CHARLES UNIVERSITY
Faculty of medicine in Pilsen
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THESIS

Microbial genomics for population studies of multiresistant gram-negative bacteria

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Declaration

It hereby declare that this thesis is the result of my own independent research and has not been submitted, in whole or in part, for any degree or qualification at any other institution. All of the work contained in this thesis is entirely my own, with the exception of particular references or acknowledgments to the contributions of others. I accept full responsibility for the content and confirm that any external sources or assistance were properly mentioned and credited.

In Pilsen, 19.08.2024

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Marc Finianos

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Abstract

Carbapenemase producing Enterobacterales (CPE) has become a global health burden, they are mostly associated with hospital acquired infections, thus limiting treatment options given that they confer resistance to multiple antibiotics. In many situations, resistance is manifested by enzymes spreading between species and clones, facilitated by plasmids and other mobile genetic elements. Thus, the aim is to characterize the mechanism of the carbapenem resistance as well as the mechanisms of dissemination.

Methodology involves the identification of the bacterial species, identification of the genes encoding for the carbapenemase enzymes, Short-reads sequencing. This is followed up by Long-reads sequencing to obtain a complete genome with circular plasmids facilitating the analysis of the plasmids and mobile genetic elements behind the possible dissemination of the resistance genes. And finally further analysis on the detected outbreak such as SNPs analysis, phylogenetic tree with genomes available from the database.

Results have shown that the dissemination of some genes, for example *bla*$_{\text{KPC}}$ was linked to plasmid dissemination and this was due to the conjugative nature of the plasmids in question. Furthermore, For the detected *bla*$_{\text{VIM}}$ in Czech Republic it was found that the dissemination was in majority linked with the integron In110, and also it was found that plasmids helped this dissemination. On the other hand, the detected *bla*$_{\text{GES}}$ isolates were different, they highlighted a persistence of plasmids in silent reservoirs and their ongoing evolution and also found that the bacterial isolate was also able to assemble plasmids from environmental sources. Finally, *bla*$_{\text{NDM}}$ isolates detected in Lebanon, were found to be spreading with multiple clones and plasmids.

In conclusion, this emphasizes the need for implementing infection control measures to prevent transmission, as well as performing in-depth analysis to discover new patterns in plasmid evolution and other mobile genetic elements.

Abstrakt

Karbapenemázy produkující Enterobacterales (CPE) se staly globálním zdravotním problémem, zejména kvůli jejich spojení s infekcemi získanými v nemocnicích, což významně omezuje možnosti léčby, neboť tyto bakterie vykazují rezistenci vůči mnoha antibiotikům. Ve většině případů se tato rezistence šíří enzymy mezi druhy a klony, přičemž zásadní roli v tomto procesu hrají plazmidy a další mobilní genetické prvky. Cílem této práce je proto charakterizovat mechanismus rezistence vůči karbapenemům a identifikovat mechanismy jejího šíření.

Použitá metodologie zahrnuje identifikaci bakteriálních druhů a genů kódujících karbapenemázové enzymy, sekvenování krátkých úseků DNA a následné sekvenování dlouhých úseků DNA, které poskytuje kompletní genom s kruhovými plazmidy. To umožňuje detailní analýzu plazmidů a mobilních genetických prvků podílejících se na šíření genů rezistence. Další analýzy zahrnují studium SNPs, tvorbu fylogenetických stromů s genomy dostupnými v databázích a další.

Výsledky ukázaly, že šíření některých genů, jako je blaKPC, bylo spojeno s přenosem plazmidů, což je dáno jejich konjugativní povahou. Dále bylo zjištěno, že šíření blaVIM v České republice bylo převážně spojeno s integronem In110, přičemž plazmidy také přispívaly k jeho šíření. Naopak u izolátů s genem blaGES bylo zjištěno, že plazmidy přetrvávají v tichých rezervoárech a pokračují v evoluci, a také se zjistilo, že bakteriální izolát byl schopen asimilovat plazmidy z okolního prostředí. U izolátů blaNDM, detekovaných v Libanonu, bylo zjištěno, že se šíří prostřednictvím více klonů a plazmidů.

Závěrem lze říci, že výsledky zdůrazňují potřebu zavedení účinných opatření pro kontrolu infekcí k zabránění jejich šíření, stejně jako nutnost provádění hloubkových analýz, které mohou odhalit nové vzorce evoluce plazmidů a dalších mobilních genetických prvků.

List of Abbreviations

Abbreviation	Meaning
AIM	Adelaide imipenemase
AMR	Anti-Microbial Resistance
ANI	Average nucleotide identity
CAUTI	catheter-associated urinary tract infections
CDC	Center for Disease Control
CG	Clonal Group
CLABSI	Central line-associated blood-stream infections
CPE	Carbapenemase Producing Enterobacterales
ECDC	European Center for Disease Control
EDTA	ethylenediaminetetraacetic acid
ESBLs	Extended Spectrum β-Lactamases
GBD	Global Disease Burden
GES	Guiana extended-spectrum
GIM	Germany Imipenemase
Gly	Glycine
HAI	Health-care Associated Infection
HGT	Horizontal Gene Transfer
IMP	Transferable imipenem resistance
KPC	Klebsiella pneumoniae carbapenemase
L3	Loop 3
MBLs	Metallo-β-lactamases
MENA	Middle-east and North Africa
MGE	Mobile Genetic Elements
MLST	Multi-locus sequence typing
MRSA	Methicillin resistant Staphylococcus aureus
NDM	New Delhi Metallo-β-Lactamase
NGS	Next generation sequencing
NTEKPC	non-Tn4401 elements KPC
OMP	Outer Membrane Proteins
ONT	Oxford nanopore technology
OXA	Oxacillinases
PacBio	Pacific Bioscience
PBPs	Penicillin-Binding Proteins
PGAP	prokaryotic genome annotation pipeline
Ser	Serine
SFH	Serratia fonticola carbapenem
SNP	Single nucleotide polymorphism
SSI	Surgical Site Infections
VIM	Verona Integron-encoded Metallo-β-Lactamase
WGS	Whole Genome Sequencing

Summary

1 Introduction

1.1 Enterobacterales:

Enterobacterales are the most reported group of bacteria that possesses the biggest clinical impact on the medical and public health fields. This group is a major causative agent of foodborne disease and outbreaks. According to WHO estimates from the Global Disease Burden (GBD), of the 2.39 billion cases of diarrheal diseases in 2015, 550 to 600 million were linked to foodborne infections [1, 2]. Also, given that 70% of diarrhea cases are food-related [3], this gives an estimation that 350 to 420 million cases of gastroenteritis are of bacterial origin [1, 2]. The representatives of foodborne pathogens in the family of Enterobacterales include but are not limited to; *Salmonella*, *Escherichia coli*, *Shigella*, and *Yersinia enterocolitica* [1, 3-5]. Major outbreaks have been reported worldwide like *Shigella* contaminated basil and bagged salad contaminated with *Y. enterocolitica* [4]. In 2011 a major outbreak was reported in Germany, *E. coli* O104:H4 was the pathogen responsible for this outbreak and was linked to sprout consumption [4].

In healthcare settings, European Center for Disease Control (ECDC) estimations showed that in Europe there are around 4.5 million infections (www.ecdc.europa.eu/en/publications-data/infographic-healthcare-associated-infections-threat-patient-safety-europe), of which 37000 cases resulted in death yearly (www.who.int/publications/i/item/report-on-the-burden-of-endemic-health-care-associated-infection-worldwide), and these cases were linked to healthcare-associated infections (HAI). Also, in the United States, 99000 cases of deaths were linked to HAI in 2004 (www.who.int/publications/i/item/report-on-the-burden-of-endemic-health-care-associated-infection-worldwide).

The Enterobacterales family was reported by Center for Disease Control (CDC) as being responsible for 3 out of 4 causes of HAI, these causes are Central line-associated blood-stream infections (CLABSI), catheter-associated urinary tract infections (CAUTI), and surgical site infections (SSI) (www.cdc.gov/hai/index.html).

Furthermore, the complications associated with Enterobacterales are not limited to HAI or foodborne infections, but in the past 10–15 years, a trend of increased antibiotic resistance has been reported especially against carbapenems, which drastically limited the use of life-saving antibiotics [6]. In order to assess the global burden of Anti-microbial resistance (AMR), a study collected data from 204 countries from 1990-2019. Data showed that in 2019 the Australasian continent had the lowest frequency with 6.5 deaths per 100000 attributed to AMR and 28/100 000 deaths associated with AMR [7]. On the other hand, the Western sub-Saharan region having the highest incidences of death attributable to AMR with a frequency of 27.3/100 000 and 114.8/100 000 deaths associated with AMR [7]. Moreover, in 2019 it was found that six pathogens (*E. coli*, *K. pneumoniae*, *Acinetobacter baumanii*, *Staphylococcus aureus, Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*) were responsible for 929 000 of 1.27 million deaths attributable to AMR and 3.57 million deaths of 4.95 million deaths associated with AMR (figure 1) [7].

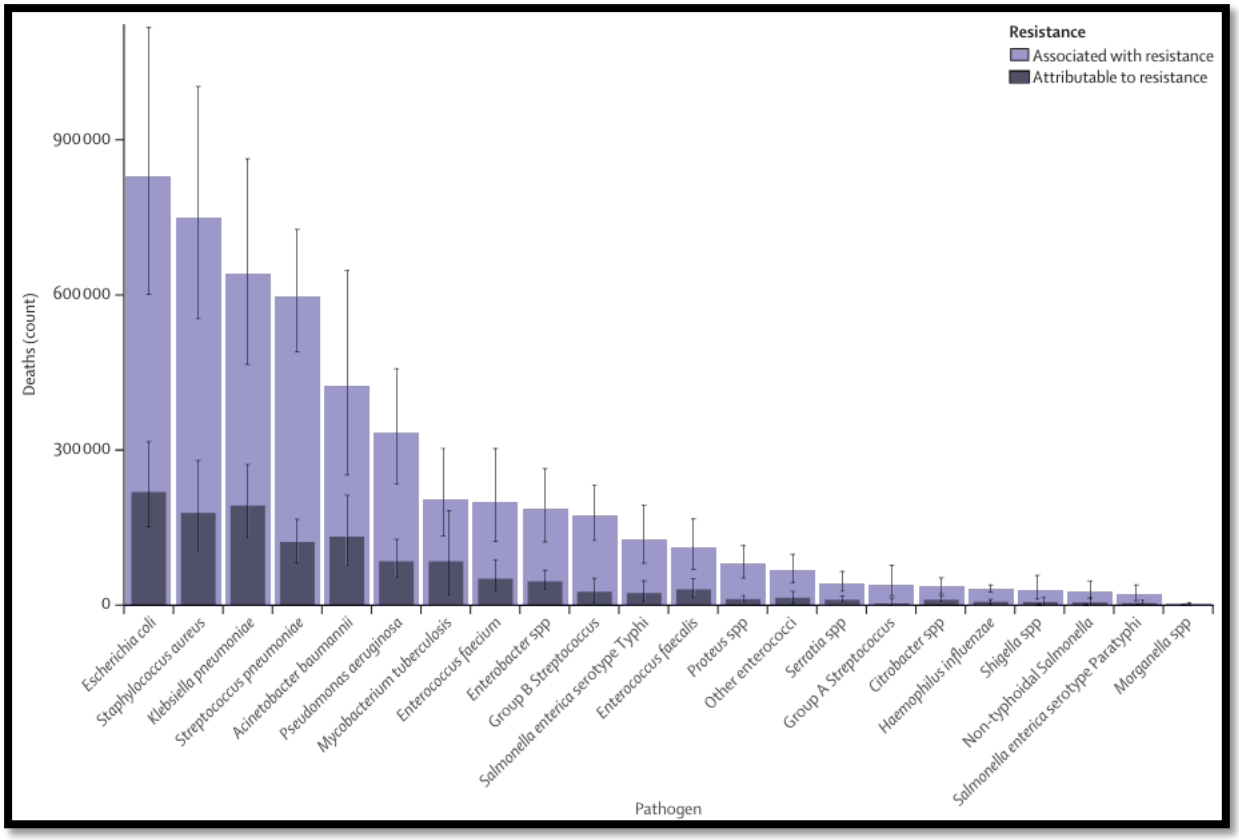

Figure 1: Pathogen related Global Death counts associated with and attributable to AMR [7].

Additionally, *E. coli*, *K. pneumoniae* and *S. aureus* were behind almost half of the deaths in both cases (death Attributable to AMR, and Death associated with AMR) generally in Central and Eastern Europe, and central Asia (Figure 2) [7].

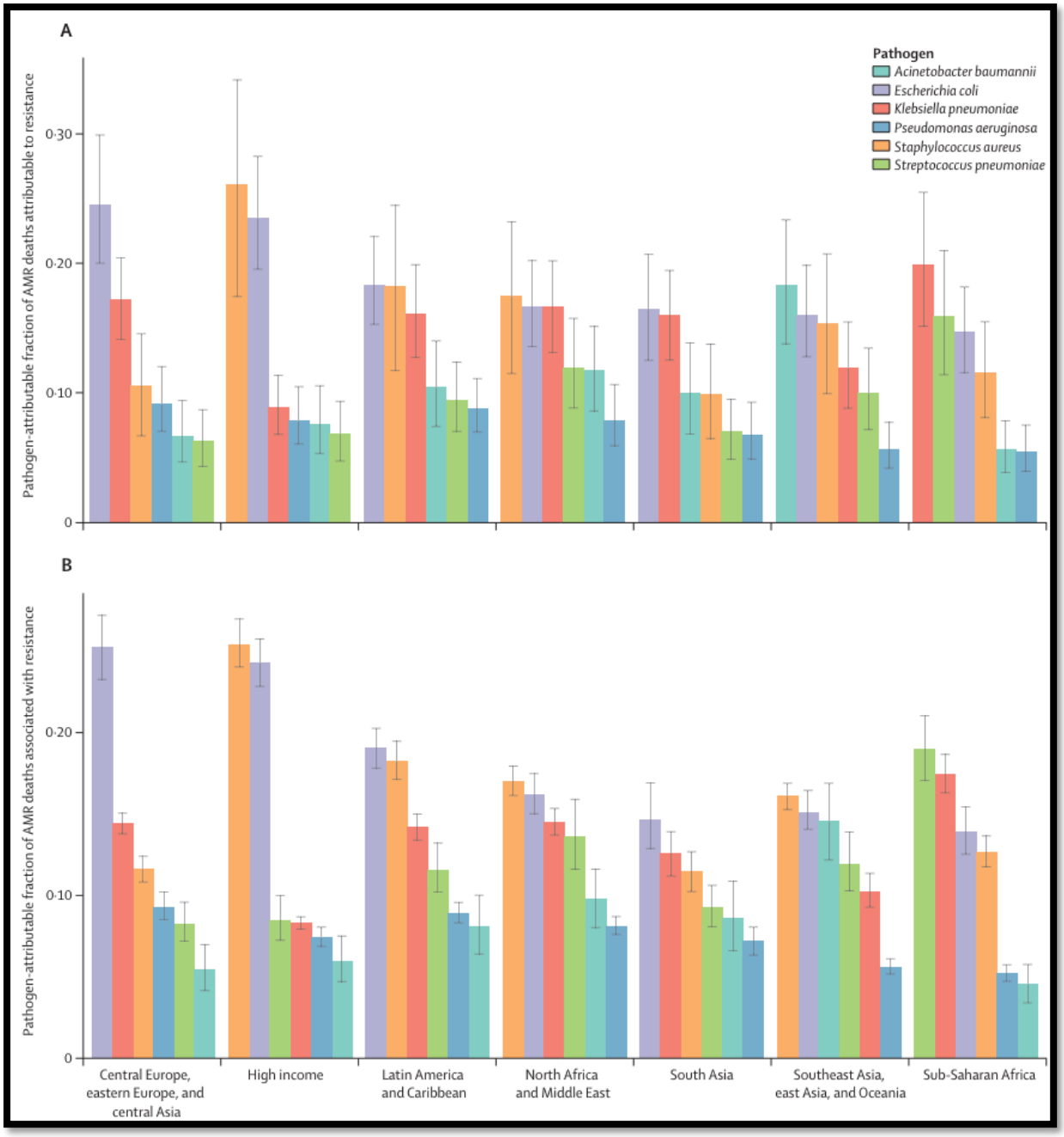

Figure 2: Six major Pathogen death associated frequencies. A, Death attributable to; B, Death associated to; AMR [7].

Furthermore, isolates with resistance against one or more antibiotic specifically resistance against fluoroquinolones and β-lactam antibiotics (used as first line empirical therapy for severe infections) resulted in more than 70% of deaths attributable to AMR (figure 3) [7]. The carbapenem Resistance mechanism is manifested in the form of enzymes called carbapenemase distributed in 3 classes A, B, and D, their role is to inactivate carbapenems, these include but are not limited to, Verona integron-encoded Metallo-β-Lactamase (VIM) a class B enzyme, the New Delhi Metallo-β-Lactamase (NDM) a class B enzyme, and *Klebsiella pneumoniae* carbapenemase (KPC) a class A enzyme [6]. Carbapenemase-producing Enterobacterales (CPE) are in majority associated with HAI, the most detected species under these criteria are, *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae* complex. These CPEs were thought to be spreading by

clonal groups not by horizontal gene transfer (HGT), this theory was distorted in a study by Cerqueira GC. *et al*. where they analyzed the phylogeny of detected CPEs from 3 different hospitals, and the results showed that there was a remarkable genetic diversity and a limited clonal expansion [8].

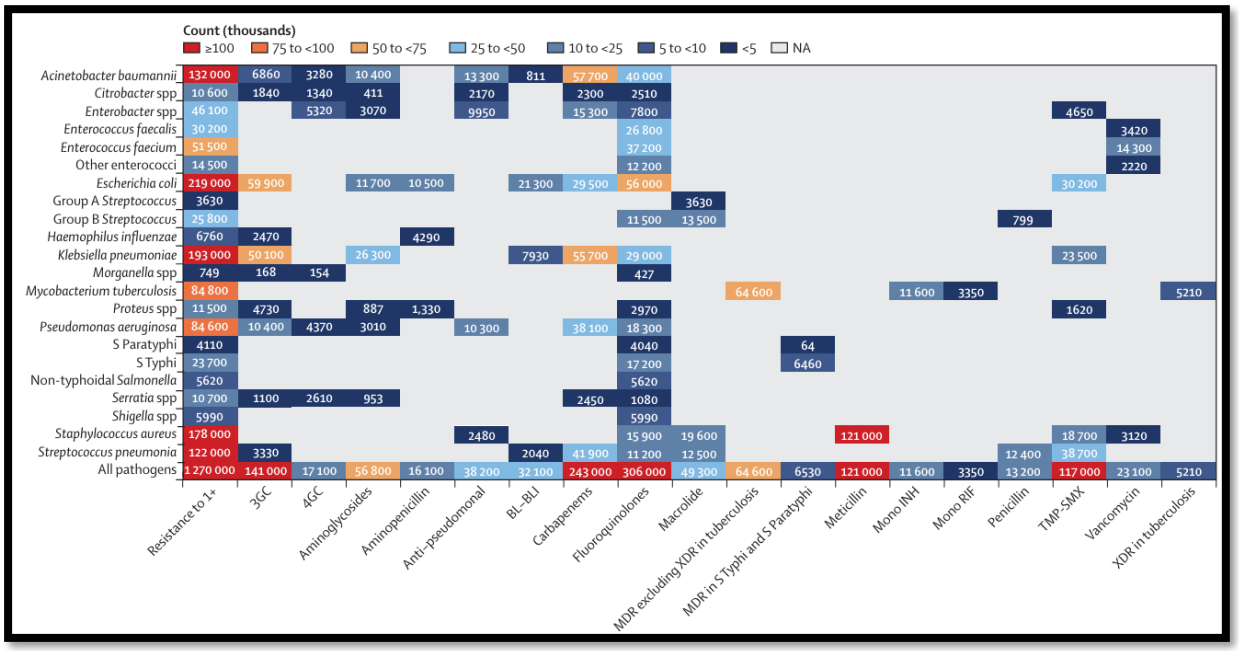

Figure 3: Global Deaths count attributable to AMR by pathogen-drug combination in 2019 [7].

1.2 Aim:

According to the data and worrisome progress of CPEs, the aim of this thesis is to investigate the mechanisms of dissemination and evolution of carbapenem resistance among Enterbacterales and clones mostly in hospital settings. Additionally, investigate the mobile genetic elements integrons, transposons, and plasmids and their role in the dissemination of the resistance genes and their evolution.

1.3 Hypothesis

We hypothesize that this dissemination is facilitated by the conjugative nature of the plasmids and their ability to undergo genetic rearrangements, by incorporating different integrons or transposons or MDR regions. We also hypothesize that without infection control the same isolates previously detected may reside in the same environment and undergo modifications. Finally, we hypothesize that the diversity of bacterial species and mobile genetic elements contribute to the success of a resistance mechanism.

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List of Figures:

2 β-lactams and Carbapenems:

Carbapenems are β-lactam antibiotics, therefore not able to diffuse easily into the bacterial cell [1]. This entry is facilitated by the presence of outer membrane proteins (OMP) referred to as porins. The cross-linkage process is a target for the β-lactam antibiotic class. The first step of the cross-linkage is to remove the terminal D-alanine, this removal releases energy due to the breakage of the bond between the dipeptides. This released energy is then used to create the bond between the second D-alanine and an amino acid present on a second cross-linked peptidoglycan, this reaction is facilitated by the transpeptidase enzyme [2]. β-lactam antibiotics (penicillins, cephalosporins, carbapenems, and monobactams) share the same mechanism, which is to bind their β-lactam ring to the transpeptidase which in return will be deactivated. However, the effect varies significantly, and this is because there are different kinds of transpeptidases. The latter is usually also known as penicillin-binding proteins (PBPs). All of these transpeptidases possess cross-linking activity but also may be involved in other essential activities like cell integrity maintenance, cell shape regulation, producing a new cross-wall in the case of cell division, and helping in segregation [3]. The primary transpeptidase of the bacterium, PBP1B, is influenced by penicillin G, ampicillin, and cephaloridine. The latter antibiotics used against *E. coli* will cause rapid lysis. PBP 4 to 6 are a different case, these enzymes do not possess a transpeptidase activity but have a high affinity to bind the β-lactams, and their inactivation does not affect the cell's viability.

Carbapenems are recognized as mechanism inhibitors of the PBP enzymes resulting in the inhibition of the peptide cross-linkage step in the cell wall synthesis process. This successful inhibition is linked to the carbapenems ability to bind to different and multiple PBPs [4]. The dynamic cell-wall formation occurs in a "three dimensional process" with autolysis and cell-wall formation happening at the same time, the resulting inhibition of PBPs by carbapenems will therefore promote the autolysis, and given no new peptidoglycan is newly synthesized, cell death will occur by osmotic pressure [5].

Due to its effectiveness against a wide range of bacteria specifically against suspected resistant organisms or severely ill patients [6-8] carbapenems have played a significant role in the treatment of these cases.

3 Mechanisms of Carbapenem resistance:

The disorganized and widespread use of the antibiotics in the past years has led to the emergence of resistant bacterial strains to every used antibiotic so far [9]. A small example is given by Davies *et al.*, penicillin was used to treat *Staphylococcus* and *Streptococcus* infections, soon after its introduction, penicillin resistant *Staphylococcus aureus* appeared. Furthermore, the treatment treatment penicillin resistant *Staphylococcus aureus* was managed with a newly synthetic penicillin based antibiotic the methicillin, similarly to penicillin Methicillin resistant *Staphylococcus aureus* (MRSA) emerged as a result [9]. This example highlights the plasticity of the bacterial genome and its ability to adapt to its environment.

There are two types of resistance, Intrinsic which involves the presence of the mechanism (efflux pumps, enzymes responsible for the inactivation, or genes responsible for cell permeability) on the bacterial genome [10, 11]. Acquired resistance, is the result of HGT that can be mediated by mobile genetic elements (MGEs) carrying efflux pumps, and enzymes [12, 13]. Therefore, the presence of the resistance genes on a

plasmid will enhance their dissemination and expression, posing a more serious threat than the intrinsic counterpart [14].

Resistance mechanisms to carbapenems associated with Enterobacterales are divided into two: 1. Reduced susceptibility caused by changes in the expression or mutations in the porins also could be associated with the expression of some β-lactamases, 2. Acquisition and expression of Extended spectrum β-lactamases (ESBLs) and carbapenemases [15].

3.1 Non-enzymatic Resistance:

The selective barrier description given to the bacterial membrane is provided; and not limited to; by the wide presence of Water filled pores, that will facilitate the uptake of certain sized and charge hydrophilic molecules (e.g. Nutritional molecules, antibiotics …) [16, 17]. This selectivity is ensured by the presence of specific loops for example, the loop 3 (L3), in addition to the hydrophilic amino acids in the β-strand, will result in a constriction inside the pore that will select according to size. As for the charge selection, the acidic residues in the L3 and the basic residues in the β-strand, will generate an electrostatic field [16]. Here we will mention only the porins that are responsible for conferring resistance in Enterobacterales against antibiotics, more specifically β-lactams.

The predominant functions for porins are to diffuse molecules according to size and charge. Normally, they are highly expressed on the bacterial outer membrane [18]. Bacterial cells have some control over the permeability of their membrane to molecules through regulating the production of porins, in response to environmental changes. Porins frequently inhibit the diffusion rate of several antibiotics, due to the exclusion limit property of the porin, which contributes to intrinsic resistance [19].

The exposure of the bacterial cells to antimicrobials can generate expression modulating mutations, therefore playing a role in the bacterial susceptibility for some antimicrobials (e.g. β-lactams, fluoroquinolones, …). In *Escherichia coli* three different mutations related to the *ompF* gene and in the regulatory genes *ompB* and *tpo*, resulted in the reduced permeability to β-lactams [20]. In imipenem resistant *Enterobacter cloacae*, the lack of the presence of OmpF was found to be the reason behind the observed resistance, and a mutation in the promoter was behind the porin loss [21].

Porin loss is not the only reason behind the resistance, as it was reported by Koebnik R, *et al.*. Mutations in the L3 (involved in the electrostatic field and the constriction zone) can lead to antimicrobial resistance [16]. In *Enterobacter aerogenes* a mutation in L3 of OmpF/OmpC-like, leads to a decrease in the susceptibility against β-lactams [22]. Other mutations inside the constriction zone of OmpC in *E. coli*, affected the passage of cefoxitime and other β-lactams [23].

A different kind of mutation affecting the expression regulators for the porins (OmpB, OmpR, SoxS, MarA, Rob and others like small RNAs (*micF*)) can lead to changes in antibiotic influx therefore resulting in a decreased sensitivity, in *E. coli* a mutated *ompB* operon (containing *ompR* and *envZ*) for example can modify the expression of the OmpF and OmpR [20, 24, 25].

According to previous research, OmpC and OmpF are the two porins changes involved in the intake of β-lactams in *E. coli* [20]. The porins OmpK35 and OmpK36 are the ones involved in *Klebsiella pneumoniae* an more specifically associated with the intake of carbapenems. Mutations associated with *ompK35, parC, and gyrA* conferred resistance to ciprofloxacin [21, 26-28]. Other porins to mention are OmpF and OmpC with modifications played a role in the susceptibility of *Enterobacter cloacae* and *Enterobacter aerogenes* respectively against carbapenems [21, 29].

3.2 Enzymatic Resistance:

β-lactamases are classified according to their functional or molecular schemes. Starting with their functional scheme, the enzymes belong to 4 groups (1-4) and later divided into subgroups [30, 31]. According to this classification, carbapenemases belong to 2d, 2f, and 3 [31, 32].

The more widely used classification is the Ambler classification, which divides the enzymes into four classes (A-D). Carbapenemases belong to classes A, B and D [33-35].

3.2.1 Class A enzymes:

Class A β-lactamases are present in several bacterial phyla, including Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria (including α-, β-, and γ-proteobacteria) [36]. These enzymes are characterized by the presence of serine70 (Ser_{70}) in their active site [37]. These enzymes efficiently hydrolyze benzylpenicillin, and ampicillin, first-generation and second-generation cephalosporins, aztreonam, and carbapenems [38]. These enzymes are recognized by their reduced susceptibility to imipenem, MICs can become mildly elevated or fully resistant [39].

3.2.1.1 The KPC enzyme:

This enzyme is of relevance because of its existence and transmission between isolates via self-conjugative plasmids, and its association with *K. pneumoniae*, a well-known species for the spread and accumulation of resistance genes [32, 40].

The first case of KPC producer was detected in North Carolina and was isolated from a *K. pneumoniae* in 1996 [41]. This enzyme is able to hydrolyze a wide variety of antibiotics which included carbapenems, cephalosporins, monobactams, and penicillins but had the highest activity against meropenem. After its first detection, more incidences of KPC producers were detected in other cities, for example New York city were KPC-2 and KPC-3 producers were detected [42, 43].

KPC-2 and KPC-3 diverged by one amino acid, and these two variants were later discovered in different Enterobacterales [43, 44]. Later, KPC producers were being reported worldwide, with endemic cases recorded in South and North of America, Greece and Italy, Middle-east and China [45]. The Bacterial Antimicrobial Resistance Reference Gene Database (NCBI) has one hundred and ninety-four variations of the bla_{KPC} gene as of January 2024, with bla_{KPC-2} and bla_{KPC-3} being the most prevalent [46, 47].

according to reports, ST258 *K. pneumoniae* is the main driver disseminating bla_{KPC}, but it differ according to the geographical location, for example in ST512 in Finland, and ST11 in Singapore, these sequence types are also part of the clonal group 258 (CG258) which is recognized worldwide as a successful group for

disseminating *bla*KPC [48-50]. Despite the link with sequence types, the *bla*KPC gene is mostly linked with a transposon the Tn*4401* Tn*3* type, this transposon was found to be very active when linked to plasmids [51, 52]. Structurally, this transposon is flanked by 2 inverted repeat sequence and carries genes encoding for transposition (transposases, resolvases), the *bla*KPC gene region is marked by the presence of two insertion sequences, the first one upstream of the gene named ISK*pn7*, and the other downstream of the gene named ISK*pn6* [52]. Variants of Tn*4401* has been characterized, up to ten variants have been identified (from a to j), a comparison between Tn*4401a* and Tn*4401b* differ by a 100bp sequence deletion in Tn*4401a* upstream of the *bla*KPC gene (figure 4.), whereas Tn*4401j* possessed a region of 2833bp harboring *bla*KPC and deriving from Tn*4401* structure; In comparison to Tn*4401b*, a deletion of 217bp upstream of the *bla*KPC gene is noticed, and the Tn*4401*-derived region is disrupted by a 111bp Tn*3*-like sequence upstream of *bla*KPC gene [50, 53, 54].

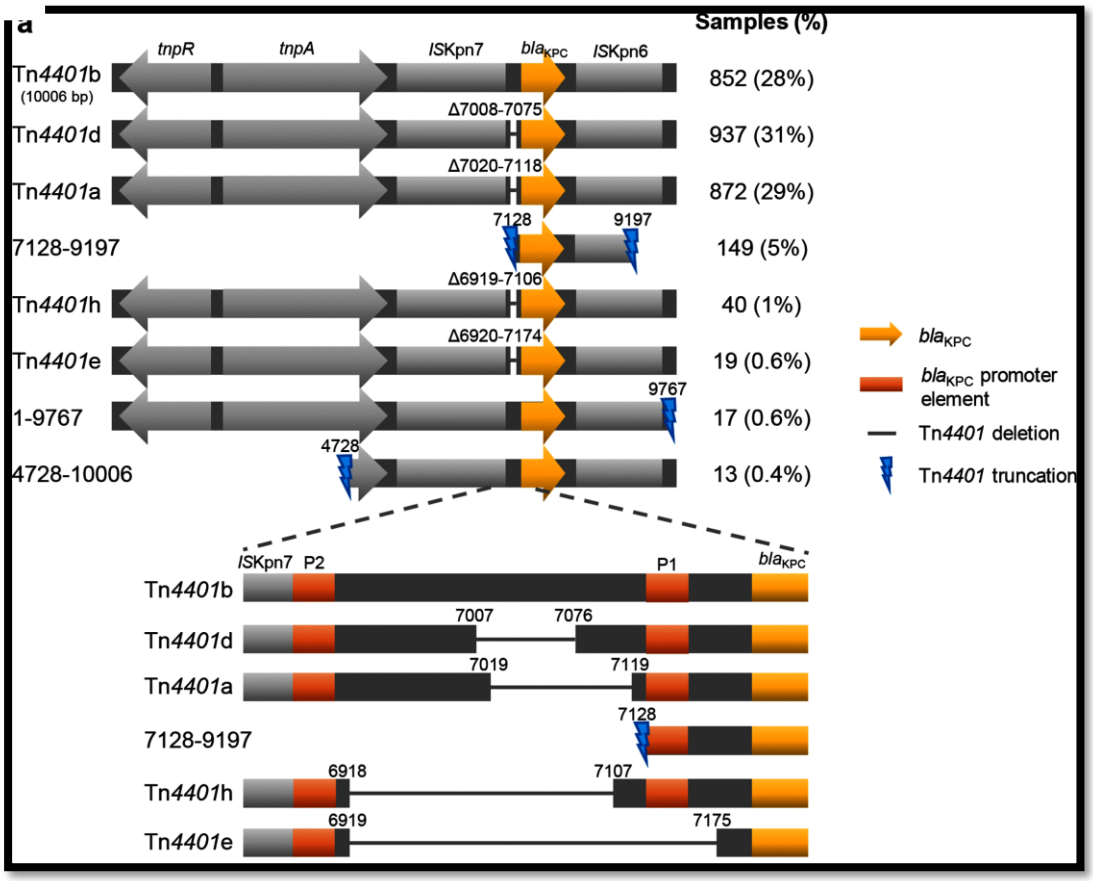

Figure 4: structure comparison between Tn4401 isoforms [53, 55].

Other reports have found *bla*KPC being carried on non-Tn*4401* elements named as NTEKPC [56]. A pKpQIL-like plasmid associated with CG258 *K. pneumoniae* is also contributing to the dissemination of *bla*KPC [57].

The first case of KPC-producing isolate in the Czech Republic was reported by Hrabak *et al.* in 2009. It was detected in a *K. penumoniae* isolate harboring *bla*KPC-2 isolated from a patient hospitalized in Greece. shortly after this report, another *K. pneumoniae* producing KPC-3 was isolated from a patient hospitalized in Italy;

these isolates carried their *bla*$_{KPC}$ genes on Tn*4401a* and on IncFII$_{K2}$ pKpQIL-like plasmids [58, 59]. Kraftová *et al.* investigated 108 KPC-producing isolates between 2018-2019 from 22 laboratories across the Czech Republic, these were found harboring *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$. remarkably in this study KPC-producers belonged to multiple species this was due to the presence of the *bla*$_{KPC}$ genes on transposons (primarily Tn*4401a* and the novel Tn*4401j*) and on self-conjugative plasmids like IncR, IncFII, pKpQIL-like, IncFII-IncR, and IncR-IncN$_3$ (previously described as carriers for *bla*$_{KPC}$) [53, 60-62]. Furthermore, this study characterized emerging plasmids such as IncN, the hybrid IncFIB/IncFII, and the IncFII/FIB/C$_2$/N plasmid. Additionally, unfolds the evolution of plasmids spreading the resistance mechanism, for instance the IncR plasmids previously reported harboring carbapenemases like VIM and NDM [63, 64].

3.2.1.2 The GES enzyme:

In 1998, the first case of Guiana extended-spectrum (GES) was a *K. pneumoniae* isolated from an infant in France and was carried by a non-conjugative plasmid of 140kp (pTK1) [65]. This first case was later referred to as GES-1, and because of its low carbapenemase activity it was classified as an ESBL [36]. Two years later, in 2000, a mutation occurred in the sequence of *bla*$_{GES-1}$ and resulted in the substitution of glycine on position 170 to asparagine resulting in the increased activity against imipenem [66]. GES-1 has Glycine 170 (Gly$_{170}$) in the active site, GES-5 is characterized by its replacement with Ser$_{170}$ therefore increasing the hydrolysis capacity to imipenem by 100-fold [67]. The enzymes of the GES family differ from each other by one to four amino acid substitutions [36].

Although *P. aeruginosa* are the main producers of GES enzymes, they have been detected in Enterobacterales. Isolates producing GES enzymes with carbapenemase activity have been collected predominantly in Europe, South Africa and the Far East [36]. Over 50 *bla*$_{GES}$ variants have been identified with only a few possessing carbapenemase activity GES-2 and GES-5, GES-6, GES-14, and GES-20 [68].

The *bla*$_{GES}$ genes were found to be harbored by class 1 integrons and can be rarely associated with class 3 integrons [69]. Matlock *et al.* studied *bla*$_{GES-5}$ transfer by downloading sequences from NCBI database deposited over 15 years. They found that the regions upstream (integrase gene *Intl1* in the case of class 1 integron) and downstream (recombination sites *attI* x *attC* and *attC* x *attC,* (Figure 5.)) of the gene shared a similar genetic environment [70]. These integron were found on genomes specially in *P. aeruginosa* on various plasmids. When these resistance genes are carried on plasmids, they can be spread to other cells. The integron in the new cell can either integrate the recipient cell's resistance genes or have its own resistance genes incorporated by other integrons. This process results in the formation of more complicated integrons. [71]. The integrons found on genomes had their *attC* sites disrupted by an IS*110* that hindered the mobility of the given integron, regardless of the *intl1* presence and activity [70].

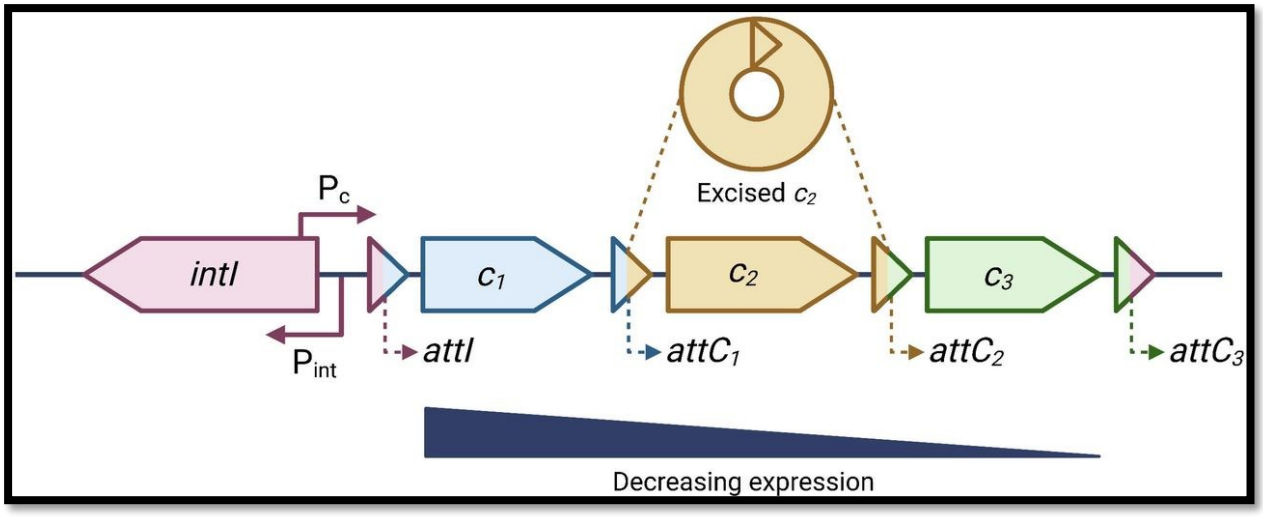

Figure 5: Integron scheme [70]. (P_c = cassette promotor, P_{int} = integrase promotor.)

In the Czech Republic the first case of GES-5 producing Enterobacterales was reported by Chudjeová *et al.* in 2018, it was detected in an *Enterobacter cloacae* ST252. The gene was carried by a novel class 1 integron In1406 [72]. Two years later, during a regular screening (in the same hospita) two isolates proved to be GES producers. These two isolates belonged to the *E. cloacae* complex but had different STs than the original (ST837 and a novel ST1622) confirming the HGT of the *bla*$_{GES-5}$. In addition, a duplication of the genes was found where one isolate carried *bla*$_{GES-1}$ and *bla*$_{GES-5}$ whereas the second isolate carried two copies of *bla*$_{GES-5}$ on untypable plasmids with 100% similarity to the backbone of the 2018 plasmid. Two new *bla*$_{GES}$ integrons were also identified, In2079 carrying *bla*$_{GES-1}$ and *bla*$_{GES-5}$ and In2081 carrying the two copies of *bla*$_{GES-5}$ [73].

3.2.1.3 Other enzymes:

IMI/NMC-A enzymes are mostly found in *Enterobacter* spp. The first identified enzyme (IMI-1) was responsible for resistance against penicillins given as a solo treatment or in combination with early generation Cephalosporins, clavulanate, and carbapenems, but it does not target broad-spectrum cephalosporins for example ceftazidime [74]. This IMI-1 enzyme was first identified in 1984 in the USA from an *E. cloacae*, since then this enzyme was identified in more *Enterobacter spp.* from France, Singapore, China, Japan, Polynesia, and Vietnam [75-78]. Despite the rarity of IMI-like enzymes, it has been detected in multiple continents. Multiple variants have been detected and characterized, as of january 2024, twenty-four variants have been detected. The *bla*$_{IMI}$ genes can be found associated with chromosomes or with plasmids; for example *bla*$_{IMI-1}$ was found to be chromosomal gene while the second variant was plasmid associated [69]. As for the integration into the genome or plasmid an EcloIMEX-like element with the involvement of XerC/XerD recombinases [79]. Even after the identification of this element, the mechanism behind the acquisition of the *bla*$_{IMI}$ remains unclear. But *bla*$_{IMI-2}$ has been found outside of the *Enterobacter spp.* And this could be due to its association with IncF-type plasmids [69]. Other variants have also been

found in other species because of the same reason, *bla*$_{IMI-3}$ associated with IncFIIY and also was found on a transposon Tn*6306* [80] *bla*$_{IMI-5}$, and *bla*$_{IMI-6}$ were carried by IncFII plasmids.

SHV-38 is the only enzyme out of more than 200 variants that possesses the mutation A146V conferring it the carbapenemase activity. All the other variants are considered as ESBLs except for SHV-1 being a narrow spectrum [69, 81]. SHV-38 is considered the natural class A β-lactamase of *K. pneumoniae* [69]. This enzyme resulted in increased MIC for imipenem when it was cloned in an *E. coli* isolate [81].

3.2.2 Class B enzymes:

This class of enzymes is also known as metallo-β-lactamases (MBLs) due to the necessity of the Zinc ion for catalysis. Enzymes belonging to this class can belong to 3 subclasses B1 and B3 characterized by having 2 Zinc ions in their active site giving them a broad-spectrum activity. Subclass B2 characterized by having one Zinc ion in its active site these enzymes have a narrow-spectrum activity but it includes carbapenems [82]. MBLs are known for hydrolyzing a wide variety of β-lactams, apart from monobactams (aztreonam) [83]. Because these enzymes activity is based on the presence of a heavy metal, they are inhibited by metal chelators such as ethylenediaminetetraacetic acid (EDTA) [39].

3.2.2.1 The NDM enzyme:

This enzyme is one of the most clinically significant MBL. In 2008, a Swedish patient showed signs of a resistant urinary tract infection after his travel to New-Delhi, later investigation linked this resistance to the presence of a novel carbapenemase NDM-1 detected in a *K. pneumoniae* [84, 85]. Variants of this enzyme have been detected, most of these variants possess at least 1 amino acid substitution that either plays a crucial role in the hydrolytic activity or does not. For instance, NDM- 2 and NDM-3 variants differ from NDM-1 with one amino acid substitution (28 Pro-Ala, and 95 Asp-Asn respectively) does not confer any activity changes. As for NDM-4 and NDM-5 mutations (shared a mutation 154 Met-Leu, and a different one in NDM-5 88 Val-Leu) have shown increased resistance to cefepime, ceftazidime, cefalotin, meropenem, cefoxitin and imipenem [86, 87]. Up till January 2024 more than 60 variants have been detected. Since its discovery, it has gained global attention, and this is due to its rapid dissemination among Enterobacterales.

Interestingly, almost all NDM producers were found to be associated with other resistance determinants, such as ESBLs and AmpC cephalosporinases, OXA-48-like enzymes, KPC, and VIM, confers resistance to Quinolones, macrolides, and aminoglycosides, greatly narrowing the treatment choices [88-93].

A study conducted by Acman *et al.* in 2022 discussed the different mobile genetic elements responsible for the *bla*$_{NDM}$ dissemination worldwide. A dataset of 6155 genomes have been used, originating from 88 countries, this includes Asian countries, European countries, USA, Thailand, and India. The major species found to be carrying the genes were *Klebsiella* and *Escherichia*. IncX3 was determined to be the most prevalent plasmid type circulating with *bla*$_{NDM}$ worldwide, other noteworthy plasmid types were IncFII/IncFI, IncC, and IncN2 [94]. The first variant *bla*$_{NDM-1}$ was found to be carried by a Tn*125* in *Acinetobacter baumanii* (Figure 6. A). This transposon had a conserved structure associated with the complete or truncated presence of the insertion sequence IS*Aba125* upstream of the gene and downstream of the *ble*$_{MBL}$ gene [85]. A truncated form of this composite transposon was identified in Enterobacterales

(the second copy of IS*Aba125* downstream of *ble* is missing), suggesting that *A. baumanii* was the initial reservoir before the transmission to Enterobcaterales [95]. This finding is confirmed by Acman *et al.* after analyzing the genetic environment of *bla*$_{NDM}$ where they found that more than 98% of the NDM producers had a ~75bp upstream flanking fragment originating from IS*Aba125*. Also they found that in the early stages of the dissemination of the gene, *A. baumanii* and *K. pneumoniae* had intact IS*Aba125* downstream of the gene which suggested that Tn*125* was the initial disseminator of the gene and later on disrupted by insertion sequences and other rearrangements [94]. This disrupted transposon was no longer able to disseminate the gene. However, A composite transposon, Tn*3000*, was identified with two copies of IS3000 flanking the disrupted transposon. This modification likely facilitated the remobilization of the *bla*$_{NDM}$ genes (Figure 6. E). The distribution of the transposons revealed that IncN2 plasmids were commonly associated with Tn*5403*. On the other hand, The IS within these transposons can vary according to the geographical region. For example, in IncX3, IS*5* upstream of the *bla*$_{NDM}$ gene was predominantly found in isolates from East Asia. Furthermore, Tn*3000* and Tn*125* had a consistent length ranging from 7 to 10 kb [94]. Moreover, downstream rearrangements are not limited to the presence of IS3000 and IS*Aba125*, different ISs were found to participate in the rearrangement for instance, IS26, IS15, ISCR27, and ISCR1, were also detected and therefore creating different types of transposons [94].

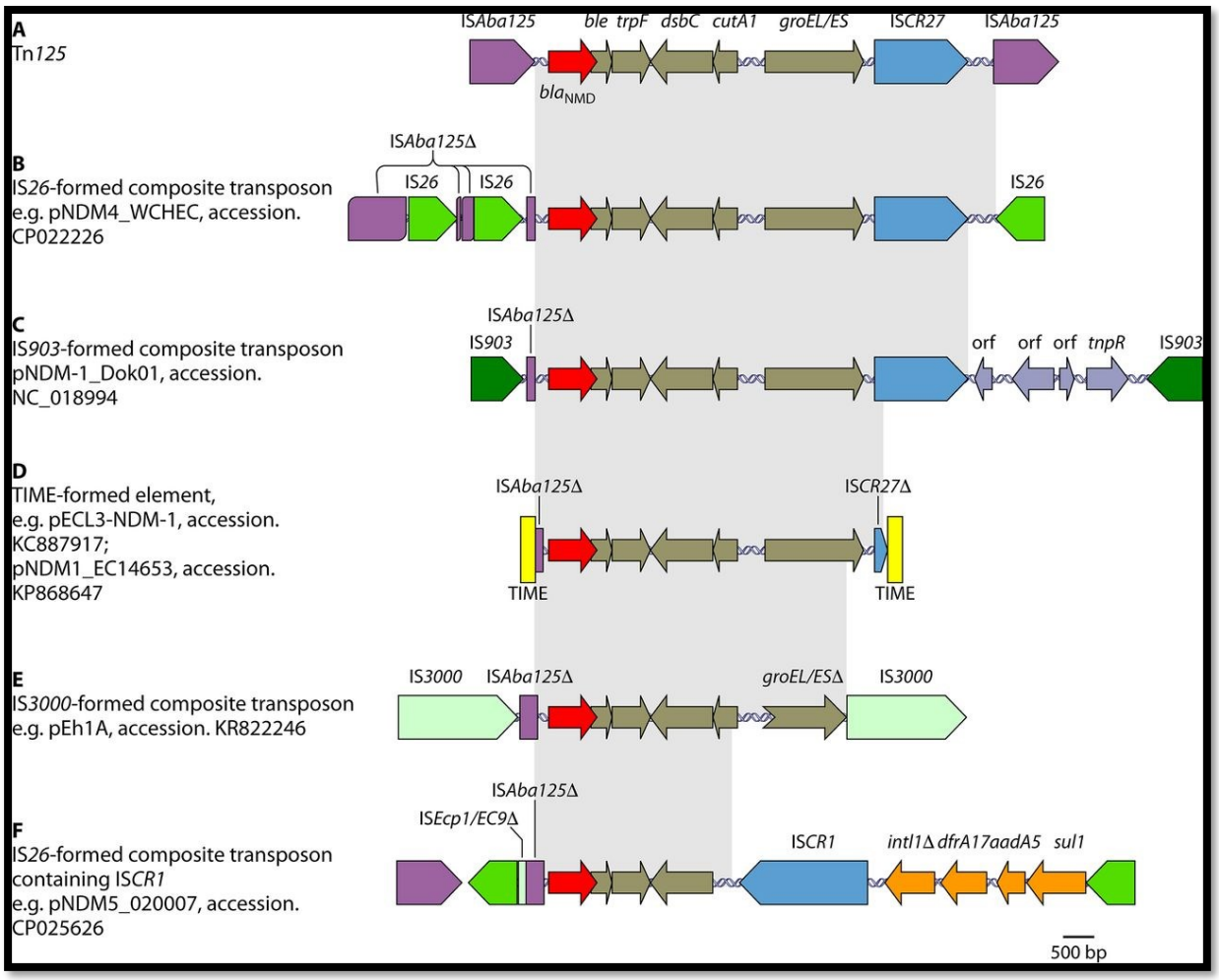

Figure 6: Differences in transposons carrying the *bla*$_{NDM}$ genes [96].

(A) Tn125 formed by two copies of ISAba125. (B) Composite transposon formed by two copies of IS26. (C) Composite transposon formed by two copies of IS903. orf, open reading frame. (D) Element formed by two copies of the TIME. (E) Composite transposon formed by two copies of IS3000. (F) Genetic contexts containing ISCR1. This element is also flanked by two copies of IS26. The plasmid names and GenBank accession numbers are shown. Δ represents truncated genes or elements.

In the Czech Republic, between 2011 and 2013 three sporadic cases were detected. The first one was an NDM-1 producing *A. baumanii* isolated from a patient repatriated from Egypt [97]. The second isolate from 2012 was an ST182 *Enterobacter cloacae* isolate producing NDM-4 isolated from a patient hospitalized in Siri Lanka [98]. The third isolate was a ST11 *K. pneumoniae* NDM-1 producing isolated from a patient hospitalized in Slovakia, this isolate was found to be carrying two *bla*$_{NDM-1}$ copies [63]. In 2016, eighteen isolates were found to be harboring *bla*$_{NDM}$, 10 isolates belonged to the *E. cloacae* complex ST182 except for the only *E. asburiae* isolate and all were NDM-4 producers, four were *E. coli* belonging to different STs, the remaining belonged to different species. seventeen of the isolates including the 2012 isolate, harbored their *bla*$_{NDM}$ gene on an IncX3 plasmid, while the remainder two carried it on IncR/IncA/C2 and IncFIB [99]. Another report of NDM-producers was in 2021, where four isolates (three *K. pneumoniae* and one *E. coli*) were found to be co-producers (NDM-like and OXA-48-like). ST147 *K. pneumoniae* harbored *bla*$_{NDM-5}$ on IncFII, and *bla*$_{OXA-181}$ on ColKP3; ST11 *K. pneumoniae* harbored *bla*$_{NDM-1}$ in an IncFIB/FII plasmid, and *bla*$_{OXA-181}$ on IncX3/ColKP3 plasmid; the third ST15 *K. pneumoniae* isolate harbored *bla*$_{NDM-1}$ on IncFIB/IncHI1B plasmid, and *bla*$_{OXA-244}$ on an IncFII plasmid. The final isolate ST167 *E. coli* harbored *bla*$_{NDM-5}$ on an IncFII/IncFIA, and a chromosomal *bla*$_{OXA-244}$ [100]. More recently, a study; published by Chudejova *et al.* in 2024; performed on forty-two ST38 NDM-5-producing *E. coli* isolates all detected from an outbreak in Brno [101]. Of which, only 26 isolates were chosen to be characterized with WGS. Briefly, data have shown that all these isolates had their *bla*$_{NDM-5}$ gene carried on their chromosome. Phylogenetic analysis on the genomes showed that all of these isolates clustered together, and SNP analysis highlighted the high similarity between them (2-16 SNPs). Therefore, these findings highlight the clonal outbreak of NDM-5-producing ST38 *E. coli* in Brno [101].

3.2.2.2 The VIM enzyme:

This enzyme was first identified in Verona, Italy. It was recovered from a *P. aeruginosa* in 1997 [102]. Further studying on the enzyme activity was achieved by cloning it in an *E. coli* which resulted in a significantly decreased susceptibility against carbapenems, ampicillin, piperacillin, cefotaxime, ceftazidime, cefepime, carbenicillin, mezlicillin, cefoxitine, and cefepime [102]. Soon after, variants of VIM enzymes started to emerge, for example the first VIM-2 producer was identified in a *P. aeruginosa* isolated in France [103]. Even though VIM-1 and VIM-2 have been reported in Enterobacterales, the known reservoir for these enzymes remains *P. aeruginosa*. More than 80 variants have been identified. According to sequence relatedness and clustering, VIM variants belong to either VIM-7-like cluster, VIM-13-like cluster, VIM-1-like cluster, or VIM-2-like cluster (Figure 7.) [104].

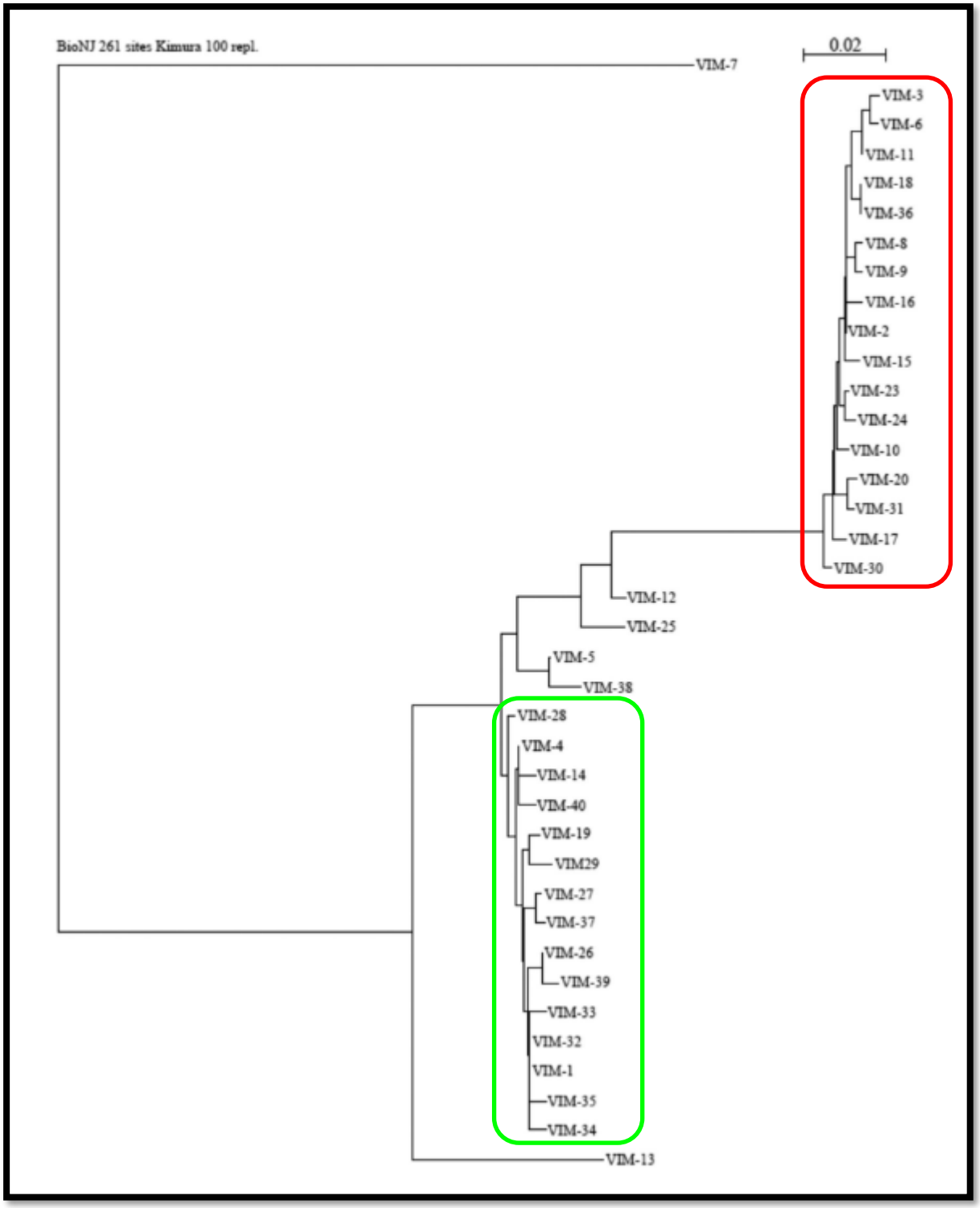

Figure 7: Phylogenetic tree of VIM enzymes [104], In green VIM-1 cluster, and in Red VIM-2 cluster.

VIM-1 and VIM-1-like enzymes and particularly VIM-4 have been reported in Enterobacterales, while VIM-2-like enzymes were mostly detected in *P. aeruginosa* isolates. The presence of the bla_{VIM} gene on integrons (mainly integron class one In110) helps it disseminate and integrate in either plasmids or chromosomes [102, 103, 105]. As previously described with the bla_{GES}, bla_{VIM} integrons can also procure gene cassettes using the integrons recombination sites (*attI* x *attC*, Figure 5). These integrons are consisted of 2 conserved

regions, the upstream beginning region consisting of the *intI1*, *attI* and a promoter, and the downstream end region consists of a truncated *qac* gene (*qacEΔ1*) fused with the *sul* gene (Figure 8) [106]. VIM-producing Enterobacterales have been detected all over the world. In Europe they have been reported extensively in Greece, Hungary, Italy, and Spain [107]. Furthermore, according to a study conducted by Matsumura *et al.* on eighty-nine global VIM-producing isolates (between 2008 and 2014). They found that *K. pneumoniae* was the most prevalent species and mostly detected from isolates originating from Greece. The second most detected species was *E. cloacae* complex mainly the *E. hormaechei*, they were identified from isolates originating from Coratia, Greece, Italy, USA, and Spain. Moreover, the most detected VIM variant was the VIM-1 giving it a global distribution. Additionally, all identified integrons were class 1 integrons, where In110 and In1209 carrying the *bla*$_{\text{VIM-1}}$ being the most predominant [107]. Another study conducted on VIM-producing Enterobacterales in Canada; eleven out of the fifteen isolates were VIM-1 producing *E. hormaechei*. Later, four VIM-producing environmental isolates were added to the study. Of these, thirteen carried the *bla*$_{\text{VIM-1}}$ on IncR plasmids, while others were associated with IncHI2, and IncFII/FIIB plasmids. Finally, they found that all clinical isolates were carrying *bla*$_{\text{VIM-1}}$ harboured by In110 class 1 integron [108]. In Italy, a study performed by Arcari *et al.* showed a new association of *bla*$_{\text{VIM-1}}$ and highly conjugable IncA plasmids. Similarly to other *bla*$_{\text{VIM-1}}$ plasmids, the gene was also found in a class 1 integron. In addition, IncA plasmids are highly conjugative therefore would contribute to the reemergence of VIM-enzymes [109, 110]. Further studies in Europe showed that the VIM-1 integron was also associated with IncN plasmids, like in Italy and Switzerland [111, 112].

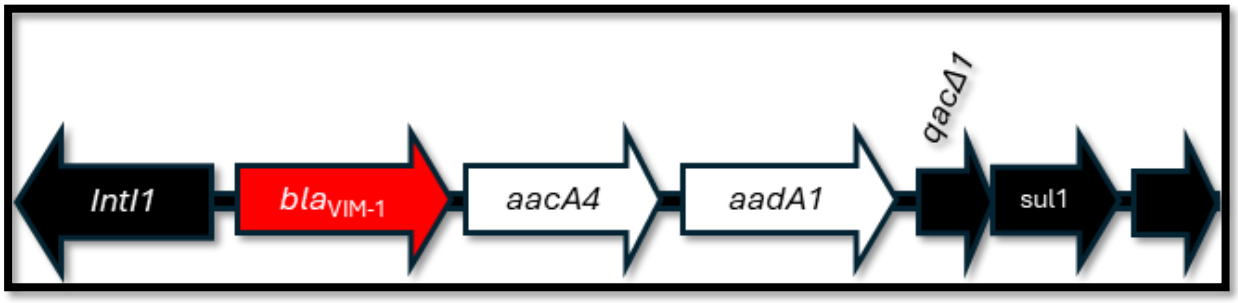

Figure 8: In110 Schematic [113].

In the Czech Republic, from 2011 till 2015 sporadic cases of VIM-producers were detected, interestingly, all of these cases carried the *bla*$_{\text{VIM}}$ genes on In*110* [113, 114]. A surge in the detection of VIM-producers spiked between 2019 and 2020. In this surveillance, 32 isolates were found to be VIM-producers, with the majority (n=23) belonging to the *E. cloacae* complex, the rest belonged to the *Citrobacter freundii* (n=5), *K. pneumoniae* (n=3), and one *K. michiganensis*. Most of the isolates were carrying *bla*$_{\text{VIM-1}}$ (n=26) while the rest were carrying the *bla*$_{\text{VIM-4}}$ variant. All the isolates carried their *bla*$_{\text{VIM}}$ genes on class 1 integrons, the majority carried by In110 (n=24) the rest of the VIM-1 producer's genes were carried by In4873. The VIM-4 isolates carried the gene on In1174, In416, In2143, and In2150. Furthermore, integrons were found on plasmids with the following types pKPC-CAV1193-like, IncHI1, IncHI2, IncFIB and IncN and were also found on non typable plasmids, and one *K. pneumoniae* with the integron on its genome [115].

3.2.2.3 Other enzymes:

Transferable imipenem resistance is known as the IMP enzyme. It was first identified in a *P. aeruginosa* from Japan, then a second report discussed the same transferable imipenem resistance was detected in a *Bacteroides fragilis* strain [116, 117]. This enzyme is active against imipenem, extended spectrum cephalosporins, and penicillins, and similarly to other class B enzymes was inhibited by EDTA. Later investigations in Japan have found that this enzyme is found in Enterobacterales on integrons [118-121]. In Europe, it is first detected in an *A. baumanii* from Italy in the modified form (IMP-2) found on a class 1 integron, then an IMP-5 porducer is detected in Portugal [122]. Shortly after, this enzyme was detected worldwide. Besides being carried by class 1 integron as previously mentioned, some studies were able to locate the bla_{IMP} genes carried by class 3 integrons [120, 123]. Interestingly, $bla_{\text{IMP-13}}$ was found to be on an integron that in his turn was embedded inside a transposon Tn*5051*, this phenomenon was reported in a *P. aeruginosa* isolate from Italy [124]. Furthermore, this transposon was hypothesized to be responsible for the dissemination of a class 1 integron later on accumulating different MBLs in its gene cassette, similar to the detection of the $bla_{\text{IMP-13}}$ found on this MGE, a $bla_{\text{VIM-2}}$ genes was found on the same backbone proving this hypothesis [124].

In 2002, a new MBL type enzyme has been detected in Germany from five *P. aeruginosa* imipenem resistant, named as *Germany Imipenemase* (GIM-1). This enzyme, classified as a subclass B1, is active against penicillins, carbapenems, and broad spectrum cephalosporins also inhibited by EDTA [125]. This enzyme is disseminating in Germany, and is detected in Enterobacterales, *Serratia marcescens*, *E. cloacae* complex, *E. coli*, *C. freundii*, *K. oxytoca*, and *C. amalonaticus* [126-130]. The bla_{GIM} gene is being detected on conjugative non typable plasmids [127, 130].

The *Serratia fonticola* carbapenem hydrolase (SFH-1) is a subclass B2 enzyme, this enzyme was only characterized in *S. fonticola* from Portugal [131, 132]. This enzyme is active against carbapenems, but it is not active against penicillins or cephalosporins.

In 2002, from Adelaide Australia, a new imipenemase was detected and named Adelaide imipenemase (AIM-1) was detected in a *P. aeruginosa* [133]. Since then this enzyme have been detected in West African country and in China specifically in waste water [134, 135]. This enzyme is resistant to carbapenems, broad spectrum cephalosporins, and to penicillins apart from piperacillin. This enzyme was classified as a subclass B3 enzyme [136]. Further investigation of the genetic context of the $bla_{\text{AIM-1}}$ gene, has found that it was associated with IS*CR5*, this is an insertion sequence that transposes with a rolling circle transposition [133].

3.2.3 Class D enzymes:

This class of β-lactamases is named oxacillinases as they efficiently target mainly isoxazolylpenicillins (oxacillin, cloxacillin, and methicillin), another target includes benzylpenicillin and first generation cephalosporins. They are thus, referred to as OXA enzymes for oxacillin being their main target [36]. This class of enzyme is also characterized by the conserved serin structure while the rest of the enzyme structure is variable [32].

Studies on the first identified OXA-1 enzyme highlighted its presence on Tn*21* transposon. This transposon was found to be incorporated between the *aad* aminoglycoside gene and its corresponding promoter [137]. The second identified OXA-2 enzyme was found to be 48% identical to OXA-1 but only the active region around Ser_{71} and around Leu_{179} was conserved, therefore this region has become the main interest while investigating these enzymes [137]. Furthermore, bla_{OXA-2} regardless of the dissimilarity with the bla_{OXA-1} was found to be carried and incorporated in the same spot [137]. More variants were discovered, and further characterized, more than 1100 variants of bla_{OXA} were detected, the enzymes were classified according to their activity, for example OXA enzymes possessing an ESBL activity (OXA-10, OXA-11, OXA-28, OXA-35, OXA-145, OXA-147, and other OXA-10-like) or carbapenemase activity (OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, OXA-48-like).

3.2.3.1 The ESBL OXA enzymes:

The Early identified OXA enzymes were found in *P. aeruginosa* strains, these isolates were still susceptible to third generation cephalosporin. Until 1991, where a *P. aeruginosa* in Türkiye was detected and had a multi resistant phenotype including the third-generation cephalosporin [138]. This detected resistance was transferrable, sequencing of the isolate have shown that this enzyme is 2 amino acid different than OXA-10 affecting the binding of ceftazidime resulting in a more active hydrolysis, this new enzyme was assigned as OXA-11 becoming an ESBL enzyme after a series of mutations [139].

Other ESBL OXA enzymes detected were also derivatives of the OXA-10, namely OXA-13, OXA-14, OXA-16, OXA-17, OXA-19, OXA-28, OXA-35, OXA-145, and OXA-147 [137]. Further studies on these enzymes have shown that all of them possesses the same mutation from OXA-10 the Arg_{157} instead of Gly_{157}, emphasizing this mutation as the primary mutation responsible for the conversion to ESBL enzymes. [140].

Another group of ESBL OXA enzymes is known as the OXA-2-like enzymes. This group is of higher diversity. Each enzyme has a different mutation, for instance, OXA-15 the substitution is a Gly_{149} instead of an Asp_{149}, while in OXA-36 the substitution is a Tyr_{149} [137].

3.2.3.2 The OXA-48 and OXA-48-like enzymes:

The OXA-48 and OXA-48-like enzymes are characterized by their ability to hydrolyze carbapenems. First identified in 2001 in Turkey from a *K. pneumoniae* isolate that was resistant to multiple antibiotics including carbapenems [141]. OXA-48 enzyme had a higher activity against imipenem rather than meropenem, this resulted in a moderate increase of the MIC value against carbapenems. These enzymes are also characterized by their low activity against extended spectrum cephalosporins, cefepime and ceftazidime. In Enterobacterales, OXA-48 enzymes can be found associated with other resistance determinants, example porin loss as explained in a study by Potron *et al.* where they cloned OXA-48-like enzymes (OXA-232, and OXA-181) in OmpF and OmpC deficient *E. coli* susceptible to carbapenems, this resulted in an increase in the MIC values on carbapenems [142]. In addition to being associated with porin loss it can also be co-present with other ESBL enzymes and/or AmpC enzymes and even with other carbapenemases [32].

The most common OXA-48 variations are OXA-164, OXA-163, OXA-181, OXA-232, OXA-204, OXA-244, OXA-245, OXA-247, OXA-436, OXA-484 and OXA-519 (Figure 9) [143]. Furthermore, research on the activity of

these variations have revealed that OXA-181 and OXA-232 appear to be substantially comparable to OXA-48 [137]. Epidemiologically, The OXA-48 and OXA-48-like producers have been recovered from many Enterobacterales with majority belonging to *K. pneumoniae, E. cloacea, E. coli, C. freundii, P. mirabilis* and more [144-146]. Turkey, North Africa, and India are the endemic sites for OXA-48 production. Nosocomial outbreaks caused by OXA-48 producers have been reported in several European nations, including France, Germany, Switzerland, Spain, Holland, and the United Kingdom [143, 147]. Endemic incidences of such diseases are recorded in Middle Eastern countries and North Africa [143, 147]. The OXA-181 is a widespread enzyme in India and has also been found to be associated with the production of NDM-5 and VIM-5 [148]. Interestingly, OXA-48-like enzymes have been also isolated from birds and wild mammals from Catalonia (Spain), and even form pet animals in Germany [149, 150].

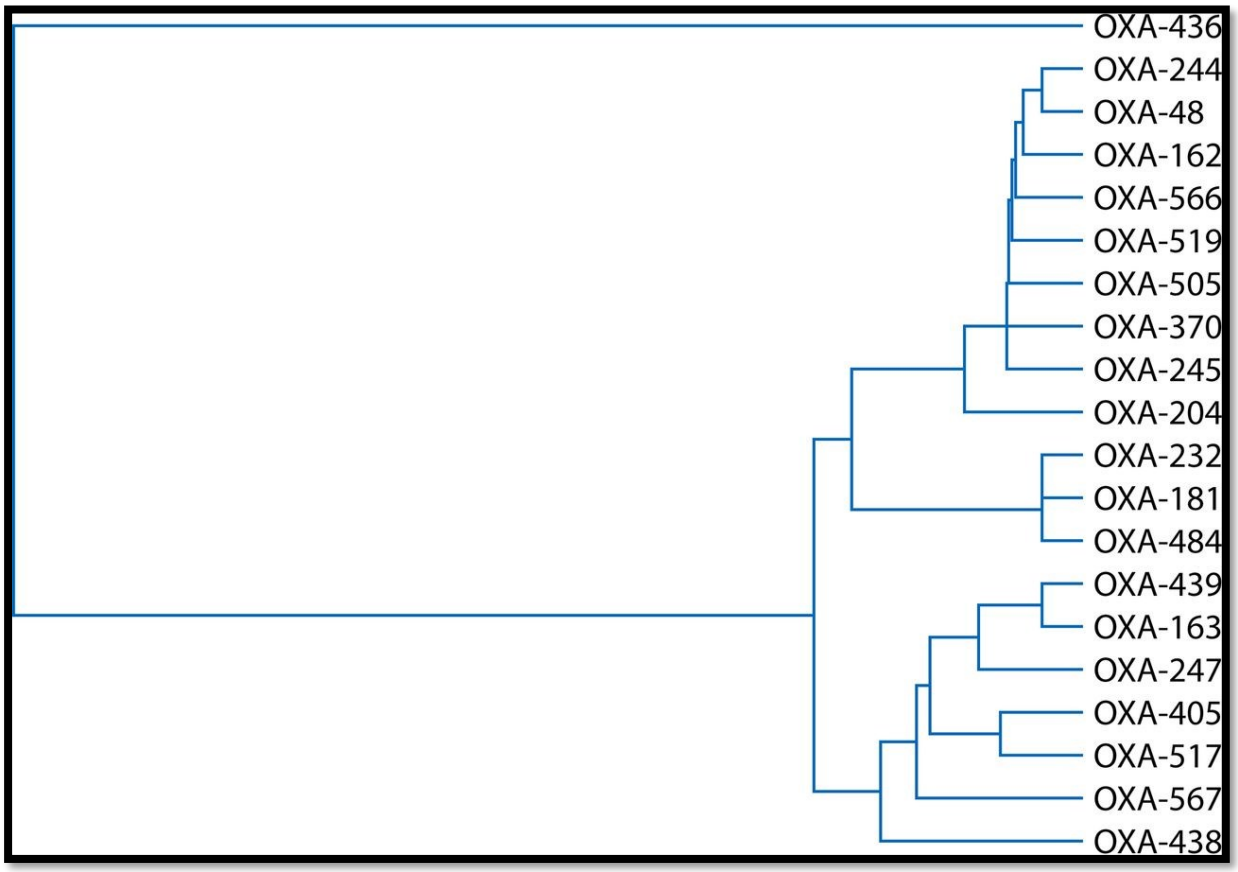

Figure 9: Phylogenetic tree on OXA-48-like [143].

Since the initial identification, studies on the *bla*$_{\text{OXA-48}}$ dynamics have been conducted and found that this gene was associated with IS*1999* on the upstream region and another copy of IS*1999* was later discovered in the downstream region, this combination resulted in a composite transposon named Tn*1999* (Figure 10 A) [141, 151]. Furthermore, different variants of these transposons were being detected. For example, Tn*1999.2* which had an IS*1R* inserted within the upstream IS*1999* (Figure 10 B). Another example, Tn*1999.3* characterized by an additional IS*1R*; compared to Tn*1999.2*; but inserted downstream of the *bla*$_{\text{OXA-48}}$ gene. As a result of these ISs, these variants contain a hybrid promoter (IS*1R*/IS*1999*) that increased by 2-folds the hydrolysis against imipenem when conjugated in an *E. coli* strain [152, 153]. Furthermore, o*bla*$_{\text{OXA-48-like}}$

genes detected on plasmids possessed different insertion sequences. For example, the *bla*$_{OXA-181}$ was found downstream of IS*Ecp1*, the resulting transposon (Tn*2013*) performs a single ended transposition [148].

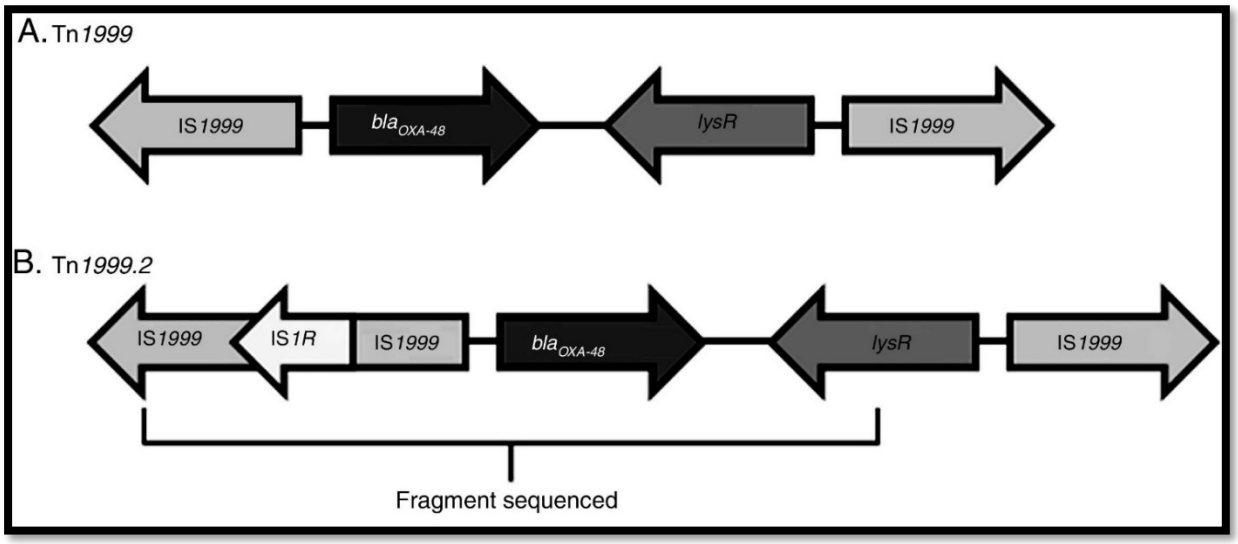

Figure 10: Tn*1999* and Tn*1999.2* structures [154].

Moreover, a variant of Tn*2013* (Figure 11 B), Tn*2016* (Figure 11 C) was characterized and found to be carrying *bla*$_{OXA-204}$, this transposon had an IS*Kpn15* inserted in the upstream IS*Ecl1*, but this insertion did not generate a hybrid promoter therefore not altering the expression of the given gene [137, 155]. Besides being able to transpose, there have been instances where the *bla*$_{OXA}$ genes are stabilized on the plasmids, for example *bla*$_{OXA-232}$ had a big part of the upstream IS*Ecp1* deleted but the promoter was unaffected, this deletion immobilized the transposon [142].

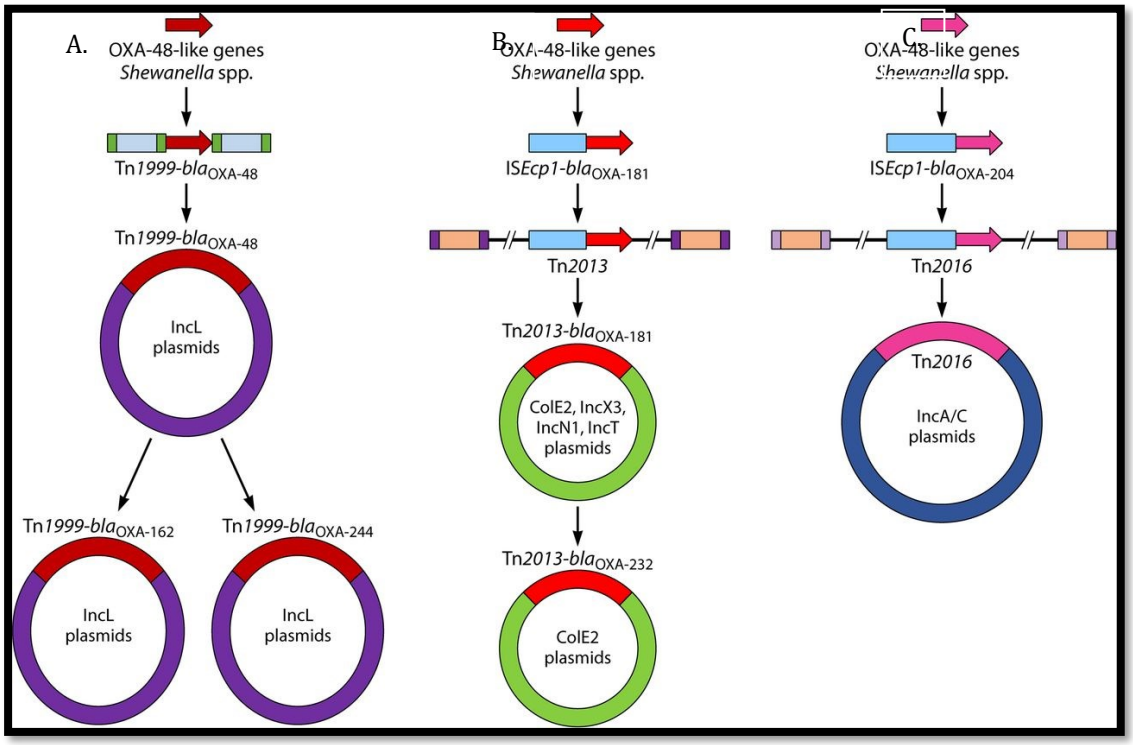

Figure 11: Evolution and origin of OXA-48-like enzymes.

A, Data presentation for OXA-48, OXA-162, and OXA-244; B, for OXA-181 and OXA-232; C, OXA-204 [143].

Plasmids were found to be the main driver for disseminating the *bla*$_{OXA}$ genes, for instance, Tn*1999* carrying the *bla*$_{OXA-48}$, or *bla*$_{OXA-162}$, or *bla*$_{OXA-244}$ (found to be derivatives of *bla*$_{OXA-48}$ given that they only diverge by one amino acid substitution) genes were found to be predominantly transposing into IncL plasmids [143]. Moreover, Tn*2013* carrying *bla*$_{OXA-181}$, or *bla*$_{OXA-232}$ (derivative of *bla*$_{OXA-181}$ by a single amino acid substitution) were found to be predominantly transposing in ColE2, IncN1, IncX3, and IncT plasmids [156]. Finally, Tn*2016* was harbored mainly on plasmids named as p204-B or p204-B-like belonging to IncA (A/C$_1$), IncA (A/C$_2$) or IncC plasmids groups [155, 157-160].

In the Czech Republic, a study conducted between 2013 and 2015 a total of 52 CPEs were detected of which 24 were OXA-48-like producers, 20 were identified as *K. pneumoniae*, 3 were identified as *E. coli*, and 1 was identified as *E. cloacae*. All isolates were resistant to piperacillin-tazobactam and piperacillin, 17 isolates demonstrated resistance against to ciprofloxacin, 16 to Gentamicin, for tigecycline 6 showed resistance, while against amikacin only 5 demonstrated the resistance. Furthermore, the *K. pneumoniae* sequence types belonged to STs that were previously reported to be associated with OXA-48-like production. Interestingly in this study, the first incidence of OXA-48-like/NDM co-production was reported in the Czech Republic, where the isolate was an ST15 *K. pneumoniae* co-producing OXA-232 and NDM-1. All *bla*$_{OXA-48}$ carrying isolates harbored the gene on an IncL plasmid, the isolate carrying *bla*$_{OXA-181}$ was found to be linked with IncX3, and finally the isolate positive for *bla*$_{OXA-232}$ was harbored by a ColE2-type plasmid [161]. This study also detected a new Tn*1999* variant Tn*1999.5* carrying the *bla*$_{OXA-48}$ [161].

3.2.3.3 Other Carbapenemase OXA enzymes:

The OXA-23 enzyme, the main carbapenemase in *Acinetobacter* spp. This enzyme confers resistance to all β-lactams. Furthermore, the *bla*$_{OXA-23}$ was detected on conjugative plasmids of the GR6 incompatibility type and found to be associated with IS*Aba1* (Tn*2006*, or Tn *2008*), and also was associated with IS*Aba4* like in transposon Tn*2007* [162-165]. Although this enzyme has been associated mainly with *Acinetobacter* spp, in 2002 this enzyme has been reported in 10 *P. mirabilis* isolates from a French hospital over the period spanning from 1996-1999. All the detected isolates were clonally similar [166]. In most cases of *P. mirabilis* harboring *bla*$_{OXA-23}$, the gene was found to be carried by the chromosome, but in 4 isolates, the gene was found on an untypable plasmid carried by an *Aba*R4-like element [167]. This element contains a Tn*2006* creating a complex transposon that is commonly found on *A. baumanii* chromosome [168].

The OXA-58 enzyme, also frequently identified in *Acinetobacter* spp. It was first identified in 2003 in France, later reported worldwide in *Acinetobacter* spp [162, 169]. Furthermore, Reports of *bla*$_{OXA-58}$ in Enterobacterales from Belgium, Germany, and Poland were published [170-172]. Further analysis showed that the isolates from Germany and Poland carried the gene on untypable plasmids of ~6.2 kb size [170, 171]. The Belgian isolate, the gene was reported on the chromosome but remarkably was a tandem repeated region with *ampC* genes [172]. In all of these isolates, similarly to Acinetobacter spp. the *bla*$_{OXA-58}$ gene was

found to be flanked by IS*Aba3*-like elements, these mobile elements were previously described as containing the promoter of *bla*$_{\text{OXA-58}}$ in *Acinetobacter* spp [173].

The *bla*$_{\text{OXA–24/–40}}$ gene was discovered in Acinetobacter baumannii isolates from Spain and later characterized in many countries. Till now, just one description of P. mirabilis that produces OXA-24 has been published. The genetic background of *bla*$_{\text{OXA–24/–40}}$ remains unknown, save for the inability to transmit carbapenemase by conjugation [174, 175].

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List of Figures:

4 Materials and Methods:

Isolates are identified by matrix-assisted laser desorption ionization-time of fight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper sofware (Bruker Daltonics, Bremen, Germany), Carbapenemase activity is tested by MALDI-TOF and 5 disk synergy test, Carbapenemase genes detection by PCR for the major carbapenemases (KPC, VIM, IMP, OXA-48, NDM), antibiotic MIC tested and evaluated according to the EUCAST, Conjugation assays are performed to check for the ability of the plasmids to conjugate, Transformation assays are performed in the case of non-conjugative plasmids to test the impact of the plasmid on the MIC values, WGS was performed on specific isolates either Short-reads (Illumina) or Long-reads (PacBio). Downstream analysis after WGS includes Integron analysis, Phylogeny, Single nucleotide polymorphism detection, and Comparison of the MDR region.

Method	**Publication number**
Bacterial isolates, Carbapenemase production and MIC breakpoints	1, 2, 3, 4
Conjugation and Transformation assays	1, 2
Short-reads sequencing and analysis	1, 3, 4
Long-reads sequencing and analysis	1, 2, 3, 4
Integron analysis	3
MDR region analysis	1, 2, 3, 4
Phylogeny	1, 4
SNPs calling	1, 4

5 List of publications included in the Thesis

5.1 Publication nb.1: Evidence of an epidemic spread of KPC-producing Enterobacterales in Czech hospitals

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Evidence of an epidemic spread of KPC-producing *Enterobacterales* in Czech hospitals

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The aim of the present study is to describe the ongoing spread of the KPC-producing strains, which is evolving to an epidemic in Czech hospitals. During the period of 2018–2019, a total of 108 KPC-producing Enterobacterales were recovered from 20 hospitals. Analysis of long-read sequencing data revealed the presence of several types of *bla*$_{KPC}$ -carrying plasmids; 19 out of 25 *bla*$_{KPC}$-carrying plasmids could be assigned to R (n = 12), N (n = 5), C (n = 1) and P6 (n = 1) incompatibility (Inc) groups. Five of the remaining *bla*$_{KPC}$ -carrying plasmids were multireplicon, while one plasmid couldn't be typed. Additionally, phylogenetic analysis confirmed the spread of *bla*$_{KPC}$ -carrying plasmids among different clones of diverse Enterobacterales species. Our findings demonstrated that the increased prevalence of KPC-producing isolates was due to plasmids spreading among different species. In some districts, the local dissemination of IncR and IncN plasmids was observed. Additionally, the ongoing evolution of *bla*$_{KPC}$ -carrying plasmids, through genetic rearrangements, favours the preservation and further dissemination of these mobile genetic elements. Therefore, the situation should be monitored, and immediate infection control should be implemented in hospitals reporting KPC-producing strains

Carbapenem-resistant Enterobacterales (CRE) incidence have increased, causing worldwide public-health concern due to their rapid global dissemination and limited treatment options. Carbapenemases are enzymes able to hydrolyse almost all β-lactam antibiotics including carbapenems, one of the last drugs of choice. Carbapenemases are divided into different groups depending on their structure and hydrolytic activity[1]. *Klebsiella pneumoniae* carbapenemase (KPC) is the most predominant β-lactamase of class A carbapenemases. The KPC-type carbapenemases hydrolyse a wide variety of β-lactam antibiotics such as cephalosporins, penicillins and carbapenems[2].

The *bla*$_{KPC}$ gene was first identified in 1996 in North Carolina, USA, harboured by a K. pneumoniae isolate[3]. Later reports presented the monoclonal dissemination of KPC-producing isolates across America that was

attributed to sequence type 258 (ST258) K. pneumoniae, as the predominant lineage[4]. Subsequently, KPC producers emerged in European countries, becoming highly endemic in some countries, especially in Greece and Italy[5, 6]. Other European countries had confirmed very few cases of *bla*$_{KPC}$ up to 2013 according to the EuSCAPE p roject7. Even though KPC-type carbapenemases have been mostly associated with K. pneumoniae isolates, there are also reports of other bacterial species harbouring *bla*$_{KPC}$ -like genes, including *Escherichia coli*, *Citrobacter freundii*, *Klebsiella oxytoca* and other Enterobacterales, and *Pseudomonas aeruginosa*[8, 9].

The *bla*$_{KPC}$-like genes are most commonly found on the 10 kb transposon Tn*4401* and its isoforms[10, 11]. Until now, there are 9 isoforms of Tn*4401* (a to i)[12]. Due to the high mobilization efficiency of Tn*4401*, *blaKPC*-like genes have been identified on several plasmids belonging to different incompatibility (Inc) groups[13].

In the Czech Republic, the first case of KPC-producing *K. pneumoniae* was identified in 2009[14]. This strain producing KPC-2, was collected from a patient repatriated from a hospital in Greece. Shortly after the first report, an outbreak of KPC-3-producing *K. pneumoniae*, belonging to ST512, was observed in another Czech hospital, with the index case being a patient repatriated from Italy. All those isolates harboured transposon isoform Tn*4401a*, carried on IncFIIK2 pKpQIL-like plasmids[15]. Another ten KPC-2-producing Enterobacterales, mainly of the species *C. freundii*, were recovered in the University Hospital of Hradec Kralove (Czech Republic)[16], during the period 2014–2016. Interestingly, sequencing revealed the presence of three plasmid types with the Tn*4401a* transposon. The first type comprised an IncR backbone and a KPC-2-encoding multidrug resistance (MDR) region, while the second type were derivatives of the first type carrying an IncN3-like segment. Finally, the third type was IncP6 plasmids sharing the same KPC-2-encoding MDR region with the two other types.

However, a significant increase in the number of KPC-producing isolates, referred to our laboratory from Czech hospitals, was observed since 2018. The aim of the present study is to describe the ongoing spread of the KPC-type producers, which is evolving to an epidemic in Czech hospitals, during the period of 2018–2019.

Results:

KPC-producing Enterobacterales. During 2018–2019, a total of 490 Enterobacterales isolates with a meropenem MIC of > 0.125 µg/ml were referred to the National reference laboratory for antibiotics (Prague) or to the Biomedical Center (Pilsen) from 55 laboratories. All *bla*$_{KPC}$-positive isolates (108) were subjected to further analysis described below. Distribution of laboratories is shown in Figure S1. Among them, 26 of the isolates were identified to be *K. pneumoniae*, 24 were identified to be *C. freundii*, 18 were identified to be Enterobacter cloacae complex, 14 were identified to be *Proteus mirabilis*, 11 were identified to be *Morganella morganii* and 10 were identified to be *E. coli*. The five remaining KPC-producing isolates belonged to the bacterial species, *Citrobacter farmeri* (n = 1), *Enterobacter aerogenes* (n = 1), *K. michiganensis* (n = 2) and *Klebsiella variicola* (n = 1) (Figure S2).

Analysis of short-read sequencing results. Forty-nine out of 108 KPC producers, selected as representatives of all different hospitals, bacterial species and susceptibility profiles, were characterized by short-read

sequencing using MiSeq (Illumina) platform. Based on short-read data, 44 of the 49 sequenced isolated harboured the *bla*$_{KPC-2}$ allele (Table S1), while the five remaining isolates carried the *bla*$_{KPC-3}$ gene. The *bla*$_{KPC-3}$ allele was identified among 3 *K. pneumoniae*, *1 K. michiganensis* and 1 *E. coli* isolates. Beside species-specific chromosomal β-lactamases, most of the clinical isolates also carried genes encoding OXA-1/9 oxacillinases (n = 37) and/ or TEM-1 penicillinases (n = 34). The *bla*$_{CTX-M-15}$ gene was found among 2 *Enterobacter* and 5 *K. pneumoniae* isolates, while 4 out of 5 *P. mirabilis* harboured the *bla*$_{CTX-M-14}$ gene. Additionally, 4 out of 7 *Enterobacter* isolates cocarried the carbapenemase-encoding gene *bla*$_{VIM-4}$. All sequenced isolates exhibited a wide variety of resistance genes conferring resistance to aminoglycosides, sulfonamides, trimethoprim, macrolides, streptogramin B, fosfomycin (low-level resistance), fluoroquinolones, chloramphenicol, tetracyclines, and/or rifampicin (Table S1).

WGS data revealed that *C. freundii* isolates belonged to sequence types ST65 (n = 6), ST580 (n = 3), ST98 (n = 2) and ST8 (n = 1) (Table S1). ST98 *C. freundii* isolates producing KPC-2 carbapenemase were previously recovered from critically ill patients hospitalized in Germany[17], while ST8 *C. freundii* expressing a VIM-4 isoenzyme were identified in Poland[18], in 2013. On the other hand, the novel ST580 was a single allele variant of ST142, which was previously associated with KPC-2 production in isolates from the University Hospital of Hradec Kralove (Czech Republic)[16]. The isolates belonging to *E. cloacae* complex were assigned to ST133 (n = 4) and ST421 (n = 3), which haven't been previously associated with the production of KPC-2 carbapenemase. Additionally, in silico hsp60 typing of the genome sequences showed that four *Enterobacter* isolates belonged to the species *Enterobacter hormaechei*[19]. The *K. pneumoniae* isolates included eight STs. Seven KPC-2-producers were distributed in ST101 (n = 4) and ST11 (n = 3). The remaining KPC-2-producing K. pneumoniae isolates belonged to unique STs (ST13, ST17 and ST147), while the K. pneumoniae isolates, which produced the KPC-3 enzyme, were ST307, ST512 and ST846. ST11, ST101, ST147, ST307 and ST512 have been previously associated with the spread of KPC resistance mechanism and have been considered as 'high risk clones'[20, 21]. Finally, the *E. coli* and *K. michiganensis* (closely related to *K. oxytoca*) isolates were assigned to diverse STs, as shown in Table S1. Since MLST schemes do not exist for *M. morganii* and *P. mirabilis* isolates, phylogenetic clusters for the respective isolates were determined based on core-genome alignment (see below), using the Harvest suite[22].

Characterization of *bla*$_{KPC}$-carrying genetic units. Based on short-read data, 25 KPC-producing isolates were selected to be sequenced by Sequel I platform, in an attempt to close plasmid sequences. All the 25 isolates showed resistance to cephalosporins and ertapenem while (except for *P. mirabilis* isolates) remained susceptible to colistin. Some variation in MIC values were noticed, however it is due to the different antibiotic resistance genes content found in each isolate (Table S3). Analysis of long-read sequencing data revealed the presence of several *bla*$_{KPC}$-carrying plasmid sequences belonging to different Inc groups and presenting diverse sizes (Table S2). Based on PlasmidFinder analysis of plasmid sequences, 19 out of 25 blaKPC-carrying plasmids could be assigned to R (n = 12), N (n = 5), C (n = 1) and P6 (n = 1) incompatibility (Inc) groups (Figure S3). Five of the remaining *bla*$_{KPC}$-carrying plasmids were multireplicon, while one plasmid couldn't be typed by the database. All plasmids, except IncN replicons, contained the Tn*4401a* isoform of the Tn*4401* transposon, which is similar to that described in plasmid pNYC, lacking 100 bp upstream of blaKPC gene[11].

Three out of 12 blaKPC-carrying plasmids, belonging to IncR group, were ≃54 kb in size, while the nine remaining IncR plasmids sized ≃89 kb. The IncR plasmids that were ≃54 kb in size were derivatives of the IncR KPC-2-encoding plasmid pCfr-31816cz (Fig. 1a), which was characterized during an outbreak of KPC-2-producing Enterobacterales in a Czech hospital (Hradec Kralove)[16]. However, they differed from pCfr-31816cz by the presence of an additional 9232-bp sequence (nt 7286 to 16,517; GenBank accession no. CP070521) encoding CcdAB toxin-antitoxin system, and IncFIIA RepA and Ssb proteins. On the other hand, the IncR plasmids that were ≃89 kb in size showed high degrees of similarity to each other and to the previously described plasmid pCfr-36049cz (Fig. 1b). Plasmid pCfr36049cz was characterized during the KPC-2 outbreak that took place in Hradec Kralove[16], during 2014–2016. Similar to pCfr-36049cz, the latter plasmids were fusion derivatives of IncR

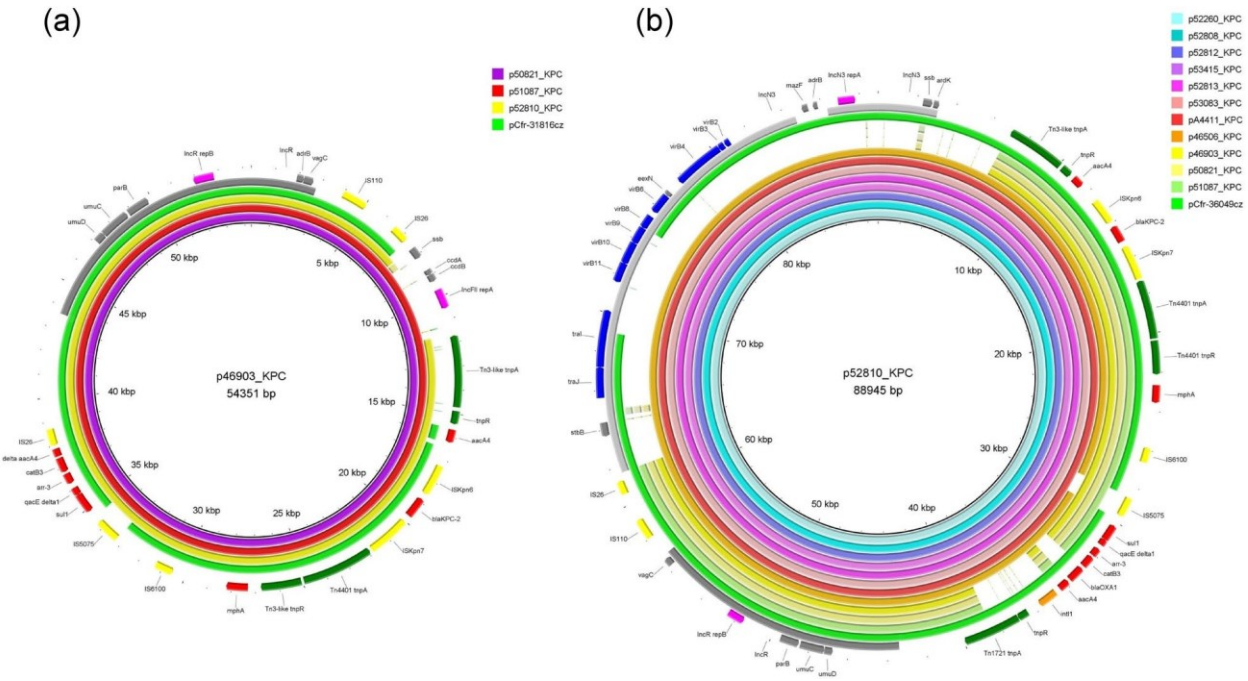

Figure 1. BRIG comparison of IncR KPC-encoding plasmids characterized from Enterobacterales isolates recovered from Czech hospitals.

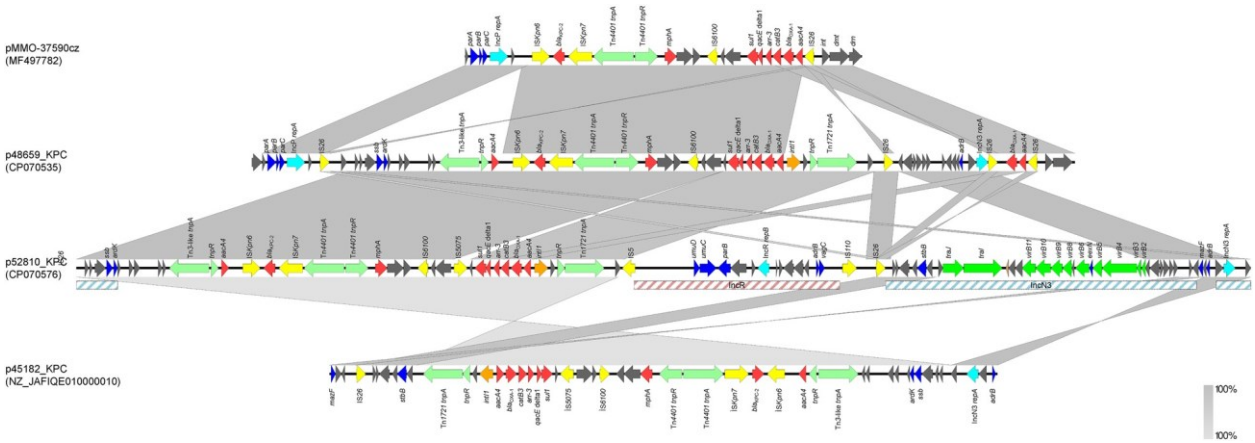

Figure 2. Linear comparisons of the KPC-encoding plasmids p48659_KPC and p45182_KPC. Arrows show the direction of transcription of open reading frames (ORFs). Resistance genes are shown in red. IS elements and transposases are shown in yellow and light green, respectively. *intI1* genes are shaded purple. Genes encoding replication, stability and transfer systems are shown in aqua, blue and green colors, respectively. The remaining genes are shown in gray. Homologous segments (representing ≥ 85% sequence identity) are indicated by gray shading.

bla$_{KPC-2}$-positive plasmids and an IncN3-type-derived segment. However, unlike in pCfr-36049, a complete IncN3 transfer system was found, explaining the ability of pA4411_KPC, p46506_KPC, p52260_KPC, p52808_KPC, p52810_KPC, p52812_KPC, p52813_KPC, p53083_KPC and p53415_KPC to transfer via conjugation. Of note, the IncR plasmid p52813_KPC carried the blaKPC-3 allele, indicating the ongoing evolution of the determinants encoding KPC carbapenemases.

Plasmid p48659_KPC is a fusion derivative of the p52810_KPC and pMmo-37590cz (Fig. 2). pMmo-37590cz is an IncP6 KPC-2-encoding plasmid that was also characterized during the KPC-2 outbreak in Hradec Kralove hospital[16]. Plasmid p48659_KPC contains a 50,603-bp sequence (nt 5917 to 56,519) encoding KPC-2, which is identical to a partial sequence of p52810_KPC. The remaining 11,723-bp sequence of p48659_KPC consists of one segment sharing extensive similarity with sequences carried by pMmo-37590cz. This segment included the IncP6 replication gene *repA*, the partitioning genes, *parA*, *parB*, and *parC*, and genes encoding a DNA invertase/recombinase (int), a deoxymethyltransferase (dmt), and a DNase (drn) of type II restriction module. Sequence analysis demonstrated that the plasmid p45182_KPC, which was not typed by PlasmidFinder, is 50,582 bp in size and is a derivative of p52810_KPC (Fig. 2). Only two differences between the two plasmids were observed. A 26,069-bp segment (nts 34,454 to 60,522 in p52810_KPC) including IncR plasmidic backbone, a Tn*1721*-like fragment (consisting of the 38-bp inverted repeat of the transposon, *tnpA*, *tnpR*, and *tnpM*), and *intI1* gene of the integron In37, was not present in p45182_KPC. In addition, a second fragment (nts 65,142 to 83,473 in p52810_KPC), being 18,332 bp in size, that contained *vir2/3/4/9/10/11* region of IncN3-like plasmids was also absent from p45182_KPC, probably explaining the inability of the plasmid to conjugate.

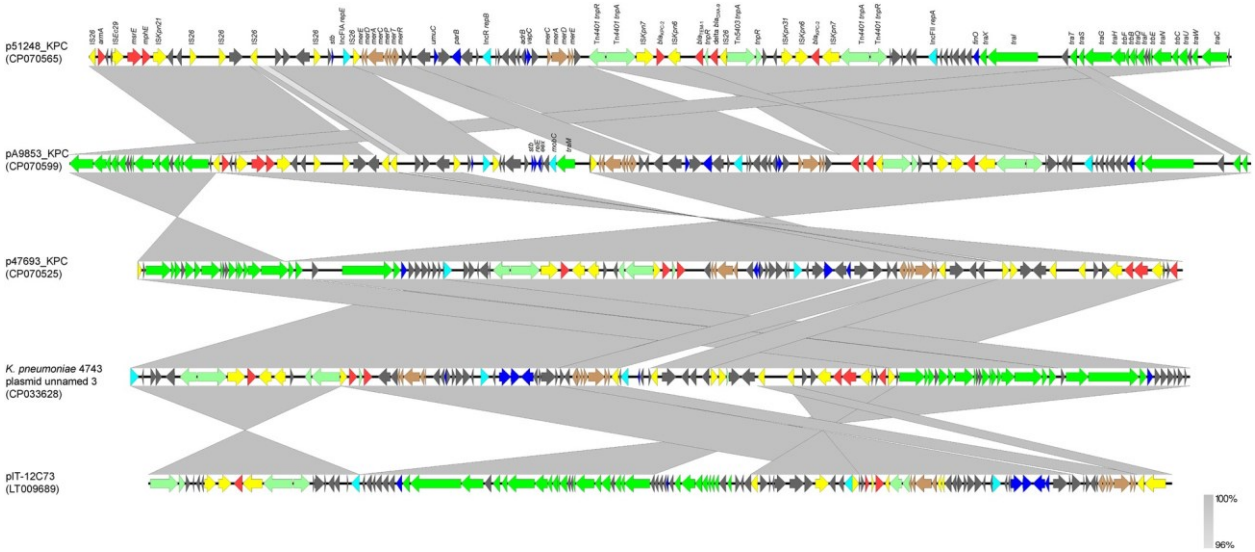

Figure 3. Linear comparisons of the multireplicon KPC-encoding plasmids p51248_KPC, pA9853_KPC and p47693_KPC. Arrows show the direction of transcription of open reading frames (ORFs). Resistance genes are shown in red, while genes involved in mercury resistance are shaded brown. IS elements and transposases are shown in yellow and green, respectively. *intI1* genes are shaded purple. Genes encoding replication, stability and transfer systems are shown in aqua, blue and green colors, respectively. The remaining genes are shown in gray. Homologous segments (representing ≥ 85% sequence identity) are indicated by gray shading.

The IncN blaKPC-2-positive plasmids, which were assigned to ST15 based on the pMLST (https://cge.cbs.dtu.dk/services/pMLST/), comprised the plasmidic backbone and a multidrug resistance (MDR) region inserted downstream the *fipA*. The IncN plasmidic backbone contained a replication region (*repA*), a transfer system (*traA/B/C/D/N/E/O/F/G*), a stability operon (*stbA/B/C*) and an antirestriction system (*ardA/B*). The MDR region of IncN plasmids, which ranged from 21,011 to 31,420 bp in size, harboured a Tn*4401*-derived fragment of 2833-bp, encoding KPC-2 carbapenemase. In comparison to Tn*4401b*, the Tn*4401*-derived sequence (designated Tn*4401j*) had a deletion of 217 bp found upstream of the bla_{KPC-2}. The Tn*4401*-derived fragment was disrupted by a Tn*3*-like sequence, 111 bp upstream of the bla_{KPC-2}. The Tn*3*-like sequence was composed of the inverted repeat (IR) of the transposon and the bla_{TEM-1} resistance gene. A similar bla_{KPC-2}-carrying genetic environment has been previously identified in the IncN plasmid pCF8698_KPC2, characterized from a *C. freundii* strain isolated in Germany (GenBank accession no. LN610760) (Figure S4). The MDR region of the IncN plasmids exhibited additional genes conferring resistance to aminoglycosides, sulfonamides, trimethoprim, macrolides, and/or fluoroquinolones (Table S2, Figure S4).

Plasmid p49969_KPC, typed as IncC based on PlasmidFinder, exhibited highest similarity with the OXA204-encoding plasmid pCf3880 (74% coverage, 99.87% identity) (Figure S5). The pCf3880, which was an IncFII/FIB/C2 hybrid plasmid, was characterized from a *C. freundii* isolated from a hospital in Canada[23]. Unlike pCf3880, plasmid p49969_KPC carried the carbapenemase-encoding gene, bla_{KPC-2}, which resulted from the acquisition of a 29,121-bp fragment (nt 111,204 to 140,234 in GenBank accession no. CP070549) showing extensive similarity to IncN plasmid p48846_KPC. The IncN-derived fragment was bound by two copies of the insertion sequence IS*26*, in parallel orientation, forming a composite transposon. Plasmid p49969_KPC carried, also, the resistance genes bla_{TEM-1B}, *aac(6')-Im*, *aac(3)-IId and aph(2'')-Ib*.

Plasmids pA9853_KPC, p47693_KPC and p51248_KPC, which were characterized from ST101 *K. pneumoniae* isolates (Table S2), exhibited extensive similarity to pIT-12C7320 and plasmid unnamed 3 (GenBank accession no. CP033628) (Fig. 3). Plasmid pIT-12C73 which was also characterized from a ST101 *K. pneumoniae*, isolated in Italy in 2011, was a multireplicon IncFIIK2-IncR KPC-encoding plasmid. Plasmids pA9853_KPC and p51248_KPC differed from pIT-12C73 by acquisition of a 3254-bp fragment containing the IncFIA *repE* gene. Plasmid pA9853_KPC harboured an additional 9295-bp sequence, being identical to ColE1-like plasmid pColRNAI-Kp1-1 (GenBank accession no. LC505458). Comparative analysis suggested that IS*26*-mediated recombination events likely played a major role in acquisition of fragments of diverse origin. Similarly, to pIT12C73, apart from *bla*$_{KPC-2}$, plasmids pA9853_KPC, p47693_KPC and p51248_KPC carried also *bla*$_{TEM-1}$, armA, *mphE*, *msrE* resistance genes (Fig. 3). Interestingly, a duplication of the KPC-2-encoding transposon, Tn*4401a*, was found in plasmid p51428_KPC.

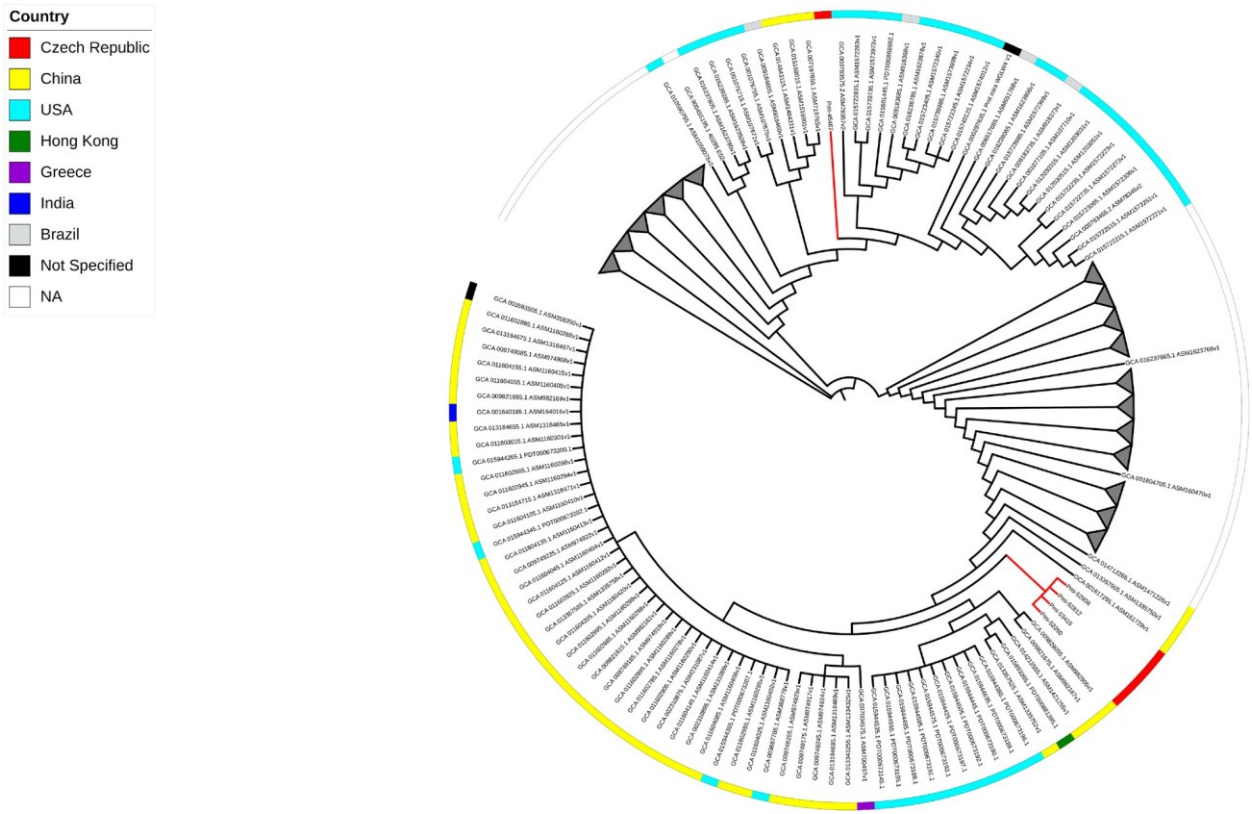

Figure 4. SNPs-based phylogeny of the five *P. mirabilis* isolates with 582 genomes downloaded from NCBI database. Red nodes indicate the Isolates from the study. Grey triangles indicate collapsed nodes.

Plasmid p51483_KPC, which contained replicons FIBK and FIIK, showed highest similarity to KPC-2-encoding plasmid pRIVM_C008981_1 (96% coverage; 99.93% identity) (Figure S6a), characterized from a *K. pneumoniae* isolate collected in the Dutch national surveillance[24]. Similarly, to pRIVM_C008981_1, apart from regions responsible for conjugative transfer (*traX/I/D/T/S/G/H/F/B/Q/C/U/W/V/P/K/Y/M*) and plasmid maintenance (*psiA/B, parA/B* and *umuD/C* operons), p51483_KPC carried genes *silE/S/R/C/B/A/P* encoding a silver exporting ATPase, *pcoA/B/C/D/R/E* involved in copper resistance, *arsH/D/A/C/B/A/D/R* conferring arsenic resistance, and *fecI/R/A/B/C/D/E* implicated in Fe (3+) dicitrate transport. In addition to *bla*$_{KPC-2}$, plasmid p51483_KPC contained *bla*$_{TEM-1}$, *aadA2*, *aph(3')-Ia*, *catA1*, *dfrA12* and *mphA* resistance

genes. Finally, plasmid p51059_KPC, isolated from a ST512 *K. pneumoniae*, showed high similarity to IncFIIK2 KPC-2 encoding plasmids pGR-1504 (99% coverage; 99.97% identity) and pIT-01C22 (coverage 99%; identity 99.96%) (Figure S6b) characterized from two endemic settings, Greece and Italy, during 2010–201120. Plasmids pGR-1504 and pIT-01C22 were derivatives of the archetypal IncFIIK2 KPC-encoding plasmid pKpQIL, originally described in the ST258 K. pneumoniae Kpn557 isolate[25].

Genomic comparison and relatedness. Sequence data from the 49 sequenced isolates, have been used to investigate their genomic relatedness with global isolates and SNPs based phylogenies have been constructed accordingly. For *P. mirabilis*, the five sequenced isolates were compared against 582 genomes found in the NCBI database (Fig. 4). Four isolates (Pmi-52808, Pmi-52812, Pmi-53415 and Pmi-52260) clustered together forming a clade. These isolates were isolated from the same hospital (NY) and using Pmi-52260 as reference for SNPs detection, Pmi-52808 and Pmi-53415 shared 14 and 9 alterations (snps, del/ins), respectively, while Pmi-52812 shared 117 alterations (Table S4). Lastly, Pmi-45467, isolated in hospital HK, was relatively distant from the rest of the isolates.

Similarly, 92 *M. morganii* available genomes in NCBI database were downloaded to compare them with the five isolates sequenced in this study (Fig. 5). Mmo-48659, isolated in HK hospital, clustered alone in a unique node. However, it was closely related to an isolate from South Africa (790 alteration). On the other hand, four isolates (isolated from three different hospitals; Table S1) clustered together, with Mmo-51087 and Mmo-50821 isolated from the same hospital forming a subclade. For the detection of SNPs among the four isolates of this clade, Mmo-51087 was used as a reference. Mmo-50821 and Mmo-46544 showed 22 and 62 alterations, respectively, while Mmo-46903 222 alterations (Table S4).

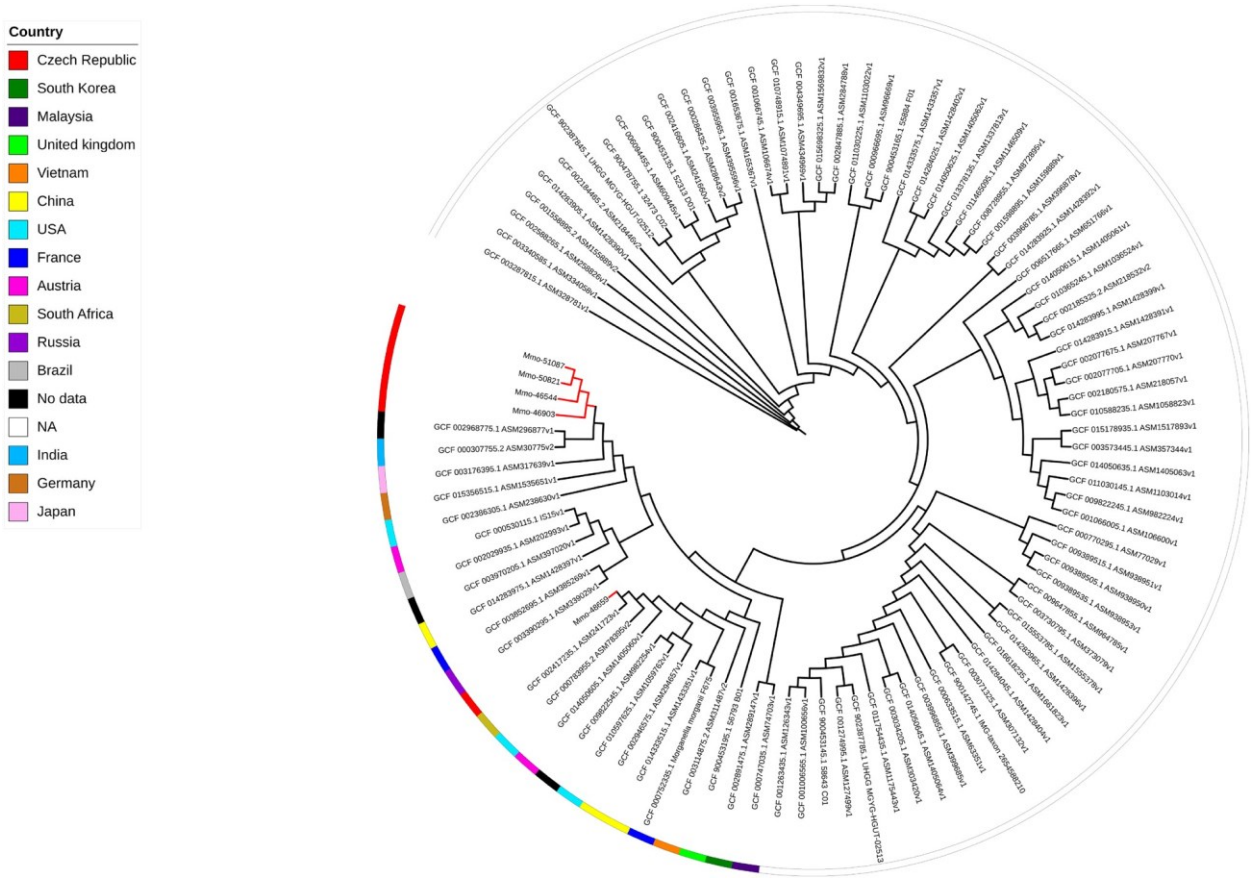

Figure 5. SNPs-based phylogeny of the five *M. morganii* with 92 genomes downloaded from NCBI database. Red nodes indicate the Isolates from the study.

For *C. freundii*, 118 genomes were downloaded from the NCBI database to compare them with the 12 sequenced isolates (Fig. 6). The genomes of three isolates (Cfr-46338, Cfr-49942 and Cfr-48658), which belonged to ST580 and were recovered from HK hospital, clustered together forming a clade. SNPs detection among these isolates showed that Cfr-49942 and Cfr-46338 had 93 and 111 alterations, respectively, compared to Cfr-48658. In a closely related clade, another six genomes from ST65 isolates Cfr-50935, Cfr-48846, Cfr-51238, Cfr-47462,

Cfr-48294 and Cfr-47299 clustered together. SNPs detection when compared to Cfr-50935 showed that Cfr48846 had 25 alterations while Cfr-51238, Cfr-47462, Cfr-47299 and Cfr-48294 had 47, 57, 88 and 99 respectively (Table S4). On the other hand, the genomes of the two ST98 isolates, Cfr-48736 and Cfr-49141, clustered together in a considerable distant clade. These isolates are clustered together with other ST98 *C. freundii* isolates from the USA and UK. SNPs detection showed that Cfr-48736 had 28 alterations when compared to Cfr-49141. Finally, the isolate Cfr-49969, which was assigned to ST8, resulted in a unique node.

For *Enterobacter hormaechei*, 126 genomes were downloaded from the NCBI database and were compared with the seven isolates sequenced during this study (Fig. 7). The isolates clustered in two clades. The first clade contained four isolates (Ecl-49142, Ecl-48587, Ecl-48293 and Ecl-49583). Ecl-48293 was used as a reference genome for SNPs detection among these four isolates. Ecl-49142 had 26, Ecl-48587 had 34 and Ecl-49583 had 32 alterations. All ST133 isolates recovered from Czech Republic, South Africa, Japan, Australia and Egypt were grouped in a unique cluster. Additionally, the other cluster contained the three

isolates (Ecl-51693, Ecl-51846 and Ecl-52075) which were ST421. For SNPs detection, Ecl-51846 was used as a reference, showing that Ecl-52075 and Ecl-51692 had 12 and 14 alterations, respectively (Table S4).

For *K. pneumoniae*, 732 genomes were downloaded from the NCBI database to compare them with the 13 sequenced isolates (Fig. 8). Isolates Kpn-51835, Kpn-47158, Kpn-51483, Kpn-53027, Kpn-52813 and Kpn-51069, which were assigned to diverse STs, formed a unique distinct node each. One clade consisting of Kpn-47693, Kpn-O141 and Kpn-A9853 was in close proximity with Kpn-51248 in the neighbouring cluster. Using Kpn-47693 as a reference genome for SNPs detection, Kpn-O141, Kpn-A9853 and Kpn-51248 had 25, 22 and 100 alterations, respectively. The above isolates were grouped together with other ST101 isolates from Italy, USA, Japan, India and South Africa. The last three *K. pneumoniae* isolates (Kpn-52810, Kpn-A4411 and Kpn-45128) clustered together. Using Kpn-52810 as a reference genome for SNPs detection, Kpn-A4411 had 27 alterations, while Kpn-45128 exhibited 816 alterations (Table S4). Interestingly, the last isolates were clustered with isolates from China, Switzerland, India and the USA, which belonged also to ST11.

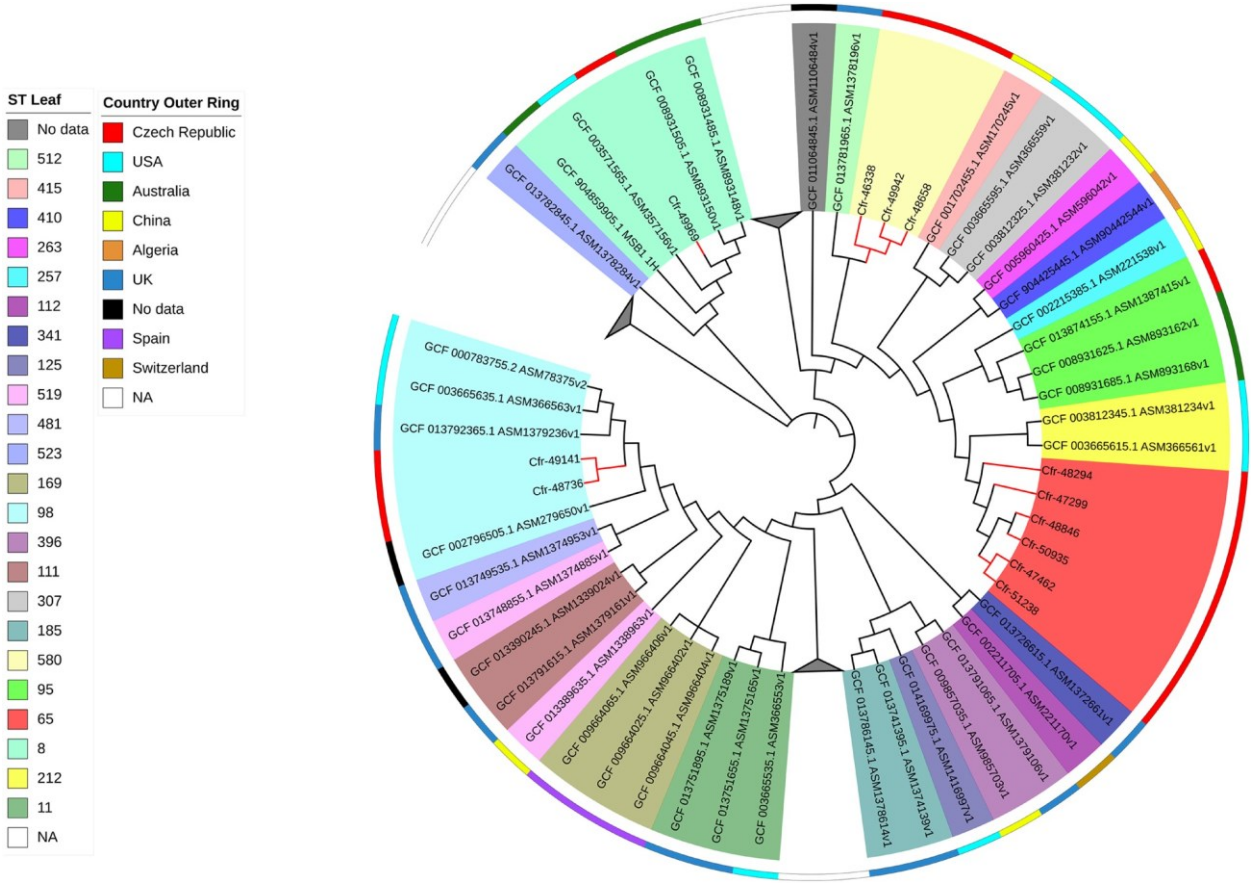

Figure 6. SNPs-based phylogeny of the 12 *C. freundii* with 118 genomes downloaded from NCBI database. Red nodes indicate the Isolates from the study. Grey triangles indicate collapsed nodes.

Discussion:

KPC-producing Enterobacterales represent a major threat of global dimensions for public health. The current study described the change of the epidemiological situation in Czech hospitals, from the sporadic cases or outbreaks to the epidemic spread of KPC-producing isolates (Figure S1). During 2018–2019, 108 KPC producers were recovered from 22 different Czech hospitals located throughout the country.

Additionally, the *bla*$_{KPC}$ gene was found among diverse species and clones of Enterobacterales family (Figure S2).

Phylogenetic analysis indicated that *P. mirabilis* and *M. morganii* isolates, carrying the *bla*$_{KPC}$-like gene, didn't exhibit close relationship with isolates characterized previously from other geographical areas. Additionally, phylogenetic analysis showed that the KPC-2-producing *E. hormaechei* isolates belonged to two distinct clones (Fig. 7), assigned as ST133 and ST421 based on MLST. The ST421 isolates weren't closely related with other isolates analysed, using parsnp software, while ST133 isolates clustered together with isolates from South Africa, Japan, Australia and Egypt. However, ST133 isolates, recovered from other geographical areas, weren't associated with the production of KPC-2 carbapenemase. Regarding *C. freundii* isolates, phylogenetic analysis revealed two main clones, which were assigned to ST65 and ST580 based on MLST. These two clones were distinct to each other and to the isolates included in the analysis. Isolates Cfr-48736 and Cfr-49141 grouped together with other ST98 isolates from the UK and USA, while the isolate Cfr-49969 was clustered with other ST8 isolates from Australia and the USA. However, the later clones have been associated with the production of KPC-2 and VIM-4 carbapenemases in Germany and Poland[17, 18], respectively. On the other hand, 6 out of 13 *K. pneumoniae* isolates characterized by WGS belonged to unique STs. Interestingly, parsnp phylogenetic analysis clustered these isolates with other *K. pneumoniae* isolates, belonging to the same STs, from worldwide. Additionally, the 7 remaining *K. pneumoniae* isolates belonged to two distinct clades. The later clades included ST11 and ST101 isolates from different geographical origins. Among *K. pneumoniae*, the 'high risk' clones, ST11, ST101, ST147 and ST512, that have been previously associated with the spread of KPC resistance mechanism were found[20, 26]. In agreement with recent reports, those data confirm that high-risk clones, other than CC258, currently contribute to spread of KPC resistance mechanism in Europe[24, 25]. Finally, the KPC-producing *E. coli* and *K. michiganensis* isolates belonged to unique STs. These findings underline the ongoing spread of the KPC resistance mechanism among different species and clones.

The analysis of the genetic units carrying the *bla*$_{KPC}$-like genes revealed the presence of a wide variety of plasmids involved in the spread of the KPC resistance mechanism. Some of the observed plasmid-types, like IncFIIK2 pKpQIL, IncFIIK2-IncR pIT-12C73, and IncR-IncN3 pCfr-36049cz, have been previously described to be responsible for the spread of the *bla*$_{KPC}$-like genes[16, 20, 25]. Additionally, some novel emerging plasmid-types, as the IncN pCF8698_KPC2 originally described from Germany (GenBank accession no. CP070521), the IncFIBK/ FIIK pRIVM_C008981_1 firstly characterized from a Dutch collection[24], and the hybrid IncFII/FIB/C2/N plasmid p49969_KPC characterized during this study, were identified to disseminate the *bla*$_{KPC}$-like genes. A few fusion derivatives of the *bla*$_{KPC}$-carrying plasmids described above were observed. These data verify the presence of some successful plasmid lineages spreading the KPC resistance mechanism, but also highlight the ongoing evolution of the mobile genetic elements involved in the dissemination of clinically important resistance mechanisms. For example, IncR plasmids carrying *bla*$_{KPC}$ genes have played a significant role in the spread of the specific resistance mechanism, in the Czech Republic. But, IncR plasmids have also been involved with the spread of other important carbapenemases, like NDM and VIM[27, 28]. Additionally, in agreement with previous studies[20, 25, 29], IncF plasmids are one of the major factors contributing to the worldwide spread of KPC carbapenemases. Moreover, the distribution of the

different plasmid types detected suggests local dissemination with IncR plasmid spreading in middle part of the map especially in Hradec Kralove and Nymburk (Figure S3), while the IncN plasmid spreading in the North West of the Bohemian region. However, Prague seems like the melting pot, in which all plasmid families were detected, indicating the transient admission of patients from surrounding districts to Prague for specialized treatment. Moreover, this is confirmed by the fact that most of the isolated strains in Prague comes from private labs which provides services for many hospitals and long-term care facilities in and outside Prague. This route of dissemination could be explained by the spread of specific plasmid families within the same region, like IncR plasmids, or crossing the borders via travelling, like IncN plasmid from Germany.

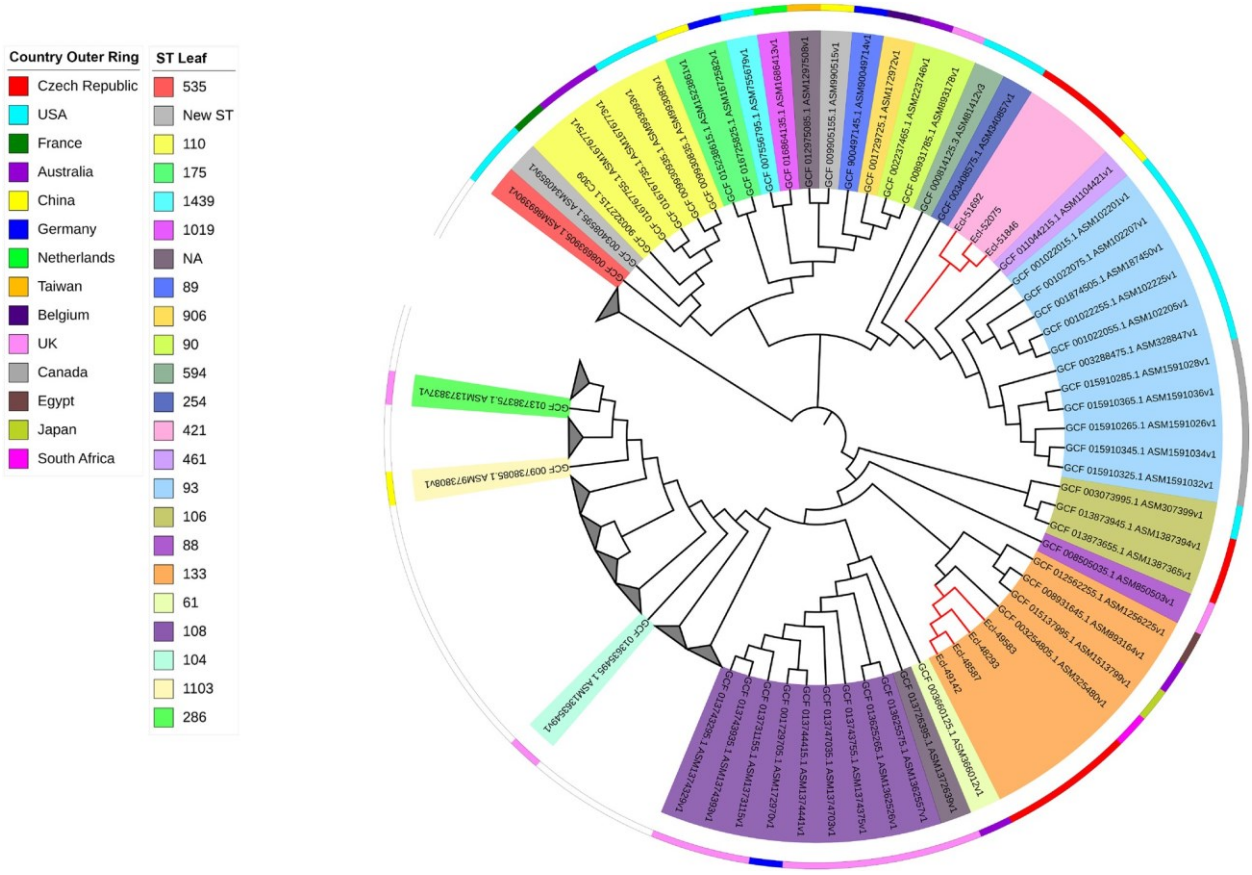

Figure 7. SNPs-based phylogeny of the seven *E. homaechei* with 126 genomes downloaded from NCBI database. Red nodes indicate the Isolates from the study. Grey triangles indicate collapsed nodes.

In conclusion, our results show that the increased prevalence of KPC-producing isolates was due to plasmids being conjugative and spreading among different species and clones. Additionally, the ongoing evolution through genetic rearrangements, observed in *bla*$_{KPC}$-carrying plasmids, favour the preservation and further dissemination of these mobile genetic elements. Therefore, the situation should be monitored, and immediate infection control should be implemented in hospitals reported.

Material and methods:

Bacterial isolates, carbapenemase production and susceptibility testing. From 2018 to 2019, National reference laboratory for antibiotics referred a total of 108 Enterobacterales isolates being PCR positive for

*bla*KPC. Species identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany).

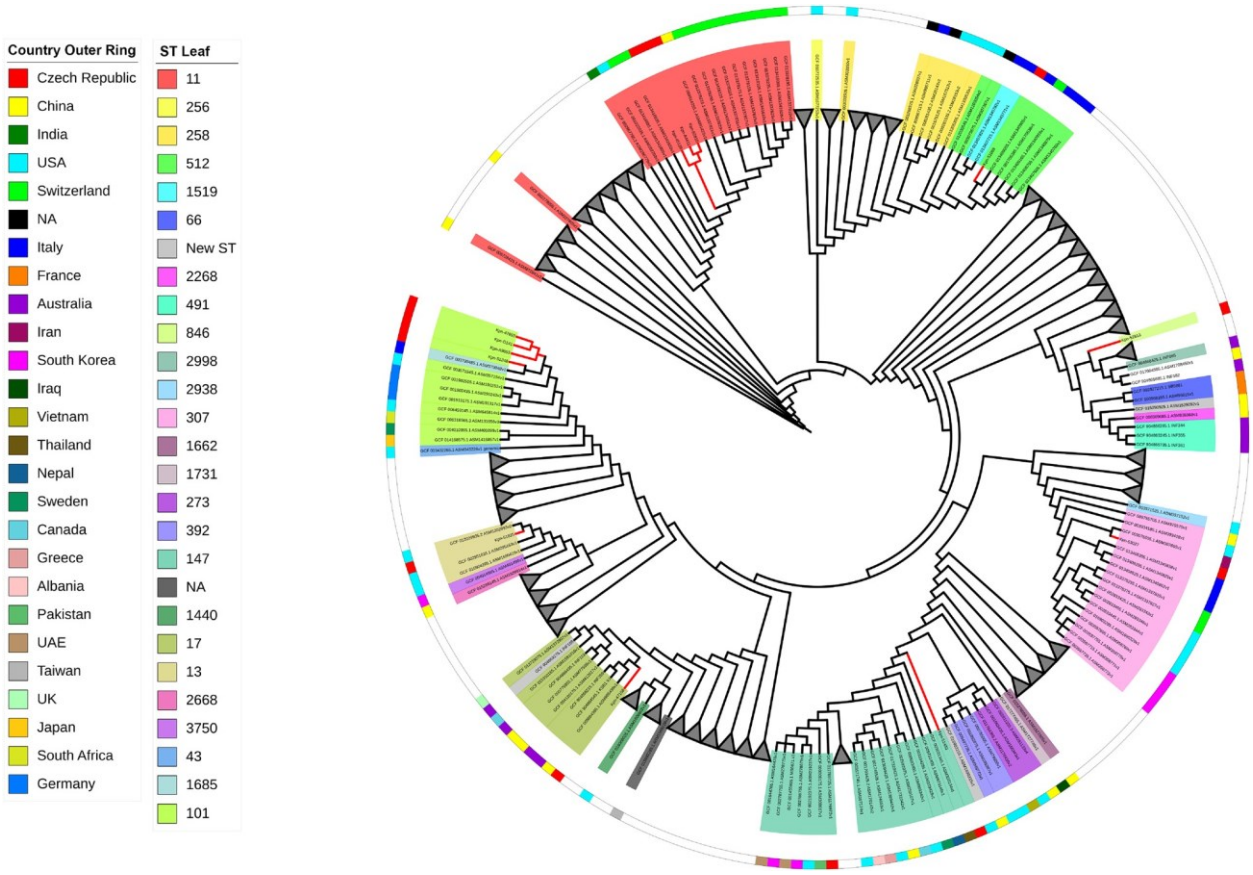

Figure 8. SNPs-based phylogeny of the 13 *K. pneumoniae* with 732 genomes downloaded from NCBI database. Red nodes indicate the Isolates from the study. Grey triangles indicate collapsed nodes.

All isolates were tested for carbapenemase production by the MALDI-TOF MS meropenem hydrolysis a ssay[30]. Additionally, the presence of carbapenemase-encoding genes (*bla*KPC, *bla*VIM, *bla*IMP, *bla*NDM, and *bla*OXA–48-like) was confirmed by PCR amplification[11, 31–33]. Antimicrobial susceptibility was performed using broth microdilution according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Susceptibility data were interpreted according to the criteria (version v11.0) of the EUCAST (http://www.eucast.org/).

Short-read whole genome sequencing. Forty-nine KPC-producing Enterobacterales were selected for complete sequencing, using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). These isolates were selected as representatives of all different hospitals, bacterial species and susceptibility profiles.

The genomic DNAs of the clinical isolates were extracted using the 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[34] and then, assembled by use of the de Bruijn graph-based de novo assembler SPAdes v3.14.0[35].

Map. Maps of the Czech Republic was created using the Leaflet package[36] in R-studio[37] from R-project[38].

Long-read whole genome sequencing. Based on the results of short-read sequencing (see below), twenty-five KPC producers were selected to be sequenced using long-read sequencing technology, to help close the whole plasmid sequences. These isolates were selected as representatives of all different hospitals, bacterial species, STs, replicon profiles and KPC alleles.

Genomic DNA was extracted from the clinical isolates using NucleoSpin Microbial DNA kit (Macherey–Nagel, Germany). Whole genome sequencing (WGS) was performed on the Sequel I platform (Pacific biosciences, Menlo Park, CA, United States). Microbial multiplexing protocol was used for the library preparation according to the manufacturer instructions for Sheared DNA. DNA shearing was performed using the Megaruptor 2 (Diagenode, Liege, Belgium) using long hydropores producing 10 kb long inserts. No size selection was performed during the library preparation. The Microbial Assembly pipeline offered by the SMRT Link v9.0 software was used to perform the assembly and circularization with minimum seed coverage of 30X. Assembled sequences were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP).

Analysis of WGS data. Antibiotic resistant genes, plasmid replicons and multilocus sequence types (MLST) were determined through uploading the assembled sequences to ResFinder 4.1 and CARD[39, 40], PlasmidFinder[41], and MLST 2.0[42], respectively.

For sequence analysis, 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 alignment was done using Mauve v.2.3.1. (http://darlinglab.org/mauve/mauve.html) and BLAST Ring Image Generator (BRIG)[43]. Diagrams and gene organization were sketched using Easyfig v.2.2.2[44].

Transfer of *bla*$_{KPC}$-like genes. Conjugal transfer of *bla*$_{KPC}$-like genes from the clinical strains was carried out in mixed broth cultures[45], using the rifampicin-resistant E. coli A15 laboratory strain as a recipient. Transconjugants were selected on MacConkey agar plates supplemented with rifampicin (150 mg/l) and ampicillin (50 mg/l). Transconjugants were confirmed to be KPC producers by PCR[11] and the MALDI-TOF MS meropenem hydrolysis assay[30].

Phylogenetic analysis. Genetic diversity and phylogenetic relationship between the sequenced samples and global genomes were studied. All phylogenies were created using core genome, recombination and single nucleotide polymorphisms (SNPs) using parsnp v1.2, available in harvest suite[22] using a corresponding reference genome. SNPs identified in local collinear blocks were subsequently used for reconstructing an approximate maximum-likelihood tree using FastTree2[46] while including the general time reversible (GTR) model of nucleotide substitution. The Shimodaira–Hasegawa test implemented in FastTree2 was used to assess the support for significant clustering in the observed phylogeny. The interactive tree of life or iTOL (https://itol.embl.de/)[47] was used for the graphic illustration of the trees along with relative annotations.

For the construction of the SNPs-based phylogenies, 582 *Proteus mirabilis* genomes were downloaded from NCBI assembly database including complete and draft genomes, using ASM6996v1 as reference. Similarly, 92 genomes for *Morganella morganii* (ASM1428397v1 as reference), 118 genomes for *Citrobacter freundii* (Cfr49,969 as reference), 126 genomes for *Enterobacter hormaechei* (Ecl-48,293 as reference) and 732 genomes for *K. pneumoniae* using Kpn-48,293 as reference.

Moreover, isolates from the study that clustered together forming a clade or/and subclade were investigated further. SNPs among the isolates within the clade/subclade were compared to a reference genome within the selected set using snippy v.4.4.3 (https://github.com/tseemann/snippy).

Nucleotide sequence accession numbers. The nucleotide sequence of the genomes and plasmids were deposited and available in GenBank under the BioProject number PRJNA700516; all accession numbers can be retrieved from Table S5.

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Author contributions:

C.C.P. and I.B. played an important role in interpreting the results and in writing the manuscript. K.C., V.J,. H.Z., V.S. and J.H. helped to acquire data. L.K., M.F., and I.B. carried out experimental work. I.B. and C.P. supervised the experiments and revised the final manuscript, which was approved by all authors.

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5.2 Publication nb. 2: Genomic characterisation of three GES-producing Enterobacterales isolated in the Czech Republic

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Genomic characterisation of three GES-producing Enterobacterales isolated in the Czech Republic

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Abstract:

Objectives: The aim of the study is to characterise the genomic features of three GES-producing Enterobacterales isolates from Czech hospitals.

Methods: In 2020, during a routine screening of the hospital's surfaces in Prague General Hospital, two strains (CZ862 and CZ863) that belonged to the *Enterobacter cloacae* complex were found to be *bla*$_{GES}$ positive. Another *bla*$_{GES}$ positive strain identified as *Klebsiella oxytoca* was recovered from a patient hospitalized in Pilsen. Antibiotic susceptibility profiling was done with broth microdilution assay. Conjugation/transformation experiments were performed on all three strains. Genomic DNA of the three isolates was subjected to whole genome sequencing using PacBio platform.

Results: Multilocus sequence types typing of CZ862 and CZ863 identified the strains as ST837 and a novel ST (ST1622). Both *bla*$_{GES}$ harbouring plasmids showed high sequence similarity and complete query coverage (100% and 99.98%) with pEcl-35771cz. Both plasmids had two copies of *bla*$_{GES}$ instead of one copy as found in pEcl-35771cz. The clinical isolate CZ598 belonged to ST180. The plasmid harboured *bla*$_{GES-7}$ gene, cat and aac(6')-lb and the novel variant *bla*$_{OXA-1011}$. No similar sequences were observed, suggesting a novel plasmid.

Conclusion: The detection of the two *bla*$_{GES}$-positive plasmids in the same hospital environment, the first report after 3 years, suggests a hidden source. This highlights the importance of the hidden sources and evolution of such plasmids on the route of spreading into clinical settings. Also, the detection of the new *bla*$_{OXA-1011}$, which is thought in this case to be associated with carbapenem resistance, imposes a health risk if disseminated, limiting therapeutic options.

1. Introduction:

The first case of a GES family enzyme (GES-1) was reported in France in 1998 in a *Klebsiella pneumoniae* strain isolated from a 1-month-old female patient. The gene was harboured on a 140- kb non-transferable

plasmid (pTK1) [1]. The first single point mu- tation, causing the substitution of Gly with Asp in GES-1, generated a new variant GES-2, detected in a *Pseudomonas aeruginosa* isolated from a 38-year-old patient from South Africa [2]. A few years later, the *Escherichia coli* strain was recovered in Greece that harboured the *bla*$_{GES}$ gene with a single amino acid mutation in increased hydrolysis activity against cefoxitin, while another mutation that substituted Glu at position 104 by Lys (in the following variants: GES-3, GES-4, GES-6, GES-7 and GES-13) increased the activity against oxyimino-β-lactams, mainly ceftazidime and aztreonam [4–6]. Up until 19 July 2021, 48 *bla*$_{GES}$ variants were detected and available on the National Database of Antibiotic Resistance Organisms (NDARO). Over the last few years, there has been an increased incidence of GES-producing Enterobacterales isolates in Europe [7–9]. In the Czech

Table 1
WGS data of the three isolates producing *bla*$_{GES}$ recovered from the Czech hospitals

ID	Species	ST	Replicons	Size (bp)	Plasmid name	Inc group	*bla*$_{GES}$	Other resistance genes
CZ862	*E. cloacae*	837	Chr	5419017	-	IncHI2, IncHI2A,IncY	-	*bla*$_{CMH-3}$, *bla*$_{TEM-1B}$,*fosA*,, *aph(3′)-la**, *aac(6′)-lb3*, *sul2*, *carA2*,
			Plasmid	35630	pCZ862_IncFIB	IncFIB	-	*qnrB2*, *aadA2B*, *sul1**, *qacE*
			Plasmid	8282	pCZ862_ColRNAI	ColRNAI	-	-
			Plasmid	7973	pCZ862_GES1_GES5	NT	***bla*$_{GES-5}$**, ***bla*$_{GES-1}$**	*aadA15*
			Plasmid	1334	pCZ862_1	NT	-	-
CZ863	*E. asburiae*	1622	Chr	4581263	-	-	-	*bla*$_{ACT-3}$, *fosA*
			Plasmid	171570	pCZ863_IncFII	IncFII,IncFIB	-	*aph(6)-ld*, *aac(6′)-lb-cr*, *aph(3″)-lb*, *sul2*, *dfr14*, *catB3*, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M-15}$
			Plasmid	72524	pCZ863_IncM1	IncM1	-	*aac(6′)-lb-cr*, *aac(6′)-lb3*, *catB3*, *bla*$_{TEM-1D}$, *bla*$_{OXA-1}$
			Plasmid	7973	pCZ863_GES5_GES5	NT	***bla*$_{GES-5}$**, ***bla*$_{GES-5}$**	*aadA15*
			Plasmid	2495	pCZ863_Col	Col	-	-
CZ598	*K. michiganensis*	180	Chr	6473323	-	IncHIA	-	*qnrS1*, *aph(3′)-la*, *bla*$_{CTX-M-14}$, *bla*$_{OXY-1-4}$
			Plasmid	12242	pCZ598_GES7	NT	***bla*$_{GES-7}$**	*aac(6′)-lb-cr*, *cat*, *bla*$_{OXA-1011}$
			Plasmid	4096	pCZ598_Col	Col	-	-
			Plasmid	3223	pCZ598_1	NT	-	-
			Plasmid	1697	pCZ598_2	NT	-	-

NT, non-typable.
* More than one copy.

Republic, the first case was reported in 2018 in a diabetic patient hospitalised in Prague General Hospital. An *Enterobacter cloacae* strain isolated from a wound swab harboured the *bla*$_{GES-5}$ gene on an un-typable plasmid (pEcl-35771cz) [10]. The detected *bla*$_{GES-5}$ was also found on a novel class 1 integron accompanied by a novel allele of *aadA15* gene cassette.

The aim of this study is to characterise the genomes and plasmids of three Enterobacterales isolates producing GES, isolated between 2019 and 2020 in the Czech Republic.

2. Materials and methods

2.1. Case study

In 2020, during a routine screening of the hospital's surfaces in Prague General Hospital, two strains that belonged to the *Enterobacter cloacae complex* were *bla*$_{GES}$ positive through PCR (10). The two strains were isolated from the sinks of two different rooms of the same hospital. Another strain identified as *Klebsiella oxytoca* was recovered from a urine sample of a patient hospitalised in Pilsen and it was also positive for

bla$_{GES}$. Initially, the patient suffered from a malignant neoplasm of the kidney and underwent a kidney transplant. Five years later, the patient was hospitalised due to chronic kidney disease and severe fungal infection (Aspergillosis).

2.2. Species identification and antibiotic susceptibility testing

The three isolates were sent to the biomedical centre in Pilsen for further genomic analysis. The strains species identification was confirmed with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). The two strains from Prague General Hospital, CZ862 and CZ863, belonged to the *Enterobacter cloacae complex* (*E. cloacae* and *E. asburiae,* respectively), whereas CZ598 was identified as *K. oxytoca*. The presence of the *bla*$_{GES}$ genes was confirmed using PCR and Sanger sequencing as described elsewhere [10]. Antibiotic susceptibility was detected with broth microdilution assay using EUCAST 2021 breakpoints (https://www.eucast.org/clinical_breakpoints/). The three isolates expressed multidrug resistance (MDR) profile, showing resistance against cephalosporins, carbapenems and ceftolozane-tazobactam, yet susceptible to gentamycin, amikacin, colistin and tigecycline (Supplementary Table S1).

2.3. Conjugation/Transformation experiments

The three strains were subjected to conjugation experiment using the strain *Escherichia coli* A15 strain (Azd^{R}) as recipient as de- scribed elsewhere [11]. None of the plasmids harbouring the *bla*$_{GES}$ were conjugative; therefore, plasmid DNAs of the three isolates were extracted using ZymoPure II Plasmid Midiprep Kit (Irvine, CA, USA), and transformation was performed using *E. coli* DH5 α as a recipient as described elsewhere [11]. Transformants did not retain the carbapenem resistance, except for CZ598 (Supplementary Table S1), nor resistance against ceftolozane-tazobactam due to the Pc hybrid 1 promotor associated low expression [12].

2.4. Whole genome sequencing (WGS) and data analysis

Genomic DNAs were extracted from the three isolates using a NucleoSpin microbial DNA kit (Macherey-Nagel, Duren, Ger- many). The DNA was then sheared, obtaining 15Kb fragments, using Hydropore-long on the Megaruptor 2 (Diagnode). Library preparation, using the sheared DNA, was done using the Express Kit 2.0 (Pacific Biosciences, Menlo Park, CA, USA) for microbial multiplexing protocol without size selection according to the manufacturer's recommendations. Using the SMRT Link v.9.0, microbial assembly was used to assemble the sequences with a minimum seed coverage of 30. Annotation of the assembled genomes and plasmids was done using Prokaryotic Genome Annotation Pipeline (PGAP). Multilocus sequence types (MLST), plasmid replicons and antibiotic resistance genes were detected through uploading the assembled data to MLST 2.0 [13], PlasmidFinder [14] and ResFinder 4.1 [15, 16], respectively. Moreover, plasmids were typed according to the plasmid relaxases as described previously [17]. Mauve v.2.3.1 and Blast Ring Image Generator (BRIG) was used to perform comparative genomic alignment [18]. Inkscape was used to execute the diagrams and gene organisation. WGS analysis confirmed species identity, showing that CZ862, CZ863 and CZ598 were identified as *E. cloacae, E. asburiae* and *K. michiganensis,* respectively.

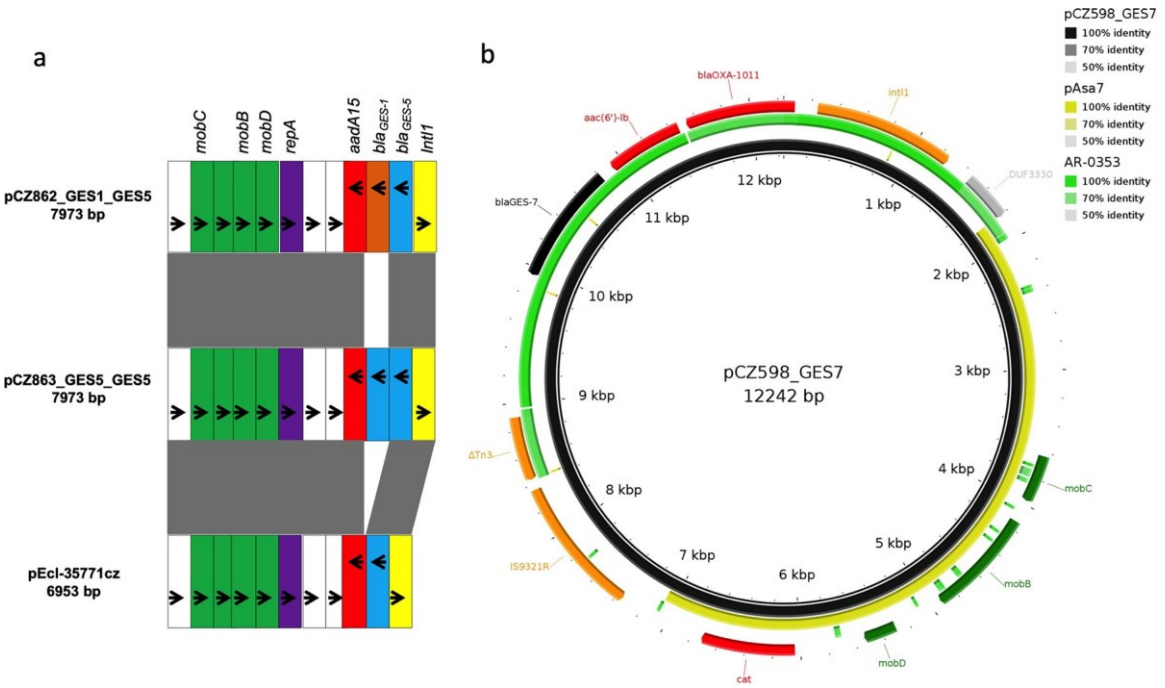

Fig. 1. (a) Linear map of pCZ861_GES1_GES5, pCZ863_GES5_GES5 and pEcl-35771cz. Arrows show the direction of transcription of ORFs. Replicons, mobile elements, mobility transfer genes, antibiotic resistance, hypothetical proteins, blaGES-1 and blaGES-5 are designated by violet, yellow, green, red, white, orange and blue, respectively. (b) Circular map of pCZ598_GES7 compared to pAsa7 and AR-0353. Mobility transfer regions, antibiotic resistance genes, mobile genetic elements hypothetical proteins and blaGES-7 were designated by green, red, orange, grey and black curves, respectively.

3. Results and discussion

The WGS sequence analysis of CZ862 produced complete circular sequence for the chromosome and four complete circular plasmids (Table 1). MLST typing identified the strain belong to ST837. The results showed that the chromosome harboured three plasmid replicons (IncHI2, IncHI2A, IncY), suggesting partial integration. The analysis showed partial integration of the plasmids' backbones, whereas the rest of the acquired antibiotic resistance genes were dispersed in the genomes. The plasmid harbouring the *bla*$_{GES}$ was designated as pCZ862_GES1_GES5 (7973 bp). On the other hand, WGS analysis of CZ863 showed that it belonged to a novel ST (ST1622) and produced the chromosome in two pieces and four complete circular plasmids with the one harbouring the *bla*$_{GES}$ designated as pCZ863_GES5_GES5 (7973 bp) (Table 1). Both plasmids were un-typable by PlasmidFinder and plasmid relaxases schemes.

Both plasmids, when blasted against the NCBI database, showed high sequence similarity (100% and 99.98%) and complete query coverage with pEcl-35771cz (MF370188) [10]. Of note, the three plasmids were isolated from the same hospital within a 3-year time difference (2017–2020). Sequence comparison showed that those plasmids had the same sequence except in the *bla*$_{GES}$ position; the plasmid had a replication initiation site (r *epA*), genes associated with plasmid mobility (*mobA/B/C/D*) and antibiotic resistance genes (*aadA15* and *bla*$_{GES}$). In the antibiotic resistance coding region, pCZ862_GES1_GES5 had *bla*$_{GES-1}$ and *bla*$_{GES-5}$ on a novel integron designated as In2079, while pCZ863_GES5_GES5 had two copies of the *bla*$_{GES-5}$ on a novel integron In2081instead of one copy of *bla*$_{GES-5}$ on In1406 in pEcl-35771cz (Fig. 1a).

The clinical isolate, CZ598, identified as *K. michiganensis,* belonged to ST180. WGS analysis produced complete circular chromosome and four complete circular plasmids (Table 1). The chromosome partially

harboured the backbone of an IncHI1A. The plasmid harbouring the *bla*$_{GES}$ was designated pCZ598_GES7 (12242 bp). The plasmid was not typable by PlasmidFinder and the plasmid relaxases scheme. It harboured genes coding for plasmid mobility (*mobC/B/D*), chloramphenicol resistance (*cat*), fluoroquinolone resistance (*aac(6')-lb*) and the *bla*$_{GES-7}$. Moreover, a novel variant of *bla*$_{OXA-2}$ -like, designated *bla*$_{OXA-1011}$, was also harboured and is suspected to be the gene responsible for the carbapenem resistance in the transformant. Upon blasting the plasmid against NCBI database, no similar sequences were observed, suggesting a novel plasmid. Nevertheless, it showed partial similarity with two sequences: a 5200 bp sequence representing pAsa7 plasmid (accession number: KU499859) detected in an *Aeromonas salmonicida* strain isolated from a sick fish in 2016 in Canada. A second 5600 bp sequence represented a partial sequence of a clinical *Pseudomonas aeruginosa* chromosome of (AR-0353; accession number: CP027172) strain isolated in 2018 in the United Stats (Fig. 1 b).

4. Conclusion

These findings highlight some important aspects: the detection of the two *bla*$_{GES}$-positive plasmids in the same hospital environment as the first report after three years suggests a hidden source. The plasmid structure was conserved over the three years with the acquisition of another *bla*$_{GES}$ gene. Recently, more reports of plasmids carrying two or three consecutive *bla*$_{GES}$ genes with different variants suggest that there is a hotspot for acquiring these genes [7, 19]. The mechanism of acquisition is not fully understood yet, but it is suggested that it could be through recombination or integration. Moreover, the detection of the hybrid plasmid with fragments originating from environmental sources is worrisome especially after being detected clinically in this study. This highlights the importance of the hidden sources and evolution of such plasmids on the route of spreading into clinical settings. Also, the detection of the new *bla*$_{OXA-1011}$, which is thought in this case to be associated with carbapenem resistance, imposes a health risk if disseminated, limiting therapeutic options.

Nucleotide sequence accession numbers

The nucleotide sequences of the chromosome and plasmids of CZ862, CZ863 and CZ598 were deposited in GenBank and the following accession numbers have been assigned, respectively: CP073310-CP073314, JAGSXX0 0 0 0 0 0 0 0 0 and CP073305-CP073309.

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Competing interests

We have no conflicts to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at "https://doi.org/10.1016/j.jgar.2022.02.016"

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5.3 Publication nb. 3: Implication of different replicons in the spread of the VIM-1-encoding integron, In110, in Enterobacterales from Czech hospitals

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SPECIALTY SECTION

Implication of different replicons in the spread of the VIM-1-encoding integron, In110, in Enterobacterales from Czech hospitals

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Abstract:

Background: VIM metallo-β-lactamases are enzymes characterized by the ability to hydrolyze all β-lactams. Usually, *bla*$_{VIM}$-like genes are carried by class 1 integrons. In the Czech Republic, only sporadic cases of VIM-producing

Enterobacterales have been reported in which those isolates carried the VIM1 carbapenemase-encoding integron In110. However, during 2019–2020, an increased number was reported. Therefore, the aim of the current study was to characterize the genetic elements involved in the increased spread of *bla*$_{VIM}$ genes.

Materials and methods: 32 VIM-producing Enterobacterales collected between 2019 and 2020 were subjected to: antimicrobial susceptibility testing, integron analysis, and short reads sequencing. Based on the results, 19 isolates were selected as representative and sequenced using Sequel I platform.

Results: The 32 VIM-producing isolates exhibited variations in the MICs of carbapenems. Based on short-read data, 26 of the 32 sequenced isolates harbored the *bla*$_{VIM-1}$ allele while six isolates carried the *bla*$_{VIM-4}$ gene. The most prevalent was the In110 integron ($n = 24$) and two isolates carried the In4873 class 1 integron. The *bla*$_{VIM-4}$ allele was identified in class 1 integrons In1174 ($n = 3$), In416 ($n = 1$), In2143 ($n = 1$)

and In2150. Long reads sequencing revealed that the *bla*$_{VIM}$ was carried by: pKPC-CAV1193-like (*n* = 6), HI1 (pNDM-CIT; *n* = 4), HI2 (*n* = 3), FIB (pECLA; *n* = 2) and N (*n* = 1) incompatibility groups. Two *bla*$_{VIM}$-carrying plasmids could not be typed by the database, while another one was integrated into the chromosome.

Conclusion: We observed the spread of VIM-encoding integrons, mainly of In110, among Enterobacterales isolated from Czech hospitals, but also an increased number of novel elements underlining the ongoing evolution.

Introduction:

Mobile elements, such as integrons, transposons and plasmids, have played an important role in the spread of antimicrobial resistance genes among Enterobacterales. Integrons are genetic elements able to acquire and express genes in the form of cassettes. They can move due to their association with insertion sequences, transposons and plasmids (Mazel and Davies, 1999). Class 1 integrons, which are the most common integrons among clinical isolates, have been involved in the dissemination of more than 60 different gene cassettes conferring resistance to almost all antimicrobial categories (Mazel, 2006). One of the most recently characterized cassettes encodes VIM metallo-β-lactamases (MβLs), which are enzymes characterized by the ability to hydrolyze all β-lactams, including carbapenems (Laraki et al., 1999). Usually, *bla*$_{VIM}$-like genes are carried by class 1 integrons, like In-e541 identified in Greece (Miriagou et al., 2003), In110 and In113 in Spain (Tato et al., 2010) or In416 in Italy (Colinon et al., 2007).

In the Czech Republic, only sporadic cases of VIM-producing Enterobacterales have been reported (Papousek et al., 2017; Papagiannitsis et al., 2018), from 2011 till 2015. Interestingly, those isolates carried the VIM-1 carbapenemase-encoding integron In110 (*bla*$_{VIM-1}$-*aacA4*-*aadA1*; Lombardi et al., 2002). However, during 2019–2020, an increased number of VIM-producing Enterobacterales was isolated from Czech hospitals. Therefore, the aim of the current study was to characterize the genetic elements involved in the increased spread of *bla*$_{VIM}$ genes, and to examine if In110 was the only/ main integron associated with the expression of VIM carbapenemases.

Materials and methods

1. Bacterial isolates, susceptibility testing and confirmation of carbapenemase production

In 2019 and 2020, Czech hospitals referred a total of 32 VIM-producing Enterobacterales isolates with a meropenem MIC of >0.125 µg/ml (2012; using *E. coli* ATCC 25922 as a quality control strain) to the National Reference Laboratory for antibiotics. However, five of those isolates have been previously published as they carried an *mcr*-like gene (Bitar et al., 2020). 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 (Rotova et al., 2017). Additionally, the presence of carbapenemase-encoding genes (*bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$-like) was confirmed by PCR amplification (Poirel et al., 2004; Ellington et al., 2007; Naas et al., 2008; Yong et al., 2009). PCR products were sequenced as described below. Isolates positive for *bla*$_{VIM}$-like genes were further studied. Antimicrobial susceptibility

was performed using broth microdilution according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2012) guidelines. Susceptibility data were interpreted according to the criteria (version v12.0) of the EUCAST.[1]

Integron analysis

Variable regions of class 1 integrons with *bla*$_{VIM}$-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 (Papagiannitsis et al., 2013). Wholegene arrays were sequenced using an ABI 3500 sequencer (Applied Biosystems, Foster City, CA). The integron database, Integrall[2] (Moura et al., 2009) was used to analyze and assign integron sequences.

Short-read whole genome sequencing

All VIM-producing Enterobacterales were sequenced, using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States). The genomic DNAs of the clinical isolates were extracted using the 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, United States) using the MiSeq v3 600-cycle reagent kit. Initial paired-end reads were quality trimmed using the Trimmomatic tool v0.33 (Bolger et al., 2014) and then, assembled by use of the de Bruijn graph-based *de novo* assembler SPAdes v3.14.0 (Bankevich et al., 2012).

Long-read whole genome sequencing

Based on the results of short-read sequencing (see below), 19 VIM producers were selected to for long-read sequencing, to help close the whole plasmid sequences. These isolates were selected as representatives of all different hospitals, bacterial species, STs, replicon profiles and *bla*$_{VIM}$ alleles.

Genomic DNA was extracted from the clinical isolates using NucleoSpin Microbial DNA kit (Macherey–Nagel, Germany). Whole genome sequencing (WGS) was performed on the Sequel I platform (Pacific biosciences, Menlo Park, CA, United States). Microbial multiplexing protocol was used for the library preparation according to the manufacturer instructions for Sheared DNA. DNA shearing was performed using the Megaruptor 2 (Diagenode, Liege, Belgium) using long hydropores producing 10 kb long inserts. No size selection was performed during the library preparation. The Microbial Assembly pipeline offered by the SMRT Link v9.0 software was used to perform the assembly and circularization with minimum seed coverage of 30X. Assembled sequences were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP).

Nucleotide sequence accession numbers

[1] http://www.eucast.org/

[2] http://integrall.bio.ua.pt/

The nucleotide sequences of the genomes and plasmids were deposited and are available in GenBank (Supplementary Table S1) under the BioProject number https://www.ncbi.nlm.nih.gov/bioproject/PRJNA772913

Results

VIM-producing Enterobacterales

During 2019–2020, a total of 32, Enterobacterales isolates with a meropenem MIC of >0.125 μg/ml were referred to the National reference laboratory for antibiotics from 15 laboratories. Among them, 23 isolates were identified to be *Enterobacter cloacae* complex, 5 were identified to be *Citrobacter freundii*, and 3 were identified to be *Klebsiella pneumoniae*. The one remaining VIM-producing isolate belonged to the bacterial species *Klebsiella michiganensis*.

All 32 VIM-producing isolates exhibited resistance to piperacillin, piperacillin-tazobactam and cephalosporins, while the variations in the MICs of aztreonam and carbapenems that were observed (Supplementary Table S1) might reflect the presence of additional resistance mechanisms in some of the isolates. Twenty-six of the VIM-producing isolates also exhibited resistance to tetracycline, 25 were resistant to chloramphenicol, 23 were resistant to gentamicin, 19 were resistant to ciprofloxacin, 3 were resistant to tigecycline, 6 were resistant to amikacin, whereas three of the isolates were resistant to colistin.

Analysis of short-read sequencing results and VIM-encoding integrons

Based on short-read data, 26 of the 32 sequenced isolates harbored the *bla*$_{\text{VIM-1}}$ allele (Tables 1, 2), while the remaining six isolates carried the *bla*$_{\text{VIM-4}}$ gene. The *bla*$_{\text{VIM-4}}$ gene was identified among 3\u00B0*C. freundii*, 1 *K. pneumoniae* and 2 *E. hormaechei* isolates. Moreover, characterization of the regions flanking the VIM-encoding genes by PCR mapping and sequencing data showed that *bla*$_{\text{VIM}}$-like genes were located in six main types of class 1 integrons (Figures 1, 2). The most prevalent was the In110 integron identified in 24 VIM-1-producing isolates. The two remaining VIM-1-producing isolates, which belonged to *K. pneumoniae* ST54, carried the In4873 class 1 integron. In4873 integron, which is an In416-like element identified for the first time in Greece (Papagiannitsis et al., 2016), included the *bla*$_{\text{VIM-1}}$, *aacA7*, *dfrA1*, *aadA1* and *smr2* gene cassettes. On the other hand, the *bla*$_{\text{VIM-4}}$ allele was identified in class 1 integrons In1174 (n = 3), In416 (n = 1), In2143 (n = 1) and In2150 (Tables 1, 2). The class 1 integron In1174 includes an array of *aacA4* and *bla*$_{\text{VIM-4}}$ gene cassettes. The In416 element, which was firstly reported in Italy (Colinon et al., 2007), comprises *bla*$_{\text{VIM-4}}$, *aacA7*, *dfrA1*, *aadA1* and *smr2* gene cassettes. Additionally, the In2143, which was a novel class 1 integron carrying *bla*$_{\text{VIM-4}}$, *aacA7* and *aacC2c* gene cassettes, was found in a ST108 *E. hormaechei* isolate. Finally, the novel integron In2150, which comprised *bla*$_{\text{VIM-4}}$, *aacA7*, *smr2* cassettes, was identified in ST674 *C. freundii* isolate (Cfr-56322cz). Beside species-specific chromosomal β-lactamases, most of the clinical isolates also carried genes encoding TEM-1 penicillinases (n = 14) and/or OXA-1 oxacillinases (n = 13). Nine out of 22 *Enterobacter* isolates harboured the *bla*$_{\text{CTX-M-15}}$ gene, while the *bla*$_{\text{SHV-12}}$ gene was found among 2 isolates. Additionally, 2 out of 3 *K. pneumoniae* co-carried the carbapenemase-encoding gene *bla*$_{\text{KPC}}$2. All sequenced isolates exhibited a wide variety of resistance genes conferring resistance to

aminoglycosides, sulfonamides, trimethoprim, streptomycin, fosfomycin (low-level resistance), fluoroquinolones, chloramphenicol, tetracyclines, colistin, erythromycin and/or rifampicin (Tables 1, 2).

WGS data revealed that most of the isolates belonging to *E. cloacae* complex isolates belonged to sequence types ST92 (*n* = 8), ST106 (*n* = 5), and ST190 (*n* = 2; Tables 1, 2). The remaining seven *Enterobacter* isolates were ST25, ST92, ST108, ST252, ST421, ST764, and the novel STs 1734 and 1735. The isolates belonging to *C. freundii* species were assigned to ST95 (*n* = 2), ST9 (*n* = 1), and ST673 (*n* = 1) and ST674 (*n* = 1). ST673 and ST674 were novel STs. The *K. pneumoniae* isolates included two STs. The VIM-1 producers belonged to ST54, while the VIM-4-producing *K. pneumoniae* was ST11. Finally, the *K. michiganensis* (closely related to *K. oxytoca*) isolate was assigned to ST226.

Localization of VIM-encoding integrons:

Based on short-read data, 19 VIM-producing isolates were selected to be sequenced by the Sequel I platform, to close plasmid sequences. Analysis of long-read sequencing data revealed the presence of several *bla*$_{VIM}$-carrying plasmid sequences belonging to different Inc. groups and presenting diverse sizes (Table 1). Based on PlasmidFinder analysis of plasmid sequences, 16 out of 19 *bla*$_{VIM}$-carrying plasmids could be assigned to: pKPCCAV1193-like (*n* = 6), HI1 (pNDM-CIT; *n* = 4), HI2 (*n* = 3), FIB (pECLA; *n* = 2) and N (*n* = 1) incompatibility (Inc) groups (Figure 2). Two of the remaining *bla*$_{VIM}$-carrying plasmids could not be typed by the database, while in the ST11 *K. pneumoniae* isolate the VIM-4-encoding integron, In1174, was integrated into the chromosome.

TABLE 1 WGS data of the 19 isolates sequenced using both short (illumina) and long reads sequencing platform (PacBio).

Isolate	Species	ST	VIM plasmid size	Inc	Integron type	Other replicons	Resistance genes
48212*	E. cloacae complex	106	55,220	pKPC-CAV1193-like	In110	Col(pHAD28), IncFIB(pECLA), IncHI2	mcr-9, aac(6′)-IIc, aadA2b, aph(6)-Id, dfrA19, catA2, sul1, sul2, tetD, aac(6′)-Ib-cr, qnrA1, ere(A), bla$_{SHV}$-12, bla$_{TEM-1b}$
							qnrS1, *bla*TEM-1a, *bla*VIM-1, *aac(6′)-Ib3*
48411	E. hormaechei	1734	171765	IncFIB(pECLA)	In110	Col(pHAD28)	aacA4, aadA1, dfrA14, blaVIM-1, catA2, blaVIM-1B, strA, strB, tetA, qnrS1,qacE, sul1, sul2, fosA
48880*	E. cloacae complex	764	2,62,616	IncHI2	In416	Col(pHAD28), IncFIB(pECLA), IncFII(pECLA), IncR	mcr-9.2, aac(6′)-II, aadA22, dfrA1, sul1, tetA, blaVIM-4

48946*	E. cloacae complex	106	55,222	pKPC-CAV1193-like	In110	Col(pHAD28), IncFIB(pECLA), IncHI2	mcr-9, aac(6′)-IIc, aadA2b, aph(3″)-Ib, aph(6)-Id, dfrA19, catA2, sul1, sul2, tetD, ere(A), blaTEM-1b, blaSHV-12
							aac(*6′*)-*Ib3*, *qnrS1*, *bla*TEM-1a, blaVIM-1,
48947	E. hormaechei	190	55220	pKPC_49790_VIM_1	In110	IncHI2	aacA4 (n=2), aac(3)-IIa, aadA1, aadA2b, catA1, catB3, dfrA14, bla_{VIM-1}, blaCTX-M-15, blaOXA-1, blaTEM-1A, blaTEM-1B, strA, strB, qnrB1, qnrS1, sul1, sul2, tetA
49589	E. hormaechei	108	294454	IncHI2	In2143	IncFIB(pECLA), IncFII(pECLA), IncR	aac(3)-Ib, aac(6')-Il, bla_{VIM-4}, qacE (n=2), qnrA1, sul1 (n=2), tetB, aacA7, aacC2c
49790*	E. cloacae complex	106	55,220	pKPC-CAV1193-like	In110	Col(pHAD28), IncFIB(pECLA), IncHI2	mcr-9, aac(6′)-IIc, aadA2b, aph(3″)-Ib, aph(6)-Id, dfrA19, catA2, sul1, sul2, tetD, aac(6′)Ib-cr, qnrA1, ere(A), blaSHV-12, bla_{TEM-1b}, aac(6′)-Ib3, qnrS1, blaTEM-1a, blaVIM-1,
54569	E. hormaechei	92	171616	IncFIB (pECLA),IncFII (peCLA)	In110	IncQ1, Col440I, ColpVC	aacA4, aac(3)-IIa, aac(6')Ib3, aadA1, aph(3')-Via, bla_{VIM-1}, blaOXA-1, catB3, dfrA14, fosA, qacE, sul1

(Continued)

TABLE 1 (Continued):

Isolate	Species	ST	VIM plasmid size	Inc	Integron type	Other replicons	Resistance genes
57816	E. hormaechei	106	55220	pKPC-CAV1193	In110	IncFIB (pECLA), IncHI2, Col (pHAD28)	aacA4, aac(6')-Ib3, aac(6')-IIc, aadA2b (n=2),blaVIM-1, blaTEM-1A, blaTEM-1B, blaSHV-12, catA2, dfrA19, ere(A),mcr-9, qnrS1, fosA, qacE (n=3), strA, strB, sul1 (n=3), sul2, tet(D)

58983	E. cloacae	421	64556	untypable	In110	IncFIB (pECLA)	aacA4 (n=2), aac(3)-IIa, aadA1 (n=2), bla_{VIM-1}, blaCTX-M-15, blaOXA-1, bla_{TEM-1B}, catA1, catB3, dfrA14, qacE, qnrB1, strA, strB, sul1, sul2, tetA, fosA
59732	E. hormaechei	1735	54956	pKPC-CAV1193	In110	IncFIB (pECLA), Col (pHAD28)	aacA4, aadA2b, aac(6')-Ib3, blaVIM-1, blaTEM-1A (n=2), qnrS1, fosA, qacE, sul1
60214	E. hormaechei	92	311801	IncHI1 (pNDM-CIT)	In110	IncFIB (pECLA), IncFII (pECLA), Col (pHAD28)	aacA4 (n=2), aac(6')-Ib3, aac(3)-IIa, aadA1 (n=2), aph(3')-Ia, catB3, bla_{VIM-1}, blaCTX-M-15, blaOXA-1, bla_{TEM-1B}, dfrA1, dfrA14, qnrB1, fosA, qacE, strA, strB, sul1, sul2, tet(A)
51929*	C. freundii	95	3,69,945	IncHI2-/IncM1	In1174		mcr-9, aac(6′)-II, aac(3)I, aac(6′)-Ib3, ant(2″)-Ia, aadA1, aadA2b, aph(3′)Ia, dfrA19, catA2, cmlA1, sul1, tetA, aac(6′)-Ib-cr, qnrA1, bla_{VIM-4}
52323	C. freundii	9	318136	IncHI1 (pNDM-CIT)	In110		aacA4, aac(6')-Ib3, aadA1 (n=2), aph(3')-Ia, bla_{VIM-1}, catA1, dfrA1, qnrB75, qacE, sul1, tet(A)
56322	C. freundii	674	344532	IncHI1A(NDM-CIT), IncHI1B(NDM-CIT)	In2150		aacA7, aac(3)-IId, aadA2, blaVIM-4, blaTEM-1B, dfrA12, qnrB75, qacE, sul1 (n=2), sul2, tetA
56415	C. freundii	673	106850	untypable	In1174	IncHI2	aacA4, aac(6')Ib3, aadA1, aadA2b (n=3), aadB (n=2), aph(3')-Ia (n=2), bla_{VIM-4}, catA2, cmlA1, dfrA19, mcr-9, qacE (n=4), sul-1 (n=4),

(Continued)

TABLE 1 (Continued)

Isolate	Species	ST	VIM plasmid size	Inc	Integron type	Other replicons	Resistance genes

51135	K. pneumoniae	11	chromosome	NA	In1174	IncFIB(K), IncM1	aacA4, aac(6')-Ib3, blaVIM-1,blaTEM-1B, blaSHV-182, qacE, cmx, oqxA, oqxB, fosA
59062	K. pneumoniae	54	56199	IncN	In4873	IncFIB (K) (pCAV1099-114), IncH1B (pNDM-MAR),IncC, IncFIB (pQil) IncFII(K)	aacA7, aac(6')-Im, aadA22, aph(3')-Ia, aph(2'')-Ib, bla_{VIM-1}, bla_{KPC-2}, catA1, dfrA1, oqxAB, qnrS1, fosA, qacE, sul1, tet(D)
53828	K. michiganensis	226	364473	IncHI1 (pNDM-CIT)	In110	IncFII(Yp)	aacA4, aph(3')-Ia, aadA1 (n=2), dfrA1, bla_{VIM-1}, catA1, qacE, sul1, tetA

*These isolates were already published in Bitar et al. (2020).

All pKPC-CAV1193-like plasmids (*n* = 6) carried the VIM-1 encoding integron In110. These plasmids, which were ~ 55,220-bp in size (except p59732CZ_VIM), were identical to plasmid p48212_VIM (Supplementary Figure S1) characterized previously from ST106 *E. hormaechei*, carrying *mcr-9* gene, isolated from a Czech hospital (Bitar et al., 2020). Plasmid p59732CZ_VIM lacked a 266-bp fragment in the ORF encoding a GNAT family N-acetyltransferase.

Also, three (p52323cz_VIM, p53828cz_VIM and p60214cz_VIM) out of four IncHI1 (pNDM-CIT) plasmids carried the In110. These plasmids were identical to VIM-1-encoding plasmid pLec-476cz (Supplementary Figure S2), which was previously characterized from a *Leclercia adecarboxylata* isolate (Papousek et al., 2017) recovered during a survey study focused on compliance with hand hygiene among the staff of a different Czech hospital in May 2011. On the other hand, the fourth IncHI1 (pNDM-CIT) plasmid (p56322_VIM), was carried by a ST674 *C. freundii* isolate. This plasmid harbored the VIM-4-encoding integron In2150. Plasmid p56322_VIM showed moderate similarity to IncHI1 plasmids, encoding VIM-1 (like p53828cz_ VIM [72% coverage, 98.68% identity]), while the highest similarity was observed for the IncHI1 plasmid pRHBSTW-00135_2 (80% coverage, 100% identity; GenBank accession no. CP056828; Supplementary Figure S3) that was characterized from a wastewater influent sample collected in the United Kingdom. Of note was that plasmid pRHBSTW-00135_2 carried no resistance genes. p56322_VIM was composed of a partial IncHI1 backbone and a MDR region. Segments of the IncHI1 backbone, encoding proteins involved in the conjugative transfer system, were duplicated in the p56322_VIM plasmid. Furthermore, beside In2150, the MDR region contained the In27 integron, consisting of *dfrA12*, *gcuF* and *aadA2* gene cassettes, the bla_{TEM-1} and *aac*(3)-*IId* resistance genes, and regions conferring resistance to macrolides, mercury and chromate.

Two IncHI2 VIM-4-encoding plasmids (p48880_MCR_VIM and p51929_MCR_VIM), which also carried the *mcr-9* resistance gene, have been previously characterized (Bitar et al., 2020). The third IncHI2 plasmid, p49589_VIM, which was 294,454-bp in size, carried the novel VIM-4-encoding integron In2143. It exhibited moderate similarity to p48880_MCR_VIM (76% coverage, 99.48% identity) and p51929_MCR_VIM (76% coverage, 99.97% identity), while it was almost identical (99% coverage, 100% identity) to IncHI2 plasmid p48293_VIM (Supplementary Figure S4), which was previously sequenced from the *E. hormaechei* strain

Ecl-48293co-producing KPC-2 and VIM-4 carbapenemases, during a study describing the ongoing spread of KPC-type producers in Czech hospitals (Kraftova et al., 2021). p49589_VIM was typed as sequence type 1 (ST1) following the IncHI2 pDLST scheme (Garcia-Fernandez and Carattoli, 2010). In agreement with other IncHI2 replicons, plasmid backbone was composed of regions responsible for replication (*repHI2*), conjugative transfer (*trh* genes), and plasmid maintenance (*par* gene). Additionally, similarly to other IncHI2 plasmids, it carried genes conferring resistance to tellurium (*terZABCDEF*), while genes conferring resistance to arsenic (*arsCBRH*) were not found. Moreover, one multidrug resistance (MDR) region was identified, in which the integron In2143 was embedded in a Tn*1696*-like transposon, also carrying a *qnrA1* resistance gene and a mercury (*mer*) resistance operon. In IncFIB plasmids, p48411_VIM and p54569_VIM showed limited similarity (57% coverage, 100% identity) to each other (Supplementary Figure S5), despite being both typed as IncFIB (pECLA) by PlasmidFinder analysis. Plasmid p48411_VIM, which contained only the IncFIB replicon of pECL_A (Ren et al., 2010), seemed to be a fusion derivative of plasmids pLec-476cz and pECL_A. It contained a 46,500-bp segment (1–32,410 and 157,676–171,765) being identical to a sequence of pLec-476cz including a part of the plasmidic backbone and a part of the MDR region, which contained the class 1 integron In110. The remaining 125,265-bp sequence, which contained a region encoding a type-F conjugative transfer system, carried regions responsible for resistance to tellurium, copper and silver, and a second MDR region carrying *dfrA14*, *catA2*, *sul2*, *strA*, *strB* and $bla_{\text{TEM-1}}$ resistance genes. This MDR region resembled the MDR region in pECL_A and p60214_IncFII. The pECL_A-like plasmid, p60214_ IncFII, was sequenced from a ST92 *E. hormaechei* isolate, characterized during this study. On the other hand, plasmid p54569_VIM was a derivative of pECL_A, which acquired a Tn*1721*-like transposition module (9986–24,830 bp) carrying In110 (Supplementary Figure S5). An identical transposition module has also been observed in plasmids p58983_VIM and pEncl-30969cz (as seen below). Direct repeats of 5 bp (TCCGG) were found at the boundaries of the Tn*3*-like element, suggesting its transposition into the pECL_A-like backbone. Unlike p48411_ VIM, no *tra* region was found on p54569_VIM. Additionally, it contained both IncFIB and IncFII replicons of pECL_A (Ren et al., 2010).

The IncN plasmid p59062_VIM, which carried the VIM-1encoding integron In4873, was typed as ST7 based on plasmid MLST (pMLST) scheme for rapid categorization of IncN plasmids (Garcia-Fernandez et al., 2011). It showed extensive similarity with other IncN plasmids (Supplementary Figure S6), like pTE_C_1 (83% coverage, 99.99% identity; GenBank accession no.MW574936), pNL194 (81% coverage, 99.22% identity; Miriagou et al., 2010) and p3846_IncN_VIM-1 (88% coverage, 100% identity; Marchetti et al., 2021). The In4873 integron was inserted between the genes, encoding EcoRII endonuclease and resolvase, of the IncN plasmidic backbone. Furthermore, a Tn*21* fragment consisting of *tniB* and *tniA* was found next to the 3'CS, 108 bp downstream of *orf5*, as in In2-like integrons. The *qnrS1* resistance gene was also found in p50962_VIM, located downstream of *fipA*.

The non-typeable plasmid p58983_VIM, which was characterized from a ST421 *E. cloacae* complex isolate, carried the VIM-1-encoding integron In110. It comprised a plasmidic backbone which was identical (100% coverage, 100% identity) to plasmid p54569CZ_2 (characterized from a ST92 *E. hormaechei* isolate in this study; Supplementary Figure S7). Additionally, it contained a MDR region being identical to the respective regions of plasmids p54569_VIM (characterized from a ST92 *E. hormaechei* isolate in this study) and pEncl-

30969cz (sequenced from a VIM-1-producing ST92 *E. cloacae* isolated, in 2015, in a Czech hospital; Papagiannitsis et al., 2018). Similar to pEncl-30969cz, the MDR of p58983_VIM was a Tn*1721*-like transposon structure containing In110, a Tn*21* fragment, a Tn*3*like transposon, and a *qnrB*-like gene conferring resistance to quinolones (Halova et al., 2014). Two copies of an IS*5075* element, which was shown previously to target the IRs of Tn*21*like transposons (Partridge and Hall, 2003), disrupted the IRs of the Tn*3*-like. The remaining part of the Tn*21 mer* module was probably deleted due to insertion of the Tn*3*-like transposon, but in contrast to pEncl-30969cz the Tn*3*-like was in an opposite orientation. Target site duplications of 6 bp (CAATAC) were found at the boundaries of Tn*1721*-like transposon, suggesting its transposition into the p58983_VIM backbone.

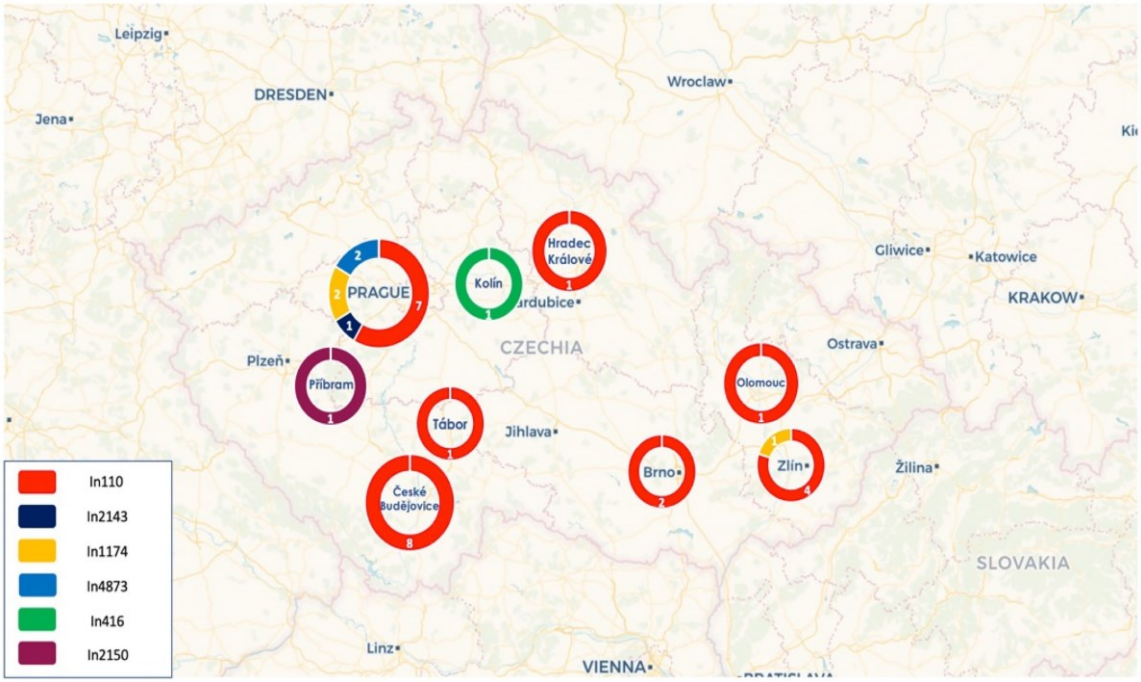

Figure 1: Distribution of the different integrons across the cities in the Czech Republic.

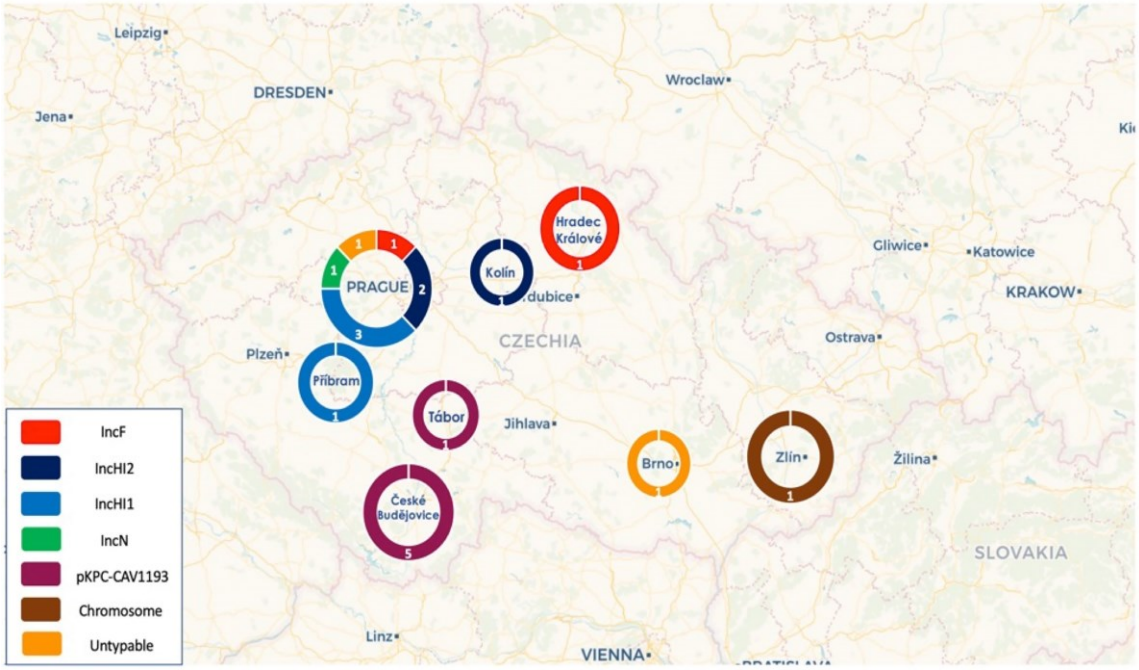

Figure 2: Distribution of the different plasmid types across the cities in the Czech Republic.

TABLE 2 WGS data of the isolates sequenced only using short reads sequencing platform (illumina).

Isolate	Species	ST	Replicons	*bla*VIM-positive Integron	Resistance genes
49049	E. cloacae	92	IncFIB(pECLA), IncFII(pECLA), IncQ1, ColpVC, Col440I	In110	aac(6′)-Ib-cr, aac(3)-IIa, aph(3′)-VIa, aadA1, bla_{VIM-1}, bla_{ACT-16}, catB3, dfrA14, sul1, fosA, qacE
51524	E. cloacae	92	IncFIB(pECLA), IncFII(pECLA), IncQ1, ColpVC, Col440I	In110	aac(6′)-Ib-cr, aadA1, aac(3)-IIa, aph(3′)-VIa, bla_{VIM-1}, bla_{OXA-1}, bla_{ACT-16}, catB3, dfrA14, sul1, qacE, fosA
52089	E. cloacae	92	IncFIB(pECLA), IncFII(pECLA), IncHI1A(NDM-CIT), IncHI1B(NDM-CIT), Col(pHAD28)	In110	aac(6′)-Ib-cr, aac(3)-IIa, aadA1, aph(3′)-Ia, blaVIM-1, blaCTX-M-15, bla_{OXA-1}, bla_{TEM-1B}, catB3, dfrA1, dfrA14, strA, strB, sul1, sul2, tetA, qnrB1, qacE
54680	E. asburiae	25	IncFIB(pECLA), IncFII(pECLA), IncHI2, IncHI2A	In110	aac(6′)-Ib-cr, aac(3)-IIa, aadA24, arr-3, blaVIM-1, blaCTX-M-15, blaOXA-1, bla_{TEM-1B}, bla_{ACT-6}, catA1, catB3, dfrA14, strA, strB, sul1, sul2, tetA, qnrB1, qacE, fosA
54818	E. cloacae	92	IncFIB(pECLA), IncFII(pECLA), IncHI2, IncHI2A, IncQ1, ColpVC	In110	aac(6′)-Ib-cr, aac(3)-IIa, aph(3′)-VIa, aadA1, blaVIM-1, blaCTX-M-15, blaOXA-1, bla_{TEM-1B}, bla_{ACT-16}, catA1, catB3, dfrA14, strA, strB, sul1, sul2, tetA, qnrB1, qacE
54822	E. cloacae	92	IncFIB(pECLA), IncFII(pECLA), IncQ1, ColpVC, Col440I	In110	aac(6′)-Ib-cr, aac(3)-IIa, aph(3′)-VIa, aadA1, dfrA14, bla_{VIM-1}, bla_{OXA-1}, bla_{ACT-16}, catB3, sul1, qacE, fosA
55614	E. cloacae	92	IncFIB(pECLA), IncFII(pECLA), IncQ1, ColpVC, Col440I	In110	aac(6′)-Ib-cr, aac(3)-IIa, aph(3′)-VIa, aadA1, blaVIM-1, blaOXA-1, blaACT-16, catB3, dfrA14, sul1, qacE, fosA
56501	E. cloacae	190	IncHI2, IncHI2A, pKPC-CAV1193, Col(pHAD28)	In110	aac(6′)-Ib-cr, aac(3)-IIa, aadA1, aadA2b, blaVIM-1, blaCTX-M-15, blaOXA-1, bla_{TEM-1B}, bla_{ACT-7}, catA1, catB3, dfrA14, strA, strB, sul1, sul2, tetA, qnrB1, qacE, fosA
57689	E. cloacae	106	IncFIB(pECLA), IncFII(pECLA), IncHI2, IncHI2A, pKPC-CAV1193, Col(pHAD28)	In110	aac(6′)-Ib-cr, aac(6′)-IIc, aph(6)-Id, aac(3)-IIa, aadA1, aadA2b, bla_{VIM-1}, blaCTX-M15, blaOXA-1, blaSHV-12, blaTEM-1B, bla_{ACT-15}, catA1, catB3, ereA, dfrA14, strA, strB, sul1, sul2, tetA, tetD, qnrB1, qnrS1, qacE, fosA

61347	E. cloacae	1735	Col(pHAD28), pKPC-CAV1193, Col(pHAD28)	In110	aac(6′)-Ib-cr, aadA2b, bla_{VIM-1}, $bla_{TEM}1_A$, blaACT-15, sul1, qacE, fosA
61503	E. cloacae	252	IncFIB(pECLA), IncFII(pECLA), repA(pENTd4a)	In110	aac(6′)-Ib-cr, aac(3)-IIa, aadA1, blaVIM-1, blaOXA-1, blaACT-3, catBe, dfrA14, sul1, qnrE1, qacE, fosA1

(Continued)

TABLE 2 (Continued)

Isolate	Species	ST	Replicons	*bla*VIM-positive Integron	Resistance genes
50714	C. freundii	673	IncFII(SARC14), IncN	In110	aac(6′)-Ib-cr, aph(3″)-Ib, aadA1, bla_{VIM-1}, bla_{CMY-78}, dfrA14, sul1, sul2, qacE, qnrS1
59343	K. pneumoniae	54	IncC, IncN, IncFIB(pQil), IncFII(K), IncFIB(K) (pCAV1099-114), IncHI1B(pNDM-MAR)	In4873	aac(6′)-Im, aacA27, aph(3′)-Ia, aadA2, blaVIM-1, blaKPC-2, blaSHV-178, catA1, dfrA1, sul1, tetD, qnrS1, qacE, oqxA, oqxB, fosA

The non-typeable plasmid p56415_VIM, which carried the VIM-4-encoding integron In1174, was composed of two parts: the plasmidic backbone and the MDR region. The plasmidic backbone was identical to plasmid p51929, which was characterized from a VIM-4-encoding *C. freundii* isolate (Cfr-51929cz; Bitar et al., 2020). Even though the plasmid p51929 carried no resistance genes, Cfr-51929cz also harbored the VIM-4-encoding integron In1174 in the *mcr-9*-positive plasmid (p51929_MCR_VIM that belonged to IncHI2 group). The MDR region of p56415_VIM consisted of a Tn*3*-like element carrying the In1174 integron (Supplementary Figure S8). The same Tn*3*-like transposon was inserted into the chromosome of the *K. pneumoniae* isolate Kpn51135cz (as seen below). Unlike Kpn51135cz, the IRmer of the Tn*3*-like element was intact, whilst the IRtnp was disrupted by IS*5075*. Direct repeats of 6 bp (AATATG) were found at the boundaries of the integrated segment, suggesting its transposition into the p56415-VIM plasmid.

Finally, in *K. pneumoniae* isolate Kpn51135cz, the VIM-4encoding integron In1174 was localized in a Tn*3*-like transposon element that was integrated into the *K. pneumoniae* chromosome (Supplementary Figure S9). The IR of the Tn*3*-like *tnp* module and the IR of the *mer* module, at the boundaries of the the insertion sequences IS*4321*. Direct repeats of 5 bp (CTCAA) were found at the boundaries of the integrated segment, suggesting its transposition into the *K. pneumoniae* chromosome.

Discussion

In the current study, we characterized 32 VIM-producing Enterobacterales (including *E. cloacae*, *C. freundii*, *K. pneumoniae* and *K. michiganensis* isolates), which were isolated during the period of 2019–2020, in order to analyze the genetic determinants involved in the dissemination of *bla*$_{\text{VIM}}$ alleles in various Czech hospitals. Our findings showed the presence of two *bla*$_{\text{VIM}}$ variants, *bla*$_{\text{VIM-1}}$ (n = 26) and *bla*$_{\text{VIM-4}}$ (n = 6), carried by a significant number of integrons. The main VIM-1-encoding integron, identified during this study, was In110 (n = 24), while the In4873 was found in two ST54 *K. pneumoniae* isolates. On the other hand, three VIM-4-producing isolates included the In1174 integron, one isolate carried the In416, and the two remaining isolates carried novel integron structures. The In110 integron has been previously reported from isolates of Czech origin (Papousek et al., 2017; Papagiannitsis et al., 2018). Additionally, the presence of other integron types and the emergence of novel integron structures demonstrates the ongoing evolution of genetic determinants involved in the spread of resistance. Integrons usually carry more than one resistance gene, conferring resistance to multiple antimicrobial classes. Therefore, integrons are associated with the emergence of MDR bacteria and the fact of co-selection.

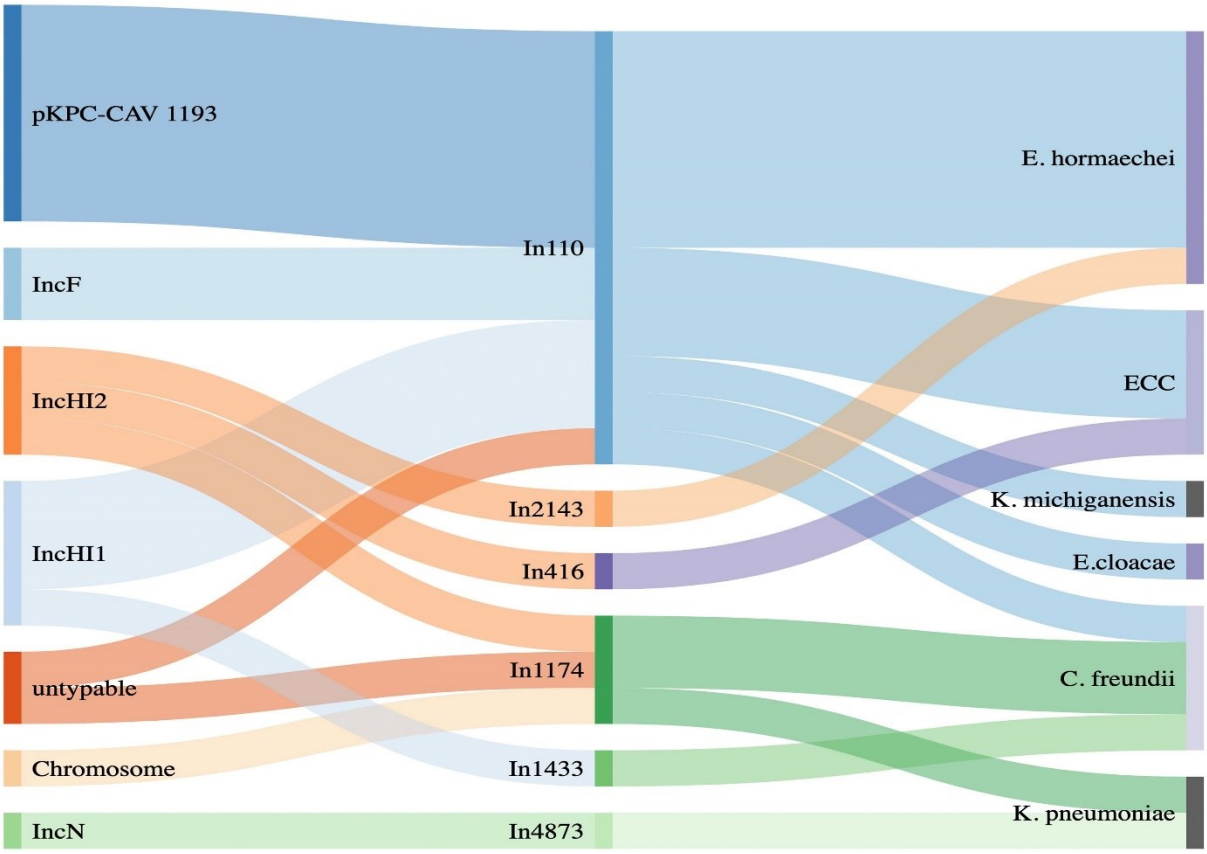

Figure 3: Sankey diagram showing the distribution of different integrons linking to the plasmid types (left) and to the bacterial species detected (right).

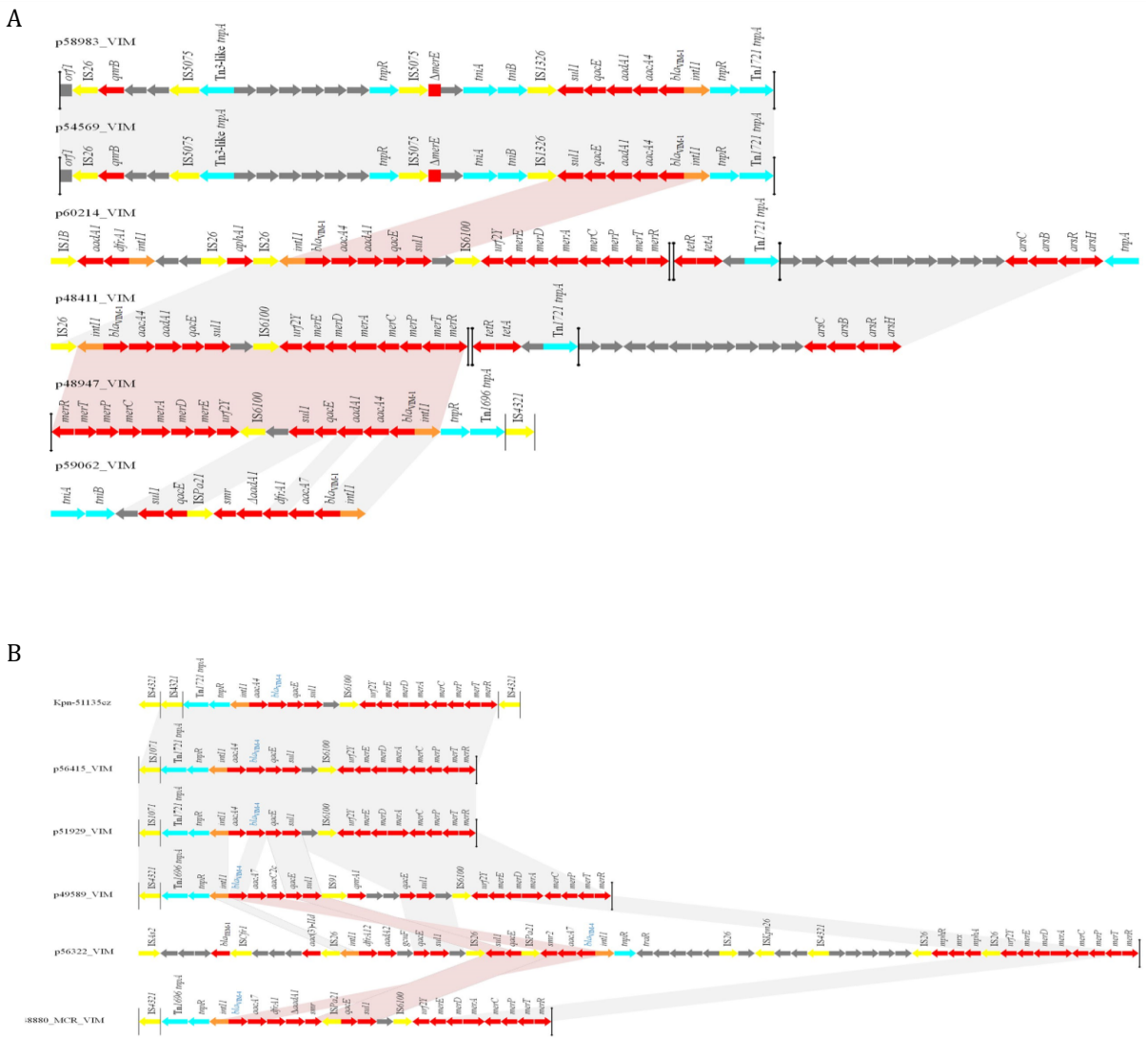

Figure 4: Linear comparisons of VIM-1- VIM-4- encoding MDR regions characterized from Enterobacterales isolated from Czech hospitals, during . Arrows show the direction of transcription of open reading frames (ORFs). Resistance genes are shown in red. IS elements and transposases are shown in yellow and aqua, respectively. intI1 genes are shaded orange. The remaining genes are shown in gray. Homologous segments (representing ≥99% sequence identity) are indicated by light gray shading, while pink shading shows inverted homologous segments.

Moreover, the analysis of WGS data showed that *bla*$_{VIM}$positive integrons were carried by several plasmids belonging to different Inc. groups (pKPC-CAV1193-like, HI1, HI2, FIB, N and non-typeable) and presenting diverse sizes (Table 1; Figure 3). Also, in one *K. pneumoniae* isolate, belonging to ST11, the VIM-4encoding integron, In1174, was integrated into the chromosome (Supplementary Figure S9). Another interesting finding is the emergence of hybrid plasmids, such as p48411_VIM and p54569_ VIM. The plurality of different plasmids, carrying *bla*$_{VIM}$ alleles, and the emergence of hybrid plasmids are two features widening the spectrum of species that these resistance determinants could be disseminated. Also, of note was the characterization of plasmids being identical to VIM-1-encoding plasmid pLec-476cz (Supplementary Figure S2), which was previously characterized from a *L. adecarboxylata* isolate (Papousek et al., 2017) recovered during a survey study focused on compliance with hand hygiene among the staff of a different Czech hospital

in May 2011. This data is worrying, since it highlights the hidden source, and the continuous spread of resistance determinants, and especially of carbapenemase-encoding genes, in Czech hospitals.

Finally, analysis of MDR regions revealed an increased divergence among these sequences (Figure 4). However, we observed the same VIM-1-encoding MDR region in IncHI1 (pNDM-CIT) plasmids (like p60214_VIM) and the IncFIB (pECLA) plasmid p48411_VIM, while a part of this structure was found in pKPC-CAV1193-like plasmids (like p48947_VIM). The presence of the same MDR region in plasmids of different Inc. groups may be the outcome of homologous recombination events. Also, we observed the presence of an identical transposition module in the IncFIB (pECLA) plasmid p54569_VIM and the non-typeable plasmid p58983_VIM. A totally different structure was identified in the IncN plasmid p59062_VIM. Regarding the VIM-4-encoding structures, we observed the presence of an identical transposition module in the IncHI2 plasmid p51929_ MCR_VIM, the non-typeable plasmid p56415_VIM and in the chromosome of the ST11 *K. pneumoniae* isolate Kpn51135cz. A similar transposition module, differing by acquisition of a different VIM-4-encoding integron (In2143 in p49589_VIM unlike In1174 in p56415_VIM) and of *qnrA1* resistance gene, was found in IncHI2 plasmid p49589_VIM. The presence of the same transposition modules into different replicons indicates the functional role of the specific transposons. On the other hand, totally diverse MDR regions were identified in IncHI1 (pNDMCIT) plasmid p56322 and IncHI2 plasmid p48880_MCR_VIM.

One limitation of the study is not investigating the ability of the detected plasmids to conjugate. This is due to the high diversity in plasmids detected and to the different conjugation dynamics between *in-vitro* and *in-vivo*. This difference is prevalent for example to IncR and pKPC-CAV1193-like plasmids. The *in-vitro* conjugation assay for these two plasmids detected has a very low conjugation rate if any (Sheppard et al., 2016; Bitar et al., 2020) yet epidemiological results state the high ability of these plasmids to conjugate.

Of note, all pKPC-CAV1193 plasmids carried the In110 integrons which seems to be spread in Cesky Budejovice implying an outbreak of this plasmid in the city (Figures 1, 2). Moreover, all IncF and most of IncHI1 plasmids (except for one plasmid carrying In1433 integron; Figure 3) also carried In110 integrons. The IncHI2 plasmids carried different types of integrons such as In2143, In416 and In1174 (which was also found on the chromosome and on one un-typable plasmid). Finally, IncN plasmid carried In4873. This shows, although there is dominance of In110, that there are multiple integrons responsible for the dissemination of *bla*$_{VIM}$ irrespective of the plasmid type. Additionally, of interest is the association of In110 with multiple replicons (Figure 3).

Conclusion

During this study, we observed the spread of VIM-encoding integrons, mainly of In110, among Enterobacterales isolated from Czech hospitals, in 2019 and 2020. Additionally, we noticed the presence of multiple mechanisms, including (i) the functional acquisition of *bla*$_{VIM}$-carrying transposons, (ii) the acquisition of *bla*$_{VIM}$-carrying MDR regions *via* homologous recombination events (iii) the ongoing evolution of *bla*$_{VIM}$-carrying integrons, (iv) and the hidden spread of *bla*$_{VIM}$-carrying replicons, involved in the

emergence and spread of MDR regions carrying carbapenemase-encoding genes. Thus, ongoing surveillance of carbapenem-resistance is of utmost importance to control the spread of these emerging pathogens.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

IB and CP designed the study, analyzed the data, and wrote the manuscript. KC collected the samples. IB, LK, HZ, VM, EP, and MF conducted the experiments. IB and JH secured the funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: "https://www.frontiersin.org/articles/10.3389/fmicb.2022.993240/full"

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5.4 Publication nb.4: Molecular Characterization of Carbapenem and Ceftazidime-Avibactam Resistant Enterobacterales and Horizontal Spread of *bla*$_{NDM-5}$ Gene at a Lebanese Medical Center

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Molecular characterization of carbapenem and ceftazidime-avibactam-resistant Enterobacterales and horizontal spread of *bla*$_{NDM-5}$ gene at a Lebanese medical center

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Abstract:

In the battle against multidrug-resistant bacterial infections, ceftazidime-avibactam (CZA) stands as a pivotal defense, particularly against carbapenem-resistant (CR) Gram-negative pathogens. However, the rise of resistance against this drug poses a significant threat to its effectiveness, highlighting the critical need for in-depth studies about its resistance mechanisms. This research focuses on the genomic characterization of CR and CZA resistant *Escherichia coli* (n= 26) and *Klebsiella pneumoniae* (n=34) strains, harboring the *bla*$_{NDM}$ and/or *bla*$_{OXA-48-like}$ genes, at a major Lebanese tertiary care medical center.

Our findings revealed a notable prevalence of *bla*$_{NDM}$ in all *K. pneumoniae* strains isolates, with 27 of these also harboring *bla*$_{OXA-48}$. On the other hand, *E. coli* strains predominantly carried the *bla*$_{NDM-5}$ gene**.** Whole Genome Sequencing (WGS) identified a predominance of ST383 among *K. pneumoniae* strains, which possessed a multi-replicon IncFIB-IncHI1B plasmid harboring the *bla*$_{NDM-5}$. Additionally, various Inc group plasmids in *K. pneumoniae* across multiple sequence types were found to carry the *bla*$_{NDM}$. Similarly, diverse STs of *E. coli* were observed to carry *bla*$_{NDM-5}$ on different plasmids.

The study underscores NDM carbapenemases as a paramount resistance mechanism in Lebanon, jeopardizing critical last-resort treatments. It also illuminates the role of varied sequence types and mobile genetic elements in the spread of NDM resistance, stressing the urgent need for strategies to mitigate this threat, especially in nosocomial infections.

Introduction

Carbapenems are the drug of choice for the treatment of complicated multidrug- resistant (MDR) bacterial infections. However, their misuse has led to an increased emergence of resistant organisms [1]. The World Health Organization (WHO) has classified Carbapenemase-producing Enterobacterales (CPE), especially *Klebsiella pneumoniae* and *Escherichia coli*, as a worldwide public health concern due to their proliferation in hospital settings (https://apps.who.int/iris/bitstream/handle/10665/312226/WHO-UHC-SDS-2019.6-eng.pdf?sequence=1&isAllowed=y). Resistance mechanisms to carbapenems include the increased activity of the efflux pumps, porin loss, and β-lactamases production, particularly carbapenemases [2]. These carbapenemases are divided according to Ambler into four classes, with only class A (i.e. *Klebsiella pneumoniae* Carbapenemase (KPC)), Class B (i.e. New-Delhi Metallo-β-lactamase (NDM)), and Class D (i.e. Oxacillinase (OXA)-48 and OXA-48-like) being associated with carbapenemase activity [3-5].

NDM is among the most common and clinically significant carbapenemase in Enterobacterales capable of efficiently hydrolyzing a wide range of β-lactams, including penicillins, carbapenems, and cephalosporins [6, 7]. It has rapidly spread across the Middle East, the Indian subcontinent, and the Balkan region [8, 9]. In the United Kingdom, in 2011, NDM-5 was first detected and two amino acid substitutions at positions 88 and 154, lead to increased NDM enzymatic activity [10].

Furthermore, there has been a notable spread of genes encoding OXA-48-like carbapenemases. In 2001, a *K. pneumoniae* strain resistant to all available β-lactams was reported in Turkey. A subsequent study by Poirel L. et al. in 2004 identified this isolate as carrying a novel *bla* variant, $bla_{\text{OXA-48}}$ [11]. Isolates harboring $bla_{\text{OXA-48-like}}$ genes have garnered attention due to their rapid horizontal transmission and increasing detection across the Middle East and North Africa showing resistance to β-lactamase inhibitors ([7, 11-13]. Co-production of NDM- and OXA-48-like enzymes has been increasingly reported, particularly in patients with travel histories from the Middle East, Southeast Asia, Europe, China, and Africa [5, 14-19].

In Lebanon, few studies highlighted the prevalence of carbapenem resistance (CR) and their mechanisms among clinical isolates in this country [20]. For example, a study conducted between 2008-2012 showed an increase in reduced susceptibility to ertapenem from 0.4% to 1.6% [21]. By 2019, resistance levels in Enterobacterales reached 3.3%, with higher rates observed in *Pseudomonas* spp. (27.3%) and Acinetobacter spp. (53.7%) [22]. In 2008, the first case of OXA-48 was detected in *K. pneumoniae* in Lebanon (24). Moreover, the first cases of NDM-1 and NDM-5, isolated in 2010 and in 2017, respectively, were identified in *K. pneumoniae* isolates [23, 24]. Between 2015-2019, carbapenemase producing *E. coli* were found to be positive for $bla_{\text{OXA-48}}$ (n=11), $bla_{\text{OXA-181}}$ (n=12), and $bla_{\text{NDM-5}}$ (n=4) and $bla_{\text{NDM-19}}$ (n=9), while *K. pneumoniae* isolates harbored $bla_{\text{OXA-48}}$ (n=6), $bla_{\text{OXA-181}}$ (n=1) and $bla_{\text{NDM-5}}$ (n=1) [22]**.** A recent study in Lebanon in 2019, following the introduction of ceftazidime-avibactam (CZA) as a treatment for Extended Spectrum β-lactamase (ESBL) and multi-drug resistant (MDR) infections, analyzed one hundred and fifty isolates (*E. coli* and *K. pneumoniae*). These were classified as ESBL (n=20), MDR (n=20), or carbapenem resistant (CR) (n=110). All CR isolates exhibited a 100% resistance to ertapenem. Moreover, 60% (n=30) of CR *E. coli* and 65% (n=39) of CR *K. pneumoniae* showed resistance to CZA, with all resistant isolates being producers of NDM and/or OXA-48-like enzymes [25]**.**

This study, thus, was warranted at expanding our understanding of the resistance and dissemination by performing a whole genome characterization of CR and CZA resistant *E. coli* and *K. pneumoniae* strains producing NDM and/or OXA-48-like carbapenemases, at a major Lebanese tertiary care medical center.

Materials and methods:

Bacterial isolates and determination of carbapenems minimal inhibitory concentrations (MICs).

This study encompassed characterization a total of 60 Enterobacterales isolates (consisted of 34 isolates of *K. pneumoniae* and 26 isolates of *E. coli*) recovered at the Clinical Microbiology Laboratory of the Department of Pathology and Laboratory Medicine at the American University of Beirut Medical Center, between 2019 and 2021. These isolates as were identified by the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using the MALDI-biotyper software (Bruker Daltonics, Bremen, Germany). The MICs determination to 3 carbapenems and CZA were carried out as reported earlier [25].

For further characterization, all isolates were sent to Charles University, Faculty of Medicine in Pilsen, Czech Republic. Carbapenemase production and activity were assessed using MALDI-TOF MS with the meropenem hydrolysis assay, as described by Rotova *et al.* [26], and the double disk synergy test incorporating EDTA, phenylboronic acid, and temocillin, following previously described methodologies [27]. In addition antimicrobial susceptibility testing was conducted using the broth microdilution method, adhering to the EUCAST guidelines, with results interpreted based on the EUCAST 2024 criteria (https://www.eucast.org/clinical_breakpoints) [28].

Gene content and multilocus sequence typing (MLST) by PCR:

The presence of *bla*$_{IMP}$-like, *bla*$_{KPC}$-like, *bla*$_{NDM}$-like, *bla*$_{VIM}$-like, and *bla*$_{OXA-48}$-like genes was tested via PCR [29]. Additionally, Multilocus sequence typing (MLST) was performed on the isolate collection as previously described [30-32].

Short reads sequencing and analysis:

Based on MLST and MIC results, 17 *K. pneumoniae* and 10 *E. coli* isolates were selected as representatives for whole genome sequencing. Genomic DNA was extracted using NucleoSpin Microbial DNA kit (Machery-Nagel, Germany). A multiplexed Library was prepared using Nextera XT library preparation kit, and sequencing was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) using the MiSeq v3 reagent kit using 600 cycle cartridges. Initial paired-end reads were quality assessed using fastqc, then trimmed and filtered to high quality using fastp [33, 34]. Subsequent reads were assembled by a de Bruijn graph-based *de novo* assembler SPAdes [35]. The assembled contigs were analyzed to detect Sequence Types (STs) using MLST software, virulence genes using VFanalyzer, and resistance genes using ResFinder and plasmid incompatibility (Inc) types using PlasmidFinder [36-38]. Additionally, these contigs were also uploaded to PHASTER to detect phage genomes, CRISPRfinder to detect CRISPR-cas systems, and ISfinder to detect insertion sequences [39, 40].

Long read sequencing and analysis:

Based on the previous short-read results, 7 and 4 representative isolates corresponding to *K. pneumoniae*, and *E. coli* isolates respectively were selected for long-read sequencing using PacBio Sequel I (Pacific Biosciences, California, United States of America). Briefly, the extracted DNA was subjected to shearing using the Megaruptor 2 (Diagenode, Liege, Belgium) this will generate sheared fragments of ~15kb each. Library preparation was performed using the Microbial multiplexing protocol according to the manufacturer recommendation. During the library preparation, no size selection was performed. Using SMRT Link v10.1, the microbial assembly pipeline was used for the assembly and the circularizing of contigs using a minimum seed coverage of 30x. The assembled contigs were then analyzed using the same procedures as the short-read assembly. Finally, all genomes were annotated using the NCBI's Prokaryotic Genome Annotation Pipeline [41-43].

Phylogeny and Single Nucleotide Polymorphism (SNP):

To study the genetic diversity and phylogenetic relationship of the studied isolates and global isolates, genomes of *K. pneumoniae*, specifically of the ST383 type, were retrieved from the NCBI assembly database, encompassing both complete and draft genomes, totaling 105 genomes. Using parsnp v1.7.4 (available in the harvest suit) a core genome phylogeny of the model General Time Reversible (GTR) based on SNP and recombination, a SNPs-based phylogeny was constructed between the sequenced genomes in this study and the 105 genomes downloaded from the NCBI database using KP1674 as a reference (complete circular genome) and *K. quasipneumoniae* as an outgroup [44-47]. Briefly, SNPs identified in local collinear blocks were subsequently used for reconstructing an approximate maximum-likelihood tree using FastTree, while including the general time reversible (GTR) model of nucleotide substitution. The Shimodaira–Hasegawa test implemented in FastTree2 was used to assess the support for significant clustering in the observed phylogeny. The interactive tree of life or iTOL (https://itol.embl.de/) was used for the graphic illustration of the trees along with relative annotations.

Moreover, the SNPs between the ST383 genomes was detected to determine the extent of clonal dissemination in the hospital. SNPs were detected using snippy multicommand (snippy-base application v4.5.0) [48]) which generates a core genome multiple alignment against the common reference (KP1674). The pipeline detects the variants and generates a single file for each isolate listing the different variations.

Comparison of Antimicrobial Multidrug-Resistant (MDR) region:

The MDR sequence from KP1633 isolate (complete circular genome) was used as a reference to compare with the rest of the isolates. Easyfig v2.2.0 was used for generating the figure [49]. It uses blastn from the package blast + by NCBI to generate a similarity report between the fragments [50]. An alignment between the short-read sequences and the MDR sequence characterized with Pacbio was used to generate a consensus and then annotated using Prokka to include in the generation of the figure [51].

Data availability:

All genomes have been submitted to the NCBI database. Accession numbers are available under the BioProject PRJNA952851.

Results:

Phenotypic Antimicrobial Resistance Profile of the Isolates:

All 34 *K. pneumoniae* isolates showed resistance against CZA, carbapenems, tazobactam, ciprofloxacin, and tetracycline. Moreover, all isolates showed resistance against amikacin and gentamicin (except one isolate) (Table 1). Similarly, all *E. coli* isolates (n=26) were also resistant against CZA, carbapenem, and showed resistance to ciprofloxacin and tazobactam and tetracycline (except two isolates). However, all isolates were susceptible against amikacin apart from two resistant isolates (Table 2).

Molecular Characteristics of the isolates:

PCR-screening showed that all the *K. pneumoniae* isolates were positive for *bla*$_{\text{NDM}}$ (n=34) while 27 isolates of them were simultaneously positive for the presence of *bla*$_{\text{OXA-48}}$. On the other hand, the *E. coli* isolates carried *bla*$_{\text{NDM-5}}$ gene.

MLST was performed on all the collection. MLST of the *K. pneumoniae* isolates revealed the presence of a major ST: ST383 (n=26), while few other STs were present as well; ST101 (n=2), ST111 (n=1), ST147 (n=1), ST307 (n=1), ST147(n=1), ST39 (n=1), and ST15 (n=1) (Table 1). Conversely, based on Achtman Scheme, MLST of the *E. coli* isolates showed several STs circulating within the hospital; ST405 (n=9), ST167 (n=4), ST617 (n=3), ST361 (n=2), ST46 (n=2), ST648 (n=1), ST2450 (n=2), ST131 (n=1), ST998 (n=1), and ST1284 (n=1) (Table 2).

Whole genome sequencing:

Based on the susceptibility profiles, antibiotic resistance genes (PCR results), and MLST results, representative isolates (17 *K. pneumoniae* and 10 *E. coli*) were selected for sequencing using short reads sequencing. WGS data showed that 13 of the 17 analyzed *K. pneumoniae* isolates carried *bla*$_{\text{NDM-5}}$ while 4 isolates carried *bla*$_{\text{NDM-1}}$. ST383 isolates co-harbored the *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-48}}$ genes, while another ST147 isolate co-harbored *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-232}}$. All the isolates harbored additional resistance genes against aminoglycoside (*armA*, and/or *aph(3')-VI*, and/or *aac(6')-Ib*), beta-lactams (*bla*$_{\text{SHV-145}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM-5/1,}}$ and/or *bla*$_{\text{TEM-1B}}$), quinolones (*OqxB*, *OqxA*, and/or *qnrS1*), streptogramin b (*msr(E)*), macrolides (*mph(A), mph(E)*), and tetracyclines (*tet(A)*) (Table 3). The genomic antibiotic resistance determinants were correlated with the resistant phenotype (Table 1). In the 10 analyzed isolates of *E. coli*, WGS data analysis revealed the presence of *bla*$_{\text{NDM-5}}$ in all isolates. Moreover, additional resistance genes were detected conferring resistance to beta-lactams (*bla*$_{\text{NDM-5}}$, and/or *bla*$_{\text{CTX-M-15}}$, and/or *bla*$_{\text{CMY-145}}$), macrolides (*mph(A)*), quinolones (the presence of efflux pump AcrAB), aminoglycoside in some isolates (*aac(6')-Ib-cr*), and tetracyclines (*tet(B)*) (Table 4 and Table S2). The genetic antibiotic resistance determinants were associated with the resistant phenotype (Table 2).

Based on the short reads sequencing results, 7 *K. pneumoniae* isolates (1 isolate each from ST307, ST15, ST147, ST39, ST101 and two isolates from ST383) and 4 *E. coli* (one isolate each from ST405, ST131, ST2450 and ST617) were selected for long reads sequencing to have complete circular chromosomes and plasmids for downstream analysis.

In *K.* pneumoniae, the long reads sequencing of the two ST383, KP1633 and KP1674, showed that the *bla*$_{\text{NDM-5}}$ was harbored on a fusion plasmid (IncFIB and IncHI1B) that was 372,845 bp (pKP1633LB_IncFIB_IncHI1B_NDM5) and 372,828 bp (pKP1674LB_IncFIB_IncHI1B_NDM5) in size, respectively. Upon blasting, the plasmid against the NCBI database it showed 100% query coverage and 99.9% sequence identity with pFQ61_ST383_NDM_5 that was 376,754 bp in size (acc.# CP091814); a plasmid isolated from a clinical *K. pneumoniae* strain in Qatar in 2016. *In-silico* plasmid analysis of the rest of the ST383 *K. pneumoniae* isolates that were sequenced using short reads, showed that all ST383 isolates carried the same plasmid type. Furthermore, pKP1633LB_IncFIB_IncHI1B_NDM5 carried a 35kb MDR sequence (from 286445 bp to 323218 bp). The MDR region was composed of a transposase from IS*1*, followed by 2 additional transposases, and ends with a transposase. Furthermore, the same MDR region was also detected in isolate KP1674 on the plasmid pKP1674LB_IncFIB_IncHI1B_NDM5 from position 52,157-85,730. In the case of short reads sequenced isolates, the MDR presence was confirmed using blast, and later a consensus was made for subsequent analysis. Data revealed that this MDR sequence was present in most ST383 isolates, with the exception of isolate KP1679 lacking 4 resistance genes (*bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-9}}$, *ant(3')-I,* and *aac(6')-Ib*). Similarly, isolate KP1655, noted the absence of *bla*$_{\text{TEM-1}}$. In addition to finding missing resistance genes across the isolates, some IS transposases were also found missing (IS*1380-like* only found in 3 isolates). Interestingly, the starting and ending IS transposases were found in all isolates (Figure. 1). Furthermore, all ST383 *K. pneumoniae* strains carried, also, the IncL plasmid harboring *bla*$_{\text{OXA-48}}$ which had the same plasmid size (68,942 bp). This plasmid has been reported extensively and high similarity scores were noticed upon blasting against the NCBI database [52-54].

Other STs such as ST111 KP1859 carried the *bla*$_{\text{NDM-5}}$ on an IncX3 plasmid, while ST307 KP1608 carried the *bla*$_{\text{NDM-5}}$ on an IncFII plasmid. On the other hand, the *bla*$_{\text{NDM-1}}$ was carried on different Inc plasmids; IncM2 plasmid (pKP1880LB_IncM2_NDM1 87,450 bp) was detected in KP1880 ST 101. This plasmid exhibited 100% query coverage and 100% sequence identity with AR_0127 plasmid (87,450 bp) identified in *a Salmonella enterica* clinical strain in USA in 2018. Moreover, ST39 KP1851 and ST15 KP1917 both carried the *bla*$_{\text{NDM-1}}$ on the same IncC plasmid (pKP1851LB_IncC_NDM1 137,593 bp and pKP1917LB_IncC_NDM1 140,300 bp respectively) (99% query coverage and 99.9% sequence identity). This plasmid exhibited 100% sequence identity and 98% query coverage with pNDM-US (acc.# CP006661.1) which was isolated from a clinical strain in 2016 in the USA. Finally, ST147 KP1734 carried the *bla*$_{\text{NDM-1}}$ on an IncFIB-IncHI1B plasmid (269,122 bp) while *bla*$_{\text{OXA-232}}$ was carried on a ColKP3 plasmid (6,141 bp). The IncFIB-IncHI1B plasmid had similar sequence to those identified in ST383 isolates, KP1633 and KP1674, and the ColKP3 showed the same nucleotide sequence as that of pC06114_4 (acc.# CP016038.1) (100% identity and 100% query coverage), a plasmid isolated in 2016 in Germany.

All the characterized *E. coli* isolates carried the *bla*$_{\text{NDM-5}}$ gene. Long reads sequencing revealed that ST131 EC1918 and ST2450 EC1811 isolates carried the *bla*$_{\text{NDM-5}}$ gene on the same IncX3 plasmid (79,672 bp). Moreover, short reads sequencing showed that ST1284 EC1733 carried an IncX3 plasmid, identical to pKP1859LB_IncX3_NDM5. Moreover, the *bla*$_{\text{NDM-5}}$ gene was carried on the IncFIA-IncFII (pEC1609LB_IncFIA_IncFII_NDM5, 118,953 bp) in ST405 EC1609 and on IncFII-IncFIA-IncFIB (pEC1856LB_IncFII_IncFIA_IncFIB_NDM5, 118 140,386 bp) in ST617 EC1856. Short-reads sequences

scaffolding showed that IncF plasmids (with different structure and sequences) carrying *bla*$_{NDM-5}$ gene was found in the rest of *E. coli* despite STs.

ST383 *K. pneumoniae* virulence factor analysis, revealed the presence of virulence genes responsible for acriflavine resistance, type VI secretion system, synthases, transporters, esterase, adherence, regulation, immune modulation, fimbriae, and delivery. Some genes were found to be missing, for example the regulatory gene for the mucoid phenotype *rmp*A2 (KP1710, KP1679, KP1663, KP1657, KP1658, KP1655, KP1645, KP1639, and KP1601). In *E. coli* the presence of the virulence genes was associated with acriflavine resistance, invasion, secretion, adherence, fimbriae, enterobactin and yersiniabactin. Notably in ST648 EC1930 in comparison with ST648 EC1726 some virulence factors were found missing, *fim* genes for fimbriae, *gsp* genes for secretion, *irp* genes for capsule and invasion characteristics, and *ybt* genes for yersiniabactin. Also, in comparison between the ST405 EC735 and ST405 EC1609, genes of *esp* variations responsible for secretion and 2 genes for adherence (*pap*E and *pap*F) were found to be missing (Supplementary Tables S1 and S2).

Phylogeny and clonality:

To evaluate the relatedness of the *K. pneumoniae* ST383 isolates in the study, global SNPs phylogeny was performed on all *K. pneumoniae* ST383 isolates in the NCBI database. The phylogenetic tree showed that our ST383 isolates clustered together along with previously detected NDM-5 producing ST383 isolates from Lebanon (Figure 2). Furthermore, SNP analysis was performed on all isolates belonging to this clade and showed high similarity with SNPs ranging from 11 to 73 (Table 5, supplementary Table S3). Plasmid analysis of the Lebanese clade showed that all the clustered isolates carried the *bla*$_{NDM-5}$ gene on the same Inc plasmid (IncFIB-IncHI1B) previously detected. Remarkably, these clustered isolates were all NDM-5/OXA-48 co-producers and their *bla*$_{OXA-48}$ gene was similarly carried by an IncL plasmid. (Figure 2).

Moreover, the clonality of the *K. pneumoniae* ST383 strains was assessed through analyzing the CRISPR array sequences. A population of any isolates that went through the same environmental conditions (subjected to phages or plasmids) will have a similar CRISPR array since these sequences (correlated with foreign sequences) are added in chronological order [55]. CRISPRCasFinder showed that all genomes have the CRISPR/Cas I-E type. The sequences were then aligned, and a SNPs-based phylogenetic tree was executed (Figure 3). The tree showed that the isolates was divided between two distinct clades (A and B) and one isolate (KP1655) that has two CRISPR arrays residing alone. The two clades were composed of both, isolates from this study and previously identified in Lebanon. This result shows that the Lebanese isolates' population is originating from at least 3 different ancestors which underwent different environmental conditions.

Discussion:

Lebanon confronts significant antibiotic resistance, notably with the recent emergence of resistance to ceftazidime-avibactam in CRE treatments, [22, 56, 57]. This study investigated CR *K. pneumoniae* and *E. coli* strains exhibiting resistance to last line antibiotic choices, including carbapenems and CZA, as reported by [25]. It intended to explore mechanisms involved in their resistance and dissemination especially through

characterizing the genetic features of *bla*$_{\text{OXA-48-like}}$ and/or *bla*$_{\text{NDM}}$-positive *K. pneumoniae* and *E. coli* isolates recovered at a major Lebanese medical center.

Sequence data showed the presence of *bla*$_{\text{NDM}}$ on plasmids of different Inc groups, in *K. pneumoniae* belonging to different STs (Table 3). Most of the *K. pneumoniae* isolates belonged to ST383 and carried a multireplicon plasmid (IncFIB-IncHI1B) with *bla*$_{\text{NDM-5}}$. This sequence type has been reported in different parts of the world, including countries around the Mediterranean basin, where it contributes to the burden of CRE [58-60]**.** The detection of *bla*$_{\text{NDM-5}}$ positive isolates raises significant concerns due to its potent ability to break down a broad spectrum of β-lactam antibiotics, including carbapenems. Recognized globally for its contribution to antibiotic resistance [61-64], the prevalence of *bla*$_{\text{NDM-5}}$ underscores the urgent challenge it poses to treating bacterial infections. The concurrent presence of *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-48}}$ in our strains severely limits treatment options with last-resort antibiotics like ceftazidime-avibactam. This synergy of resistance genes, documented across the MENA region [19, 65-68] and mirrored in findings from Italy [17], emphasizes the global nature of this threat and underscores the need for international efforts in surveillance and containment. This finding highlights the important role of horizontal transfer in the worldwide spread of *bla*$_{\text{NDM}}$ resistance determinants [69].

Additionally, the close clonal phylogenetic relationship of ST383 isolates was confirmed by SNPs phylogeny. ST383 is a high-risk clone associated with the spread of diverse resistance mechanisms [70] like *bla*$_{\text{KPC}}$ and *bla*$_{\text{VIM}}$. Nevertheless, CRISPR-Cas array analysis revealed genetic diversity within ST383 *K. pneumoniae* isolates, identifying distinct clades and indicating diverse ancestral origins, suggesting varied exposure to environmental pressures and selective forces.

On the other hand, *bla*$_{\text{NDM-5}}$ was also found in *E. coli* isolates. *E. coli* isolates, which belonged to diverse STs, harbored a variety of *bla*$_{\text{NDM-5}}$-carrying plasmids. The variation of plasmids involved in the spread of *bla*$_{\text{NDM-5}}$ underlines the important role of mobile elements, like insertion sequences and transposons, in the horizontal spread of resistance mechanisms. However, short-read sequencing data suggested that, in most of the *E. coli*, which belonged to diverse STs, the *bla*$_{\text{NDM-5}}$ gene was localized on IncF-type plasmids. Additionally, WGS data confirmed the presence of similar *bla*$_{\text{NDM-5}}$-carrying plasmids, like pKP1674LB_IncFIB_IncHI1B_NDM5 (IncFIB and IncHI1B) and pEC1609LB_IncFIA_IncFII_NDM5 (IncF-type), into different STs or species. These findings highlight the important role of plasmids in the horizontal gene transfer (HGT) of resistance genes in the institution and community where such strains exist.

Conclusion

In conclusion, the current study confirmed the fact that production of NDM carbapenemases is one of the most clinically significant resistance mechanisms among CRE isolates in Lebanon, including ceftazidime-avibactam. This finding is in line with previous studies highlighting the importance of *bla*$_{\text{NDM}}$ spread, cancelling last-line therapeutic options, worldwide [15]. Additionally, our study showed the contribution of divers STs and mobile genetic elements to the success of NDM resistance mechanism. Furthermore, the high-risk clone ST383 and the broad host range plasmids IncH and IncF, has contributed to the successful

prevalence and dissemination of NDM resistance. Thus, such findings emphasize the need of scrutinizing the implementation of infection control aspects to curb/ control the spread of these “superbugs”.

Author contributions

GA, IB, and CCP designed the study. GS, TS and MEC conducted the experiments. IB, CCP, and MF analyzed the data. All authors contributed to the writing of the manuscript. IB and JH secured the funding. All authors approved the submitted version.

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Transparency declarations

We have no conflicts to declare.

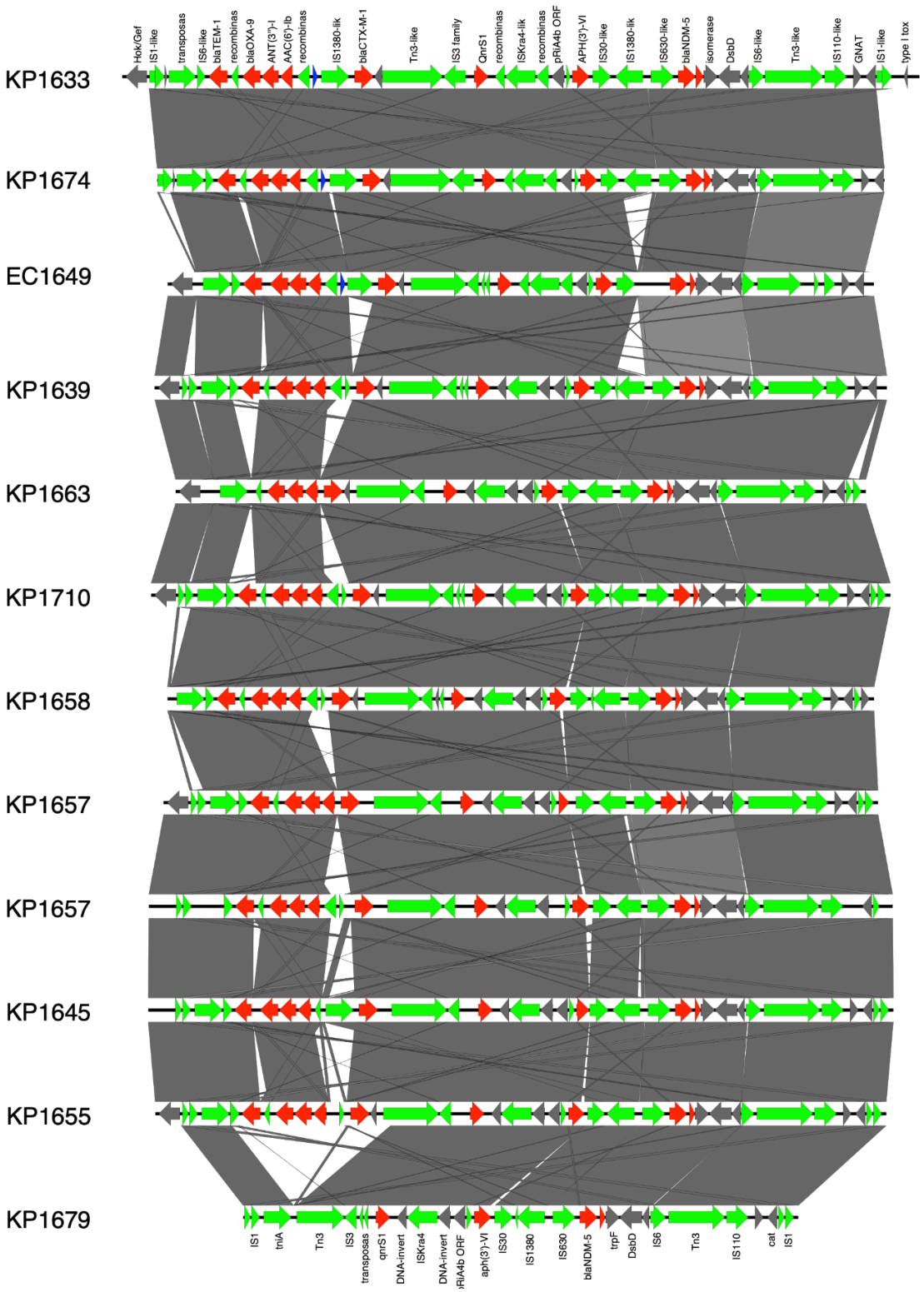

Figure 1: Prophage harboring *bla*$_{\text{NDM-5}}$ region comparison, found in ST383 *K. pneumonia* plasmids and in *E. coli* EC1609, with the prophage sequence detected in the long read sequenced KP1633 isolate. Red represents the resistance genes; green represents insertion sequences, transposases; in blue DUF-domains; in gray other genes.

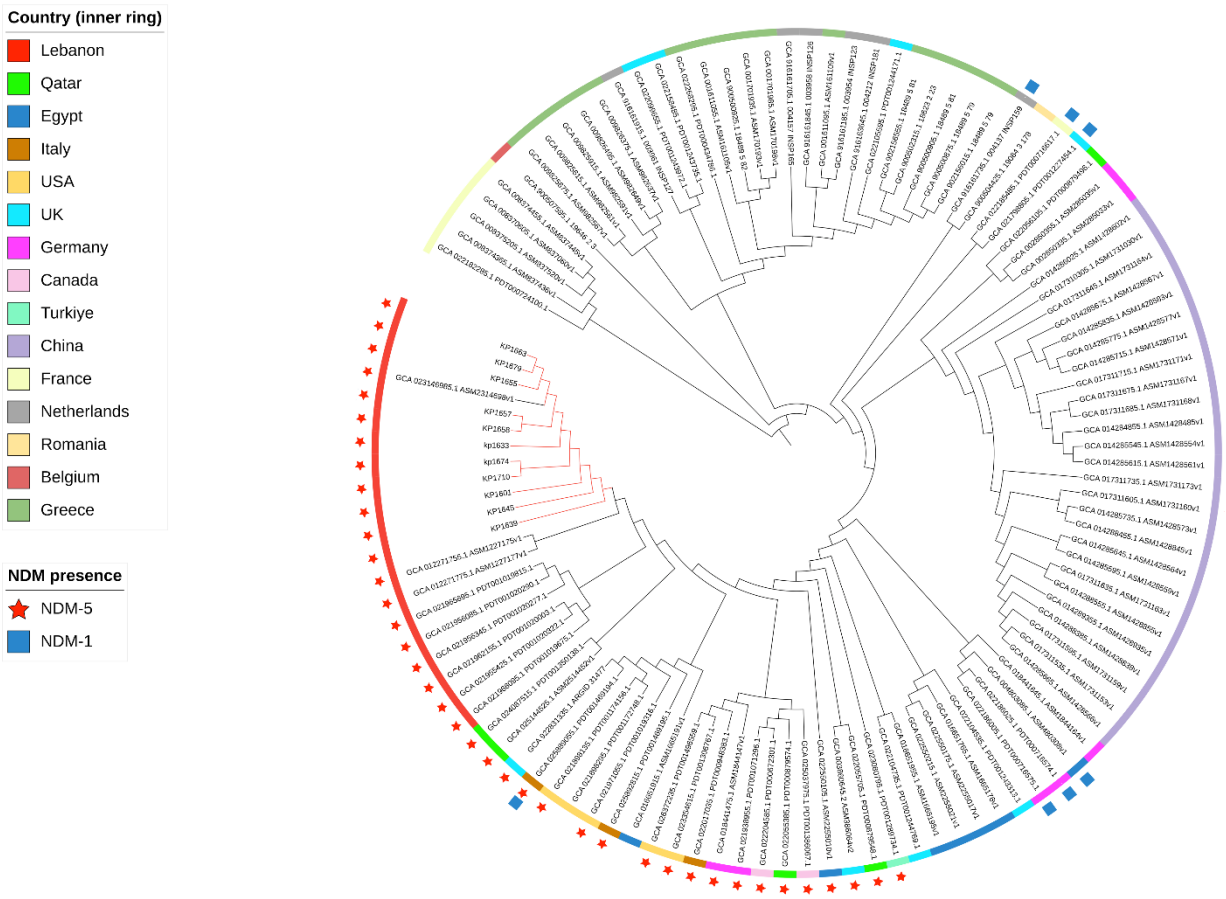

Figure 2: SNP based phylogeny for global *K. pneumoniae* ST383 with 105 isolates downloaded from NCBI database. Red nodes highlight the isolates used in this study.

Figure 3: SNPs based phylogeny for the CRISPR/Cas arrays of the Lebanese ST383 *K. pneumoniae* isolates. A represents the clade in the pink while B represents the clade in purple.

Table 1: MIC and disk diffusion test results for the K. pneumoniae isolates.

Isolate ID				MIC (µg/ml)				DD (S, I, R)								
	Date	Specimen	Ward	Ert	Imp	Mero	CZA	Amk	Cip	Gent	Tazo	Tet	Trimeth	Tige	Col	Fosfo
KP1601	10/01/2020	Urine	ICU	>32	>32	>32	>256	R	R	R	R	R	R	-	I	R
KP1608	27/01/2020	Urine	OC	>32	4	16	>256	R	R	R	R	R	R	-	I	R
KP1612	01/02/2020	Blood	ICU	>32	>32	>32	>256	R	R	R	R	R	R	S	I	I
KP1618	10/02/2020	Urine	8 north	>32	>32	>32	>256	R	R	R	R	R	R	-	I	R
KP1623	17/02/2020	DTA	ICU	>32	>32	>32	>256	R	R	R	R	R	R	S	I	R
KP1633	01/03/2020	DTA	5 south	>32	>32	>32	>256	R	R	R	R	R	R	S	I	I
KP1635	06/03/2020	DTA	ICU	>32	>32	>32	>256	R	R	R	R	R	R	S	R	R
KP1639	09/03/2020	Fluid	5 south	>32	>32	>32	>256	R	R	R	R	R	R	S	I	I
KP1642	12/03/2020	Blood	9 north	>32	>32	>32	>256	R	R	R	R	R	R	-	I	R
KP1645	20/03/2020	Urine	ICU	>32	>32	>32	>256	R	R	R	R	R	R	-	R	R
KP1648	10/04/2020	Urine	ICU	>32	>32	>32	>256	R	R	R	R	R	R	-	I	R
KP1652	20/04/2020	Blood	ICU	>32	>32	>32	>256	S	R	R	R	R	R	S	I	R
KP1655	21/04/2020	Tissue	ICU	>32	>32	>32	>256	R	R	R	R	R	S	R	R	-
KP1656	23/04/2020	Urine	ICU	>32	>32	>32	>256	R	R	R	R	R	R	-	I	S
KP1657	24/04/2020	Blood	NEUROICU	>32	>32	>32	>256	R	R	R	R	R	R	-	-	-
KP1658	30/04/2020	DTA	ICU	>32	>32	>32	>256	R	R	R	R	R	R	S	I	I
KP1661	05/05/2020	Wound	5 south	>32	>32	>32	>256	R	R	R	R	R	R	S	-	-
KP1663	17/05/2020	Urine	9 south	>32	>32	>32	>256	R	R	R	R	R	R	-	I	S
KP1669	23/05/2020	DTA	ICU	>32	>32	>32	>256	R	R	R	R	R	R	S	I	I
KP1674	25/05/2020	DTA	10 south	>32	>32	>32	>256	R	R	R	R	R	S	-	I	I
KP1679	01/06/2020	Sputum	9 south	>32	>32	>32	>256	R	R	R	R	R	R	S	I	S
KP1688	17/06/2020	Urine	BASIL IN	>32	>32	>32	>256	R	R	R	R	R	R	-	R	I
KP1690	28/06/2020	Screening	NEUROICU	>32	>32	>32	>256	R	R	R	R	R	R	S	S	R
KP1696	02/07/2020	Urine	9 north	>32	>32	>32	>256	R	R	R	R	R	R	-	I	-
KP1704	22/07/2020	Blood	BASIL IN	>32	>32	>32	>256	R	R	R	R	R	R	-	S	I
KP1710	06/08/2020	Urine	9 north	>32	>32	>32	>256	R	R	R	R	R	R	-	R	I
KP1734	31/08/2020	Blood	NEUROICU	>32	>32	>32	>256	R	R	R	R	R	R	-	I	R
KP1736	05/09/2020	Sputum	10 south	>32	>32	>32	>256	R	R	R	R	R	R	I	S	S
KP1851	26/02/2021	Screening	6 north	>32	>32	>32	>256	R	R	R	R	R	R	S	I	R
KP1859	08/03/2021	Blood	CCU	>32	>32	>32	>256	S	R	S	R	R	R	R	I	S
KP1877	08/04/2021	DTA	9 north	>32	>32	>32	>256	R	R	R	R	R	R	S	I	S
KP1878	08/04/2021	Screening	NICU	>32	>32	>32	>256	R	I	R	R	R	R	S	I	S
KP1880	08/04/2021	Screening	NICU	>32	>32	>32	>256	R	R	R	R	R	R	S	I	S
KP1917	17/06/2021	Wound	9 south	>32	>32	>32	>256	R	R	R	R	R	R	R	I	R

Ert: ertapenem, Imp: imipenem, Mero: Meropenem, CZA: Ceftazidim-Avibactam, Amk: amikacin, Cip: ciprofloxacin, Gent: Gentamicin, Tazo: Tazobactam, Trimeth: Trimethoprim, Tige: Tigecycline, Col: colistin, Fosfo: Fosfomycin, , CZA MIC: Ceftazidim-Avibactam Minimum Inhibitory Concentration.

DD: Disk Diffusion, MIC: Minimum inhibitory Concentration, S: sensitive, I: intermediate, R: resistant.

DTA: Deep Tracheal Aspirate, ICU: Intensive Care Unit, OC: Outside Patient , NEUROICU : neurology Intensive Care Unit, NICU: Neonatal Intensive Care Unit.

Table 2: MIC and disk diffusion test results for the *E. coli* isolates.

Isolates ID				MIC (µg/ml)				DD (S, I, R)								
	Date	Specimen	Ward	Ert	Imp	Mero	CZA	Amk	Cip	Gent	Tazo	Tet	Trimeth	Tige	Col	Fosfo
EC1507	04/10/2019	Tissue Culture	9 north	>32	>32	>32	>256	S	R	S	R	R	R	I	I	S
EC1609	30/01/2020	Urine	6 south	>32	>32	>32	>256	S	R	S	R	R	R	-	S	S
EC1637	07/03/2020	Urine	9 north	>32	>32	>32	>256	S	R	R	R	R	R	-	I	S
EC1649	08/04/2020	DTA	RCU	>32	>32	>32	>256	S	R	S	R	R	R	R	I	S
EC1675	29/05/2020	Urine	10 south	4	1.5	2	>256	S	R	S	R	R	R	-	I	S
EC1723	21/08/2020	Sputum	COVIDICU	32	>32	16	>256	S	R	S	R	S	R	S	I	S
EC1726	22/08/2020	DTA	ICU	>32	>32	>32	>256	S	R	S	R	R	R	S	R	S
EC1733	29/08/2020	Screening	CCU	>32	>32	>32	>256	S	R	R	R	R	R	S	I	S
EC1735	03/09/2020	Urine	8 north	>32	>32	>32	>256	S	R	S	R	R	R	-	S	S
EC1739	11/09/2020	Blood	Basil In	>32	>32	>32	>32	S	R	S	R	R	R	-	I	S
EC1811	12/11/2020	Urine	Basil In	>32	>32	>32	>256	S	R	R	R	R	R	-	I	S
EC1813	17/11/2020	Urine	ICU	>32	>32	>32	>256	S	R	S	R	R	R	-	R	S
EC1825	18/12/2020	Urine	CCU	>32	>32	>32	>256	S	R	S	R	R	R	-	I	S
EC1844	14/02/2021	Blood	Basil In	>32	>32	>32	>256	S	R	S	R	R	R	-	I	S
EC1856	05/03/2021	Urine	7 south	>32	>32	>32	>256	S	R	S	R	R	R	-	I	S
EC1886	17/04/2021	Urine	9 south	>32	>32	>32	>256	S	R	S	R	R	R	-	I	S
EC1911	07/06/2021	Urine	5 south	>32	>32	>32	>256	R	R	R	R	R	R	-	I	-
EC1918	26/06/2021	Blood	9 north	>32	>32	>32	>256	S	R	S	R	R	S	-	R	S
EC1926	15/07/2021	Screening	Basil in	>32	>32	>32	>256	S	R	S	R	R	S	S	S	S
EC1930	31/07/2021	Blood	Basil in	>32	>32	>32	>256	S	R	R	R	R	R	-	R	S
EC1934	03/08/2021	Fluids	Basil in	>32	>32	>32	>256	R	R	R	R	R	R	S	I	S
EC1961	05/09/2021	Urine	COVIDICU	>32	>32	>32	>256	S	R	S	R	R	R	-	I	S
EC1964	14/09/2021	Blood	Basil In	>32	>32	>32	>256	S	R	S	R	R	R	-	S	S
EC1976	29/09/2021	Urine	9 south	8	>32	>32	>256	S	R	S	R	S	R	S	I	S
EC1979	01/09/2021	Blood	9 south	>32	>32	>32	>256	S	R	S	R	R	R	-	I	S
EC1984	12/10/2021	Blood	PICU	>32	>32	>32	>256	S	R	S	R	R	R	-	I	S

Ert: ertapenem, Imp: imipenem, Mero: Meropenem, CZA: Ceftazidim-Avibactam, Amk: amikacin, Cip: ciprofloxacin, Gent: Gentamicin, Tazo: Tazobactam, Trimeth: Trimethoprim, Tige: Tigecycline, Col: colistin, Fosfo: Fosfomycin, CZA MIC: Ceftazidim-Avibactam Minimum Inhibitory Concentration.

DD: Disk Diffusion, MIC: Minimum inhibitory Concentration, S: sensitive, I: intermediate, R: resistant.

DTA: Deep Tracheal Aspirate, ICU: Intensive Care Unit, NEUROICU : neurology Intensive Care Unit, PICU: Pediatric Intensive Care Unit, COVIDICU: COVID-19 Intensive Care Unit, CCU: Critical Care Unit, RCU: Restorative Care Unit, Basil in: Cancer hospital patient

Table 3: WGS data representing resistance gene content and plasmid replicons of the *K. pneumoniae* isolates.

Isolate ID	Sequence Type	*bla*$_{NDM}$ plasmid	*bla*$_{OXA}$ plasmid	Resistance Genes	Other Replicons
KP1658	383	IncFIB-IncHI1B	IncL	sul1, dfrA5, OqxA, OqxB, bla$_{OXA-48}$, bla$_{CTX-M-15}$, aph(3')-Ia, bla$_{SHV-145}$, fosA, aph(6)-Id, aph(3'')-Ib, bla$_{CTX-M-14b}$, qnrS1, aph(3')-VI, mph(E), msr(E), armA, mph(A), bla$_{TEM-1B}$, bla$_{OXA-9}$, ant(3'')-Ia, aac(6')-Ib, sul2, bla$_{NDM-5}$, tet(A)	Col440
KP1679	383	IncFIB-IncHI1B	IncL	fosA, bla$_{SHV-145}$, aph(6)-Id, aph(3'')-Ib, bla$_{CTX-M-14b}$, OqxB, OqxA, aph(3')-VI, qnrS1, mph(E), msr(E), armA, sul2, bla$_{NDM-5}$, tet(A), mph(A), bla$_{OXA-48}$	Col440I
KP1601	383	IncFIB-IncHI1B	IncL	aph(3')-VI, qnrS1, bla$_{CTX-M-14b}$, aph(6)-Id, aph(3'')-Ib, mph(A), aac(6')-Ib, ant(3'')-Ia, bla$_{OXA-9}$, bla$_{TEM-1B}$, sul2, bla$_{NDM-5}$, tet(A), dfrA5, sul1, bla$_{OXA-48}$, bla$_{CTX-M-15}$, mph(E), msr(E), bla$_{SHV-145}$, OqxB, OqxA, fosA	Col440I
KP1645	383	IncFIB-IncHI1B	IncL	mph(A), sul2, bla$_{NDM-5}$, mph(E), msr(E), tet(A), dfrA5, sul1, aac(6')-Ib, ant(3'')-Ia, bla$_{OXA-9}$, bla$_{TEM-1B}$, bla$_{OXA-48}$, aph(3')-Ia, fosA, aph(3'')-Ib, aph(6)-Id, bla$_{CTX-M-14b}$, OqxB, OqxA, bla$_{CTX-M-15}$, qnrS1, bla$_{SHV-145}$	Col440I
KP1663	383	IncFIB-IncHI1B	IncL	armA, msr(E), mph(E), mph(A), aac(6')-Ib, ant(3'')-Ia, bla$_{OXA-9}$, bla$_{TEM-1B}$, sul2, bla$_{NDM-5}$, tet(A), dfrA5, sul1, bla$_{SHV-145}$, qnrS1, bla$_{OXA-48}$, bla$_{CTX-M-15}$, aph(3')-VI, aph(3'')-Ib, aph(6)-Id, OqxB, OqxA, fosA, bla$_{CTX-M-14b}$	Col440
KP1639	383	IncFIB-IncHI1B	IncL	mph(A), sul2, mph(E), msr(E), tet(A), sul1, dfrA5, aac(6')-Ib, ant(3'')-Ia, bla$_{OXA-9}$, bla$_{TEM-1B}$, bla$_{OXA-48}$, bla$_{CTX-M-14b}$, aph(3')-Ia, OqxA, OqxB, bla$_{SHV-145}$, aph(3'')-Ib, aph(6)-Id, qnrS1, bla$_{CTX-M-15}$, bla$_{NDM-5}$, fosA	Col440
KP1674	383	IncFIB-IncHI1B	IncL	fosA, tet(A), mph(A), catA1, bla$_{SHV-145}$, OqxA, OqxB, bla$_{NDM-5}$, aph(3')-VI, qnrS1, bla$_{CTX-M-15}$, aac(6')-Ib, ant(3'')-Ia, bla$_{OXA-9}$, bla$_{TEM-1B}$, aph(3')-Ia, mph(A), sul1, dfrA5, mph(E), msr(E), armA, sul2, bla$_{OXA-48}$, bla$_{CTX-M-14b}$, aph(3'')-Ib, aph(6)-Id	Col440I
KP1657	383	IncFIB-IncHI1B	IncL	fosA, qnrS1, aph(3')-VI, mph(A), sul2, bla$_{NDM-5}$, mph(E), msr(E), tet(A), sul1, dfrA5, bla$_{OXA-9}$, ant(3'')-Ia, aac(6')-Ib, bla$_{OXA-48}$, bla$_{TEM-1B}$, bla$_{CTX-M-15}$, catA1, aph(6)-Id, aph(3'')-Ib, OqxA, OqxB, bla$_{SHV-145}$, bla$_{CTX-M-14b}$	Col440I
KP1633	383	IncFIB-IncHI1B	IncL	OqxB, OqxA, bla$_{SHV-145}$, catA1, mph(A), tet(A), fosA, sul2, armA, msr(E), mph(E), dfrA5, sul1, mph(A), aph(3')-Ia, bla$_{TEM-1B}$, bla$_{OXA-9}$, ant(3'')-Ia, aac(6')-Ib, bla$_{CTX-M-15}$, qnrS1, aph(3')-VI, bla$_{NDM-5}$, aph(6)-Id, aph(3'')-Ib, bla$_{CTX-M-14b}$, bla$_{OXA-48}$	Col440I
KP1710	383	IncFIB-IncHI1B	IncL	tet(A), dfrA5, sul1, OqxA, OqxB, bla$_{OXA-48}$, bla$_{CTX-M-15}$, aph(3')-Ia, bla$_{SHV-145}$, aph(3'')-Ib, aph(6)-Id, fosA, bla$_{CTX-M-14b}$, mph(E), msr(E), qnrS1, aph(3')-VI, bla$_{TEM-1B}$, bla$_{OXA-9}$, ant(3'')-Ia, aac(6')-Ib, mph(A), sul2, bla$_{NDM-5}$	Col440I
KP1655	383	IncFIB-IncHI1B	IncL	fosA, bla$_{SHV-145}$, aph(3'')-Ib, aph(6)-Id, OqxB, OqxA, bla$_{CTX-M-14b}$, bla$_{CTX-M-15}$, qnrS1, aph(3')-VI, tet(A), sul2, mph(E), msr(E), armA, mph(A), bla$_{NDM-5}$, sul1, dfrA5, aac(6')-Ib, ant(3'')-Ia, bla$_{OXA-9}$, bla$_{OXA-48}$	Col440I
KP1608	307	IncFIB-IncFII	-	fosA6, OqxB, OqxA, bla$_{SHV-106}$, tet(A), bla$_{OXA-1}$, aac(6')-Ib-cr, bla$_{CTX-M-15}$, dfrA14, bla$_{TEM-1B}$, aph(6)-Id, aph(3'')-Ib, sul2, qnrB1, sul1, aadA2, dfrA12, rmtB, bla$_{NDM-5}$	Col440I
KP1734	147	IncFIB-IncHI1B	colKP3	OqxB, OqxA, bla$_{SHV-11}$, fosA, aph(3')-Ia, mph(A), sul1, dfrA5, bla$_{NDM-1}$, aph(3')-VI, qnrS1, mph(E), msr(E), armA, sul2, qnrS1, bla$_{CTX-M-15}$, sul1, arr-3, catB3, bla$_{OXA-1}$, aac(6')-Ib-cr, qnrS1, bla$_{CTX-M-15}$, bla$_{OXA-232}$, bla$_{TEM-1A}$, bla$_{OXA-9}$, ant(3'')-Ia, aac(6')-Ib	Col440I, IncFIB(pKPHS1), IncFIB(pQil), IncR
KP1859	111	IncX3	-	bla$_{NDM-5}$, qnrS1, catA2, OqxB, OqxA, aph(3')-Ia, bla$_{SHV-187}$, fosA6, mph(A), sul1, aadA2, dfrA12, aph(6)-Id, aph(3'')-Ib, sul2, bla$_{CTX-M-15}$	IncFIB(K)

KP1880	101	IncM2	-	OqxB, OqxA, $bla_{SHV-106}$, fosA, tet(A), dfrA14, sul2, aph(3'')-Ib, aph(6)-Id, bla_{TEM-1B}, $bla_{CTX-M-15}$, qnrS1, bla_{SHV-12}, bla_{NDM-1}, sul1, armA, msr(E), mph(E), aac(3)-IId, bla_{TEM-1B}	IncFIB(K), IncFII, IncFII(K)
KP1851	39	IncC	-	$bla_{SHV-187}$, fosA, tet(A), sul1, dfrA7, aph(3')-Ia, aph(6)-Id, aph(3'')-Ib, sul2, bla_{TEM-1B}, OqxB, OqxA, $bla_{CTX-M-15}$, aph(3')-Ia, sul2, aph(3'')-Ib, aph(6)-Id, qnrS1, bla_{CMY-6}, aac(6')-Ib, sul1, rmtC, bla_{NDM-1}	IncFIB(K), IncQ1
KP1917	15	IncC	-	fosA6, $bla_{SHV-106}$, $bla_{CTX-M-15}$, OqxA, OqxB, aph(3')-Ia, mph(A), tet(A), bla_{CMY-6}, aac(6')-Ib, sul1, rmtC, bla_{NDM-1}, qnrB1, dfrA14	Col440I, IncFIA(HI1), IncFIB(K), IncFIB(pKPHS1), IncFII(K), IncFII(pMET), IncR

Table 4: WGS data representing resistance gene content and plasmid replicons of the *E. coli* isolates.

Sample	Sequence Type	*bla*$_{NDM}$ plasmid	Resistance Genes	Other Replicons
EC1930	648	IncFIB-IncFII	dfrA12, aadA2, sul1, bla_{NDM-5}, catA1, mph(A), $bla_{CTX-M-15}$, tet(B), aac(3)-IIa, bla_{OXA-1}, aac(6')-Ib-cr, bla_{TEM-1B}	Col (BS512), Col (MG828), IncFIA
EC1726	648	IncFIB-IncFII	mph(A), bla_{NDM-5}, dfrA12, aadA2, tet(B), dfrA17, aadA5, sul1	Col (BS512), Col (MG828), Col156, Col440I
EC1609	405	IncFIA-IncFII	bla_{CMY-42}, tet(A), dfrA12, aadA2, sul1, bla_{NDM-5}, mph(A)	p0111
EC1735	405	IncFIB-IncFII	qepA4, dfrA12, aadA2, sul1, bla_{NDM-5}, mph(A), bla_{TEM-1B}, tet(B)	p0111
EC1918	131	IncX3	bla_{CMY-2}, bla_{NDM-5}, $bla_{CTX-M-27}$, tet(A), aph(6)-Id, aph(3'')-Ib, sul2, mph(A), sul1, aadA5, $bla_{CTX-M-15}$	IncFIA, IncFIB(AP001918), IncFII(pRSB107), IncI1
EC1811	2450	IncX3	$bla_{CTX-M-15}$, mph(A), aac(3)-IIa, bla_{OXA-1}, aac(6')-Ib-cr, aadA5, dfrA17, catA1, tet(B), sul2, aph(3'')-Ib, aph(6)-Id, bla_{NDM-5}	Col (MG828), IncFIA, IncFIB(AP001918)
EC1649	998	IncFIB-IncHI1B	aph(3')-VI, qnrS1, $bla_{CTX-M-15}$, tet(B), sul2, aph(3'')-Ib, aph(6)-Id, bla_{NDM-5}, sul1, aac(6')-Ib, bla_{TEM-1B}, bla_{OXA-9}, dfrA10	IncFIA, IncFIB(AP001918), IncFII(pRSB107), IncQ1
EC1733	1284	IncX3	tet(B), sul1, aadA5, dfrA17, mph(A), $bla_{CTX-M-15}$, aac(3)-IIa, aac(6')-Ib-cr, bla_{CMY-42}, bla_{NDM-5}	Col440I, IncFIA, IncFIB(AP001918), IncFII, IncI
EC1825	361	IncFIA-IncFII	bla_{NDM-5}, sul1, aadA2, dfrA12, catA1, qepA1, mph(A), bla_{OXA-1}, ant(3'')-Ia, $bla_{CMY-145}$, tet(A)	IncI, IncY
EC1739	167	IncF	aph(6)-Id, sul2, aph(3'')-Ib, mph(A), $bla_{CTX-M-15}$, aac(6')-Ib-cr, bla_{OXA-1}, $bla_{CMY-145}$, tet(A), dfrA12, aadA2, sul1, bla_{NDM-5}	Col (MG828), IncFIA, IncFIB(AP001918), IncFII, IncI
EC1856	617	IncFII-IncFIA-IncFIB	$bla_{CTX-M-15}$, bla_{OXA-1}, aac(6')-Ib-cr, aadA5, sul1, bla_{NDM-5}, bla_{OXA-1}, aac(6')-Ib-cr, aadA5, sul1, bla_{NDM-5}, mph(A), sul1, aadA2, dfrA12, bla_{OXA-1}, aac(6')-Ib-cr, aadA5, sul1, aph(6)-Id, aph(3'')-Ib, sul2	Col (BS512), IncFII
EC1507	361	IncFIA-IncFII	tet(A), bla_{NDM-5}, sul1, aadA2, dfrA12, mph(A)	IncI, IncY

Table 5: Number of SNPs in the *K. pneumoniae* ST383 isolates in this study as compared to the reference KP1674.

Isolate	Number of SNPs
KP1633	15
KP1679	26
KP1655	28
ASM2314698v1	31
KP1601	41
KP1710	50
ASM1227177v1	55
KP1658	55
ASM1227175v1	61
KP1663	61
PDT001019815.1	71
PDT001020290.1	71
PDT001020277.1	73
PDT001019675.1	78
PDT001020322.1	80
PDT001020003.1	83
KP1657	90
KP1645	91
KP1639	100

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6 Discussion

6.1 Dissemination of KPC-producing Enterobacterales in Czech hospitals

KPC-producing Enterobacterales represents a major global public health threat. This study investigated the transition in Czech hospitals from isolated cases and localized outbreaks to an epidemic dissemination of KPC-producing isolates. Between 2018 and 2019, a total of 108 KPC-producing isolates were collected from 22 hospitals across the Czech Republic. The *bla*$_{KPC}$ gene was detected in multiple species and clones within the Enterobacterales family.

The isolates responsible for the KPC-production were identified to be *K. pneumoniae* (n=26), *C. freundii* (n=24), *E. cloacae* (n=18), *P. mirabilis* (n=14), *M. morganii* (n=11), *E. coli* (n=10), *C. farmeri* (n=1), *E. aerogenes* (n=1), *K. michiganensis* (n=2), and *K. variicola* (n=1). 49 isolates were selected for WGS using illumina MiSeq, data revealed the forty-four were producers for the KPC-2 the remainder five produced KPC-3. Further investigation on the WGS revealed that in *C. freundii* ST65 (n=6) was the predominant ST detected and ST580(n=3), these two ST were phylogenetically distinct in comparison to the data used, while the ST8 (n=2) isolates phylogenetically clustered with isolates of ST98 and the remainder ST8 co-producing KPC-2 and VIM-4 clustered with other similar STs that also reported previously in Germany and Poland [1, 2]. Phylogenetic data on the *P. mirabilis* and *M. morganii* showed that the study isolates were distinct from other isolates used in the analysis. Two distinct STs were identified for *E. hormachei* isolates, ST133 (n=4) phylogenetically clustered with non KPC-2 producers, while ST421(n=3) was distinct and did not cluster with any isolates from the data. Moreover, six *K. pneumoniae* isolates belonged to unique STs and phylogenetically clustered with other similar global ST *K. pneumoniae* isolates, the remainder of the isolates (n=7) clustered into two subclusters with other *K. pneumoniae* ST11 and ST101. High risk clones of *K. pneumoniae* (ST11, ST101, ST147, and ST512) were proven by previous studies to be responsible for the spread of *bla*$_{KPC}$ [3, 4]. Finally, the remaining *E. coli* and *K. michiganensis* belonged to distinct STs. This data highlights the ongoing spread of *bla*$_{KPC}$ into different species and clones.

According to the MiSeq data, twenty-five isolates were selected for long-reads sequencing in order to investigate the *bla*$_{KPC}$ carrying units. The data showed multiple plasmid replicon types behind the dissemination. Some of these plasmids were described previously in other studies as responsible for the dissemination of *bla*$_{KPC}$, for example the IncFII$_{K2}$-IncR fusion plasmid, pKpQIL, and IncR-IncN$_3$ fusion plasmid [3, 5, 6]. Furthermore, newly emerging plasmids were detected in this study for example the IncN plasmid characterized in Germany, and another plasmid the fusion IncFIB-IncFII characterized from a Dutch collection [7]. Additionally, a new plasmid was detected the hybrid IncFII/FIB/C$_2$/N plasmid.

This Data highlighted the dissemination of the KPC mechanism through successful plasmids, and at the same time highlighted the ongoing evolution of plasmids disseminating this resistance mechanism. IncR plasmids for example, were previously reported in the Czech Republic carrying *bla*$_{KPC}$ and other resistance mechanisms like NDM and VIM, therefore this study highlighted the plasmid evolution [8, 9]. One of the main contributors in the KPC resistance mechanism dissemination is the IncF plasmid, this plasmid was also detected in this study [3, 6, 10]. Multiple plasmid types were detected during this study and correlated

with either a local dissemination for IncR plasmids specially in the center of the map (Hradec Kralove and Nymburk), and IncN plasmids in the northwest part of the Bohemian region. Furthermore, the reason behind the multiple plasmid types and their dissemination could be related either with the spread in the same region for example the IncR detected in Hradec Kralove [5], or related with a cross border spread like in the case of IncN from Germany. Finally, Prague was found to be a hotspot where the most plasmid types were detected, and this is mainly due to the presence of private laboratories that provide services for hospitals from the inside and outside of Prague. In addition to this is the admission of patients from Prague's surroundings for specialized treatments.

6.2 GES-producing Enterobacterales isolated in the Czech Republic

The first case of *bla*$_{\text{GES-5}}$ in the Czech Republic was reported from an *Enterobacter cloacae* isolated from the wound of a hospitalized diabetic patient in 2018 [11]. The gene was found in a novel integron class 1. Two years later and during a routine screening of the surfaces in the same hospital of the first detection, two GES-producing *Enterobacter cloacae complex* were isolated from two different rooms. An additional GES-producing isolate (*Klebsiella oxytoca*) was isolated from the urine of a patient hospitalized in Pilsen.

Molecular investigations on the two isolates from Prague (CZ862, and CZ863) showed that CZ862, and CZ863 belonged to ST837, and the novel ST1622 respectively, according to the MLST scheme. Furthermore, CZ862 harbored the *bla*$_{\text{GES-1}}$ and *bla*$_{\text{GES-5}}$ genes on a single plasmid untypable by PlasmidFinder and Plasmid relaxase scheme. CZ863 had similar findings but instead it harbored 2 copies of the *bla*$_{\text{GES-5}}$. These two plasmids were then compared to the original plasmid detected from the same hospital in 2018 and were of ≥99% similarity and 100% query cover. In comparison, these newly detected isolates had their *bla*$_{\text{GES}}$ genes carried by a novel integrons, In2079 for CZ862, and In2081 for CZ863. These findings highlight the presence of an underlying source for *bla*$_{\text{GES}}$ gene acquisition in the hospital's environment.

The third isolate, CZ598 was identified as an ST180 using the MLST scheme. This isolate had an interesting plasmid carrying the *bla*$_{\text{GES-7}}$, first it was identified as an untypable plasmid by PlasmidFinder and plasmid relaxases schemes. On this plasmid, a novel variation of *bla*$_{\text{OXA-2}}$ was detected and designated as *bla*$_{\text{OXA-1011}}$ and could be the reason behind the observed carbapenem resistanceFurther study of the plasmid's origins revealed that it is made up of two separate sequences: one originated from a 5200bp plasmid and the other from a 5600bp incomplete sequence identified from the genome of a *Pseudomonas aeruginosa*. This hybrid plasmid sheds light on the plasmid evolution happening in environmental hidden sources and expanding into clinical environments.

6.3 Dissemination of VIM-producing Enterobacterales through Integrons in the Czech Republic

Up till 2015, only sporadic cases of VIM-producing Enterobacterales have been detected in the Czech Republic [12, 13] all of the detected isolates carried their *bla*$_{\text{VIM}}$ genes on an integron class 1 In110 (*bla*$_{\text{VIM-1}}$-*aacA4*-*aadA1*) [14]. However, a surge in VIM-producing Enterobacterales have been detected in the years between 2019-2020, thus a study was warranted to investigate the main mechanism behind the dissemination on the *bla*$_{\text{VIM}}$ that led to the increased detection during these years.

Overall, thirty-two VIM-producing Enterobacterales were detected during this period and originated from fifteen different laboratories from the Czech Republic. These isolated were identified as *E. cloacae complex* (n=23), *C. freundii* (n=5), *K. pneumoniae* (n=3), and one *K. michiganensis*. Furthermore, all isolates were subjected to short-reads sequencing and revealed that twenty-six isolates harbored *bla*$_{VIM-1}$ and six isolates harbored the *bla*$_{VIM-4}$. Integron analysis showed that twenty-four isolates carried the *bla*$_{VIM-1}$ gene on In110 while the remainder two isolates carried *bla*$_{VIM-1}$ on In4873. Additionally, *bla*$_{VIM-4}$ gene was found on In1174 (n=3), In416 (n=1), and finally two novel integrons. This integron diversity detected highlighted the ongoing evolution of integrons carrying resistance genes. Moreover, integrons have the ability to integrate more than one resistance gene, therefore contributing to the emergence of MDR bacteria.

In order to investigate the integron location and spread, nineteen isolates were selected for long-reads sequencing to obtain complete genomes. According to PlasmidFinder analysis, multiple plasmid replicons were found to be carrying the *bla*$_{VIM}$, pKPC-CAV1193 (n=6 VIM-1 producing), IncFIB (n=2 VIM-1 producing), IncHI1 (n=3 with In110, n=1 with VIM-4 producing), IncHI2 (n=2 VIM-4/mcr9, n=1 VIM-4), IncN (n=1 VIM-1 producing, the hybrid IncHI1A/IncHI1B (n=1 VIM-4 producing), and the untypable (n=1 VIM-1 producing, and n=1 VIM-4 producing) plasmids. Remarkably, on *K. pneumoniae* ST11, the *bla*$_{VIM-4}$ was detected in the genome on integron In1174. In addition, hybrid plasmids were detected, signaling an interesting emergence.

The pKPC-CAV1193-like plasmids carried the In110, this plasmid was previously characterized from *E. hormarchei* from a Czech hospital in Cesky Budejovice [15]. The additional detection of this plasmid in the same city highlights a plasmid outbreak.

The IncHI1 VIM-1 producing plasmids were similar to previously reported plasmids also producing VIM-1 [13]. Two of the IncHI2 plasmids co-carried the *mcr-9* gene with the *bla*$_{VIM-4}$ gene on two different integrons (In416 and In1174), this plasmid was identified in a previous study [15], the remaining IncHI2 plasmid carried the novel integron the In2143 incorporated in the transposon Tn*1696*-like.

The IncN plasmid had high similarity with other plasmids from the database ranging from 99.2%-100% identity and query coverage ranging from 81%-88%, The most similar plasmid with 88% coverage and 100% identity was previously reported producing VIM-1 by Marchetti *et al.* [16], the MDR region was found to be completely different from any regions identified in the current study and the integron carried was In4873.

The first IncFIB plasmid was found to be a plasmid fusion where it contained a fragment from pLec-476cz (previously identified in 2011 [13]) containing part of the plasmidic backbone and part MDR that was consisted of In110, another fragment originated from plasmid pECL_A previously identified and only contained the replicon of IncFIB, and finally one more fragment contained the transfer system type-F and another MDR region. This plasmid analysis highlighted the presence of a hidden source where plasmids are evolving and contributing to the spread of resistance mechanisms. Furthermore, the other IncFIB plasmid was also found to be a derivative of pECL_A but acquired the In110 by transposition where Tn*1721*-like was carrying the integron in question.

Finally, the two untypable plasmids were found to be producing VIM-1 and VIM-4. The VIM-1 encoding plasmid was found to have two regions, region 1 originated from a plasmid backbone from an IncFIB plasmid characterized in this study and region 2 was categorized by the MDR region which was identical to the same MDR region from the IncFIB plasmid but also this MDR region was identical to a VIM-1-producing isolate characterized in 2015, the gene was carried by a Tn*1721*-like transposon containing the integron In110, evidence of transposition were confirmed [12]. On the other hand, The VIM-4-encoding untypable plasmid, the backbone of this plasmid was previously detected in a *C. freundii* VIM-4-producing isolate but no resistance genes were detected on this untypable plasmid [15], but the integron In1174 (element carrying the VIM-4 mechnism) was harbored by another plasmid (the IncHI2) suggesting its transposition. Interestingly, further analysis showed that a Tn3-like element was responsible for the transposition observed.

The appearance of plasmids of different Inc groups harboring the same MDR region were a consequence of homologous recombination. Furthermore, we discovered an identical transposition module in the IncFIB (pECLA) plasmid and the non-typeable plasmid. As shown by the data, IncN had a completely different MDR structure, highlighting the evolution of the vectors disseminating the *bla*$_{VIM}$ genes. In terms of VIM-4-encoding structures, In1174 was found to be a common integron for an IncHI2, St11 *K. pneumoniae*, and one of the untypable plasmids, while the remaining IncHI2 carried In416 and In2143. These multiple integron presence with the predominance of the In110, contributes to the dissemination of the *bla*$_{VIM}$ gene. Overall, the presence of the same transposons on different plasmid types highlights the important role these transposons play in the spread of the VIM-mediate resistance mechanism. Additionally, the implication of different plasmid types and the emergence of hybrid plasmids are two aspects broadening the range of species to which these resistance determinants might be transmitted.

6.4 Dissemination of NDM-5 /or OXA-48-like producing Enterobacterales in a hospital in Lebanon

Carbapenem resistance mechanisms and spread in Lebanon is not extensively studied, few studies have been conducted to present their prevalence [17, 18]. The first case of NDM-5 producer was detected in 2017 while in 2008 the first case of OXA-48 producer both cases were detected in *K. pneumoniae* isolate [19, 20]. In 2019, an increase in resistance against Ceftazidime-avibactam (CZA) was worrisome given that this drug was newly introduced in the country as treatment a surveillance study done by Sobh *et al.* revealed that the entity behind this resistance was Carbapenem isolates with a resistance of 100% to ertapenem and resistance to CZA [21]. This study aimed to investigate the previously detected NDM and OXA producing *E. coli* and *K. pneumoniae*.

A total of 60 isolates identified as *K. pneumoniae* (n=34) and *E. coli* (n=26) from the American university of Beirut were sent to Charles University for further characterization. Primary molecular data showed that *K. pneumoniae* isolates belonged in majority to ST383 (n=26) the remaining isolates belonged to unique STs. On the other hand, and based on the MLST Achtman scheme, the *E. coli* isolates belonged to multiple STs with the majority belonging to ST405 (n=9), and ST167 (n=4). Isolates were the selected for Short-reads sequencing (17 *K. pneumoniae* and 10 *E. coli*) to investigate the MDR regions and vectors responsible for the dissemination, data showed that ST383 *K. pneumoniae* isolates were carrying a hybrid plasmid the IncFIB-

IncHI1B harboring the *bla*$_{NDM-5}$ gene, this plasmid was found to be similar (100% coverage and 99.9% identity) to a plasmid previously reported from Quatar in 2016. According to epidemiological data this sequence type was found to be disseminating CRE in the world and more specifically in the Mediterranean regions [22-24]. In addition to producing NDM-5, ST383 isolates were found to be also producing OXA-48 enzymes and IncL was the plasmid type harboring the *bla*$_{OXA-48}$ genes. This co-production of NDM-5 and OXA-48 has drastically limited the treatment options specially with last resort drugs like CZA. Similar isolates have been documented in the MENA region [25-28] and similar finding were reported in Italy [29], this highlights the importance of surveillance for such isolates in order to contain the spread and further complications.

To understand the clonal relatedness of the *K. pneumoniae* ST383 isolates a global phylogeny was performed with the use of 105 genomes from the NCBI database, KP1674 was used as reference and *K. quasipneumoniae* as an outgroup. According to the branch clustering, study isolates clustered together with isolates previously reported in Lebanon, these isolates also produced NDM-5 and OXA-48. Remarkably, the Lebanese cluster had SNPs ranging from 11-73, and the plasmids encoding the NDM-5 and OXA-48 are similar to the ones detected in this study (IncFIB-IncHI1B, and IncL). Additionally, the CRISPR-Cas array was analyzed in ST383 *K. pneumoniae*, this would highlight if the isolates originated from the same environment given that isolates exposed to the same foreign sequences will add the spacer to the array in chronological order of exposure [30]. The tree was divided into 2 clades, and a single isolate that possessed 2 CRISPR arrays, both clades had study isolates and isolates from the previously identified in Lebanon. These finding suggests that ST383 in Lebanon has three different ancestors due to their exposure to environmental pressures and selective forces.

In a similar context, *E. coli* isolates in this study were all found to be NDM-5 producers, and they belonged to multiple STs. Plasmids were also diverse and belonged to multiple Inc groups, this highlights the importance of mobile elements like insertion sequences and transposons in the dissemination of *bla*$_{NDM-5}$. In most cases, the *bla*$_{NDM-5}$ genes are found on IncF plasmids. Moreover, some similar plasmids were detected in the isolates, for example the hybrid IncFIB-IncHI1B and the IncFIA-IncFII plasmids. In this context, plasmids were found to play a crucial role in the spread of resistance genes among institutions and communities.

7 Conclusion

Our results have shown the importance to characterize the carbapenem resistant isolates in order to understand the mechanism of resistance and the role of the mobile genetic elements in the dissemination of a given resistance mechanism between species and clones.

Starting with the increased detection of the *bla*$_{KPC}$ in the Czech Republic that was correlated to the conjugative nature of the plasmids detected and the ongoing plasmid evolution through genetic rearrangements that have shown preservation and further dissemination. Thus, monitoring this situation is of utmost importance, and the need of immediate infection control measures.

On the other hand, the *bla*$_{VIM}$ dissemination in the Czech Republic highlighted the outbreak of Mobile genetic element responsible for the dissemination of carbapenem resistant determinant in majority the integron class 1 In110. The emergence and spread of MDR regions carrying carbapenemase-encoding genes are facilitated by multiple mechanisms, this included the functional acquisition of *bla*$_{VIM}$-carrying transposons; the second mechanism detected was acquisition of the *bla*$_{VIM}$ MDR region by recombination; also variations of the integron were detected this highlighted the ongoing evolution; and finally the dissemination of the *bla*$_{VIM}$ MDR through plasmids. This emphasizes the significance of continuous monitoring of carbapenem resistance to control the spread of these new infections.

Other notable results were the detection of two mutated *bla*$_{GES}$-positive plasmids from the same environment three years apart. The conservation of the plasmid and the duplication of the *bla*$_{GES}$ gene highlights a hotspot for gene acquisition [31, 32]. This duplication noticed, its mechanism is not yet fully understood and can be correlated with recombination or integration. The worrying discovery lies in the third isolate where a hybrid plasmid had 2 fragments that were of environmental origins, and this is a concerning situation given that it happened in a clinical setting. These findings impose the need to understand the hidden sources and the evolution of plasmids.

In Lebanon a similar image was detected, where the finding showed that the spread of *bla*$_{NDM}$ was limiting the last resort antibiotic treatment, and the spread was linked to multiple STs and mobile genetic elements, more notably the high risk ST383 and the multiple plasmids. Therefore, these findings emphasize the need for rigorous implementation of infection control measures to curb the spread of these “superbugs”.

Overall, the studies conducted highlighted the importance of the continuous monitoring, the implementation of infection control in order to limit the spread in all cases reported either a plasmid mediated, integron mediated or an outbreak. The implementation of deeper analysis in order to detect new trends in plasmid evolution and other mobile genetic elements evolution.

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8 Curriculum vitae

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H-Index	4 (20.8.2024)
Sum of Times Cited without Self-Citations	55 (20.8.2024)
Number of Publications in Journals with IF	12 (20.8.2024)
Publications	See list of publiccations
Professional Experiences	34th ECCMID 2024 Poster presentation: Mega pipeline: an automated pipeline for easy microbial whole genome sequencing analysis JCI and FEMS Conference 2021 ePoster: Genomic characterization of three GES-producing Enterobacterales isolated in the Czech Republic

9 List of publications

Publications	Impact factor (Web of science 28.8.2024)	Citations (Web of Science, 28.8.2024)	Citations (Scopus, 28.8.2024)
Sobh, G., et al., Molecular characterization of carbapenem and ceftazidime-avibactam-resistant Enterobacterales and horizontal spread of bla (NDM-5) gene at a Lebanese medical center. Front Cell Infect Microbiol, 2024. 14: p. 1407246.	4.6	1	0
Fattouh, N., et al., Adhesive and biofilm-forming Candida glabrata Lebanese hospital isolates harbour mutations in subtelomeric silencers and adhesins. Mycoses, 2024. 67(6): p. e13750.	4.1	0	0
El Hachem, S., et al., Sequential Induction of Drug Resistance and Characterization of an Initial Candida albicans Drug-Sensitive Isolate. J Fungi (Basel), 2024. 10(5).	4.2	0	0
Mattioni Marchetti, V., et al., Polyclonal Spread of Fosfomycin Resistance among Carbapenemase-Producing Members of the Enterobacterales in the Czech Republic. Microbiol Spectr, 2023. 11(3): p. e0009523.	3.7	3	3
Bitar, I., et al., Implication of different replicons in the spread of the VIM-1-encoding integron, In110, in Enterobacterales from Czech hospitals. Front Microbiol, 2022. 13: p. 993240.	4	4	4
Reslan, L., et al., The impact of vaccination on the burden of invasive pneumococcal disease from a nationwide surveillance program in Lebanon: an unexpected increase in mortality driven by non-vaccine serotypes. Expert Rev Vaccines, 2022. 21(12): p. 1905-1921.	5.5	4	4
Finianos, M., et al., Genomic characterisation of three GES-producing Enterobacterales isolated in the Czech Republic. J Glob Antimicrob Resist, 2022. 29: p. 116-119.	3.7	4	4
Reslan, L., et al., Molecular Characterization of Candida auris Isolates at a Major Tertiary Care Center in Lebanon. Front Microbiol, 2021. 12: p. 770635.	4	12	11
Kraftova, L., et al., Evidence of an epidemic spread of KPC-producing Enterobacterales in Czech hospitals. Sci Rep, 2021. 11(1): p. 15732.	3.8	12	12
Reslan, L., et al., The Emergence of Invasive Streptococcus pneumoniae Serotype 24F in Lebanon: Complete Genome Sequencing Reveals High Virulence and Antimicrobial Resistance Characteristics. Front Microbiol, 2021. 12: p. 637813.	4	9	10
Reslan, L., et al., The origins of G12P[6] rotavirus strains detected in Lebanon. J Gen Virol, 2021. 102(3).	3.6	3	3
Mishra, N., et al., Full genome characterization of human G3P[6] and G3P[9] rotavirus strains in Lebanon. Infect Genet Evol, 2020. 78: p. 104133.	2.6	7	8

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