Bitar I, Jakubu V, Bergerova T, Zemlickova H, Papagiannitsis CC, Hrabak J. Characterization of NDM-encoding plasmids from Enterobacteriaceæ recovered from Czech hospitals. Frontiers in Microbiology.2018; Article in press.	4.076	–	–