CHARLES UNIVERSITY
Faculty of Medicine in Pilsen
Department of Microbiology

# DISSERTATION THESIS

## Development and validation of methods for typing of bacteria by MALDI-TOF mass spectrometry

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## Declaration

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

In Pilsen 29. 6. 2018

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Kateřina Chudějová

*"In everyone's life, at some time, our inner fire goes out. It is then burst into flame by an encounter with another human being. We should all be thankful for those people who rekindle the inner spirit."*

Albert Schweitzer

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Kateřina Chudějová

## Abstract

Healthcare-associated infections represent a significant cause of morbidity and mortality in hospital settings. The risk of nosocomial infection differs significantly in different group of patients, depending on the character of their primary illness, the co-morbidities, the type of care provided, the length of hospitalization, or the medical procedures used. Artificial surfaces such as central venous catheters, shunts, urinary catheters, valve and joint replacements or controlled lung ventilation play a major role. The majority of nosocomial infections is caused by several representative of *Enterobacteriaceæ* family, *Pseudomonas* spp., *Acinetobacter* spp., or some Gram-positives, especially *Staphylococcus* and *Enterococcus* spp. This is largely due to their ability to retain and transfer different types of resistance to antibiotics. The identification and subtyping of these pathogenic microorganisms is an essential tool of modern public health infectious disease surveillance not only for appropriate and efficient treatment of infections, but also in case of an outbreak. Understanding clonal continuity among investigated strains is essential to determine the source and routes of infections, confirm or rule out outbreaks, trace cross-transmission of healthcare-associated pathogens, or recognize virulent strains. For this purpose, a series of simple phenotypic methods such as determination of minimal inhibition concentration, biochemical testing or serotyping can be used, utilizing differences in morphology, biochemical and enzymatic activity, or antigenic composition of given microorganism. Furthermore, more complex genotypic methods can be used to provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeability, and discriminatory power compared with phenotypic typing methods. These techniques include not only the number of PCR-based methods, often associated with conventional, pulsed or capillary electrophoresis, but also sequenation, ranging from Sanger sequencing of individual genes to exacting whole-genome sequencing.

The present dissertation thesis summarizes the results of five selected publications published in impact factor journals, with two first-author publications. The first work led to the development and validation of a novel automatic technique for bacterial and yeast deposition on MALDI target using "wet-deposition" into a droplet of 70% formic acid. This automatic deposition was compared to conventional manual spotting using a wooden toothpick, semi-extraction routinely used for yeasts, and manual "wet-deposition". Spotting by MALDI Colonyst robot significantly increased identification score of bacteria compared with the routine diagnostic process. Following four publications are focused on the epidemiology of several of the most common carbapenemases in the Czech Republic (KPC, OXA-48, NDM, and IMP), either within hospital outbreaks or their occurring across the country. In particular, we focused on detecting the possible clonal spread and tracking the path and the source of their transmission with the use of a number of typing methods, both phenotypic and genotypic, including whole-genome sequencing.

**Keywords:** bacterial typing – phenotypic methods – genotypic methods – NGS – MALDI-TOF MS – outbreak – HAI

## Abstrakt

Infekce spojené s nemocniční péčí se ve značné míře podílejí na mortalitě a morbiditě ve zdravotnických zařízeních. Riziko vzniku nozokomiálních infekcí se významně liší u různých skupin pacientů v závislosti na charakteru jejich primárního onemocnění, komorbiditách, poskytované péči, délce hospitalizace a také typu použitých diagnostických a léčebných postupů. Velkou roli mohou také hrát umělé materiály jako jsou centrální žilní katetry, shunty, močové cévky, kloubní a chlopenní náhrady, nebo umělá plicní ventilace. Většina nozokomiálních infekcí je způsobena zástupci čeledi *Enterobacteriaceæ*, členy rodů *Pseudomonas* spp., *Acinetobacter* spp., nebo některými grampozitivními bakteriemi, zejména rody *Staphylococcus* spp. a *Enterococcus* spp. Což je především umožněno jejich schopností uchovávat a přenášet různé typy rezistencí k antimikrobním látkám. Identifikace a typizace těchto patogenních organismů je proto nepostradatelným nástrojem moderního sledování infekčních onemocnění v oblasti veřejného zdraví, nejen pro vhodnou a účinnou léčbu infekcí, ale i v případě výskytu epidemických epizod. Pochopení klonální kontinuity mezi vyšetřovanými kmeny je nezbytné pro určení zdroje a cesty šíření infekcí, potvrzení nebo vyloučení epidemických epizod, sledování zkříženého přenosu patogenů souvisejících se zdravotní péčí, nebo rozpoznání virulentních kmenů. Pro tento účel může být použita řada jednoduchých fenotypových metod, jako je stanovení minimální inhibiční koncentrace, biochemické testování nebo serotypizace, které využívají rozdílů v morfologii, biochemické a enzymatické aktivitě, nebo antigenního složení jednotlivých mikroorganismů. Kromě toho lze použít komplexnější genotypizační metody k zajištění senzitivnější diferenciace kmenů, vyšší úrovně standardizace, reprodukovatelnosti, typizovatelnosti a diskriminační síly ve srovnání s metodami fenotypizačními. Tyto techniky zahrnují nejen řadu postupů založených na PCR, často spojených s běžnou, pulzní nebo kapilární elektroforézou, ale také sekvenování, a to od sekvenování jednotlivých genů Sangerovou metodou, až po náročnou celogenomovou sekvenaci.

Tato dizertační práce shrnuje výsledky pěti vybraných prací publikovaných v zahraničních časopisech s impaktním faktorem, přičemž dvě publikace jsou prvoautorské. První práce vedla k vývoji a validaci nové automatické techniky pro depozici bakterií a kvasinek na MALDI destičku pomocí „mokré depozice“ do kapky 70% kyseliny mravenčí. Tato automatická depozice byla porovnávána s běžnou ruční depozicí pomocí dřevěného párátka, semiextrakcí – běžně používanou při identifikaci kvasinek, a ruční „mokrou depozicí“. Přičemž, použití robota MALDI Colonyst výrazně zvýšilo identifikační skóre bakterií ve srovnání s rutinním diagnostickým procesem. Následující čtyři publikace se zaměřují na epidemiologii několika nejběžněji se vyskytujících karbapenemáz v České republice (KPC, OXA-48, NDM a IMP), a to buď v rámci nemocničních epidemických epizod, nebo v rámci jejich výskytu po celé republice. Konkrétně jsme se zaměřili na detekci možného šíření klonů a sledování cesty a zdroje jejich přenosu, s využitím řady typizačních metod, jak fenotypových, tak genotypových, včetně celogenomové sekvenace.

**Klíčová slova:** typizace bakterií – fenotypizační metody – genotypizační metody – NGS – MALDI-TOF MS – epidemická epizoda – HAI

## Acronyms and abbreviations

| Abbreviation | Meaning |
|---|---|
| A | adenosine |
| AFLP | amplified fragment length polymorphism |
| AGE | agarose gel electrophoresis |
| Amk | amikacin |
| AMR | antimicrobial resistance |
| AP | alkaline phosphatase |
| AP-PCR | arbitrarily-primed PCR |
| APS | Adenosine-5'-phosphosulfate |
| AS-PCR | allele-specific PCR |
| AST | antimicrobial susceptibility testing |
| Atm | aztreonam |
| ATP | adenosine triphosphate |
| BMD | broth microdilution method |
| C | cytosine |
| Caz | ceftazidime |
| CDC | Centers for Disease Control and Prevention |
| Cip | ciprofloxacin |
| Col | colistin |
| CPP | carbapenemase-producing *P. æruginosa* |
| CRE | carbapenem-resistant *Enterobacteriaceæ* |
| Ctx | cefotaxime |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| ddATP | dideoxyadenosine triphosphate |
| ddCTP | dideoxycytidine triphosphate |
| ddGTP | dideoxyguanosine triphosphate |
| DDST | double-disc synergy test |
| ddTTP | dideoxythymidine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| dTTP | deoxythymidine triphosphate |
| EARS-Net | European Antimicrobial Resistance Surveillance Network |
| ECCMID | European Congress of Clinical Microbiology and Infectious Diseases |
| EDTA | ethylenediaminetetraacetic acid |
| emPCR | emulsion PCR |
| ESBL | extended-spectrum β-lactamases |
| Etp | ertapenem |
| EUCAST | the European Committee on Antimicrobial Susceptibility Testing |
| Fep | cefepime |
| Fos | fosfomycin |
| FTIR | infrared spectroscopy with Fourier transformation |

| Abbreviation | Meaning |
|---|---|
| G | guanosine |
| Gen | gentamicin |
| GI | genomic island |
| HAI | healthcare-associated infection |
| HD | hematology department |
| HRP | horseradish peroxidase |
| ICE | integrative conjugative element |
| ICU | internal care unit |
| IEF | isoelectric focusing |
| Imp | imipenem |
| Inc | replicon incompatibility |
| IR | infrared spectroscopy |
| IR | inverted repeat |
| KPC | carbapenemase producing *K. pneumoniæ* |
| LAMP | loop-mediated isothermal amplification |
| m/z | mass-to-charge ratio |
| MALDI-TOF MS | matrix assisted laser desorption ionization time of flight mass spectrometry |
| MDR | multidrug resistance |
| Mem | meropenem |
| MER-R | meropenem-resistant |
| MER-S | meropenem-susceptible |
| MIC | minimal inhibition concentration |
| MLST | multilocus sequence typing |
| MLVA | multiple locus variable number tandem repeat analysis |
| MNPs | multi-nucleotide polymorphisms |
| MRSA | methicillin-resistant *S. aureus* |
| MS | mass spectrometry |
| MβL | metallo-β-lactamase |
| N-CPP | non-carbapenemase-producing *P. æruginosa* |
| NA | not applicable |
| NDM | New Delphi metallo-β-lactamase |
| NGS | next generation sequencing |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PBRT | PCR-based replicon typing |
| PCR | polymerase chain reaction |
| PFGE | pulsed-field gel electrophoresis |
| Pip | piperacillin |
| PPi | pyrophosphate |
| qRT-PCR | quality real-time PCR |
| RAPD | random amplified polymorphic DNA |
| RE | restriction endonuclease |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |

| Abbreviation | Meaning |
|---|---|
| RSD | relative standard deviation |
| RT-PCR | real-time PCR |
| SBL | sequencing by ligation |
| SBS | sequencing by synthesis |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| SERS | surface-enhanced Raman spectroscopy |
| SLST | single-locus sequence typing |
| SMRT | single molecule real-time |
| SNPs | single nucleotide polymorphisms |
| ST | sequence type |
| STR | short tandem repeats |
| Sxt | trimethoprim-sulfamethoxazole |
| T | thymidine |
| Tgc | tigecycline |
| Tob | tobramycin |
| Tzp | piperacillin-tazobactam |
| VIM | Verona integron-encoded metallo-β-lactamase |
| VNTR | variable number tandem repeats |
| VRE | vancomycin-resistant enterococci |
| WGS | whole-genome sequencing |
| ZMW | zero-mode waveguide |

## Contents

# 1 Introduction

Healthcare-associated infections (HAIs) represent a significant cause of morbidity and mortality in hospital settings, affecting an estimated 2 million people in the United States annually, or about 5% of hospitalized patients. They result in an estimated 88 000 deaths and $4.5 billion in health care costs annually.[1] A similar incidence of nosocomial infections is also found in the countries of the European Union, namely in the Czech Republic these infections occur in about 5-7% of patients admitted to hospitalization and their treatment leads to a significant increase in costs.[2] In developing countries, the incidence of nosocomial infections is higher than 10%, often resulting from inadequate health care and low levels of hygiene. The risk of nosocomial infection differs significantly in different group of patients, depending on the character of their primary illness, the co-morbidities, the type of care provided, the length of hospitalization, or the medical procedures used. Artificial surfaces such as central venous catheters, shunts, urinary catheters, valve and joint replacements or controlled lung ventilation play a major role.[2, 3] Four basic groups of HAIs as follow: *(i)* central line-associated bloodstream infections, *(ii)* catheter-associated urinary tract infections, *(iii)* surgical site infections, and the most numerous *(iv)* ventilator-associated pneumonia.[4] The outbreaks of nosocomial infections are continually being reported around the world in a variety of healthcare facilities, they occur not only in intensive care units (ICU) but also in ordinary wards.[5] The majority of nosocomial infections is caused by several representatives of the microbial group ESKAPE (***E****nterococcus fæcium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniæ*, ***A****cinetobacter baumannii*, ***P****seudomonas æruginosa*, and ***E****nterobacter* species), so-called because, as a result of their multidrug resistance, they have the potential to escape the action of antimicrobial agents.[6] According to the Center for Disease Control and Prevention (CDC), other pathogens, especially *Clostridium difficile* (pseudomembranous colitis associated with the postantibiotic effect), *Candida* spp., some viruses (mostly hepatitis, influenza), and in some areas also tuberculosis, are also frequently involved in HAIs. In recent years, carbapenem-resistant *Enterobacteriaceæ* (CRE) have also been extensively involved in these infections. The CRE have developed resistance to virtually all antibiotics and are frequently susceptible only to the last-line antibiotics, such as aminoglycosides, tigecycline, fosfomycin, or colistin. Some CRE bacteria have become resistant to most of the available antibiotics. Infections with these germs are very difficult to treat and can be deadly.[7] The reservoir of these pathogens causing HAIs cannot only be the endogenous microflora of a patient which is transferred to tissue wound or surgical site during surgery, but the source may also be other patients, staff and the environment.[8]

The identification and subtyping of these pathogenic microorganisms is an essential tool of modern public health infectious disease surveillance not only for appropriate and efficient treatment of infections, but also in case of an outbreak.[9,10] Understanding clonal continuity among investigated strains is essential to determine the source and routes of infections, confirm or rule out outbreaks, trace cross-transmission of healthcare-associated pathogens, or recognize virulent strains. This significantly helps to increase the effectiveness of surveillance systems for public health control and prevent future outbreaks from occurring.[11,12]

## 2 Bacterial typing methods

Over the past two decades, plenty of novel and mostly innovative typing methods have been developed. The more commonly used identification/typing methods are phenotypically based observing characteristics of an organism as a result of the interaction of its genotype with the environment. The second option is using molecular typing methods to determine the genetic background.[6]

From a practical point of view, used typing approach should be reproducible, have a high discrimination capacity and be easy to use and to interpret the results.[12] It also depends on other aspects, such as the microorganism of interest, the type and the number of clinical samples, the target of the study (a single gene or the entire genome), the application area, the cost, the required time to obtain results, and last but not least available instrumentation at the clinical laboratory.[13] **Table 2-1** compares the main currently available techniques suitable for identification and/or typing of bacteria in clinical microbiology.

**Table 2-1** Summary and comparison of main currently available techniques for identification and typing of bacteria. *Adapted by Varadi, L.[6] and MacCanell, D.[9]*

| | Technique | | | | | | |
|---|---|---|---|---|---|---|---|
| **Characteristic** | **Chromogenic media** | **Biochemical testing** | **MALDI-TOF** | **PFGE** | **PCR** | **MLST** | **WGS** |
| **Sensitivity** | ++ | ++ | +++ | +++ | +++ | +++ | +++ |
| **Specificity** | + | ++ | +++ | ++ | +++ | +++ | +++ |
| **Cost** | + | ++ | ++ | ++ | +++ | +++ | ++++ |
| **Complexity** | + | ++ | ++ | ++ | ++ | ++ | ++++ |
| **Labor intensity** | + | + | + | ++++ | +++ | +++ | ++++ |
| **Direct detection from clinical samples** | Yes | No | No | No | Yes | No | No |
| **Time to result (h)** | >16 | 2-3[a] | 0.2-3[a] | >72 | 2 | 48 | >24 |

[a] from previously isolated colonies; + represents the least and ++++ the most.

### 2.1 Phenotypic typing methods

The classic conventional techniques used to differentiate isolates phenotypically are based on the presence or absence of biological or metabolic activities expressed by an organism. These methods are easily affordable due to their relatively low cost and simple performance without the necessary labor experience.[14, 15] However, they have a number of practical limitations which render them unsuitable for detailed studies of bacterial population structure, its dynamic, infection control, and regular surveillance.[16] Furthermore, most phenotypic methods were developed for specific bacterial species and are not generally applicable, e.g., serotyping of *Salmonella* or bacteriophage typing of *Staphylococcus aureus*.[17] Besides, any given phenotype does not always accurately reflect the genotype of a microorganism and has a tendency to vary, based on changes in growth conditions, growth phase, and spontaneous mutation.[5, 17]

### 2.1.1 Cultivation and preliminary identification on chromogenic media

Even in an era of molecular-genetic procedures, the cultivation of bacteria remains the "gold-standard" of microbiology. These methods mainly involve enrichment and/or selective enrichment agars for the overnight growth of bacteria. Identification by culture is based on growing at different incubation atmospheres, different temperatures, different morphology of colonies, pigmentation, and biochemical expression of a given bacterium on various culture media, e.g., hemolysis on the Blood agar, or lactose-fermenting on the ENDO agar. They are widely used and have the advantage that they are cheap, detect only viable bacteria and gained isolates can be further characterized and studied by others phenotypic, or genotypic approaches. However, they are laborious, relatively slow and less efficient.[18, 19]

Over the last 20 years, a range of chromogenic media has been developed those are designed to target pathogens with high specificity. Such media contain fluorogenic or chromogenic substrates that are hydrolyzed upon the effect of a specific enzyme of given bacterium (**Figure 2-1**, **Table 2-2**), thus resulting in pathogens forming colored colonies that can be easily differentiated from commensal flora. Ideally, commensal flora is either inhibited entirely by selective agents or forms colorless colonies.[6, 20]

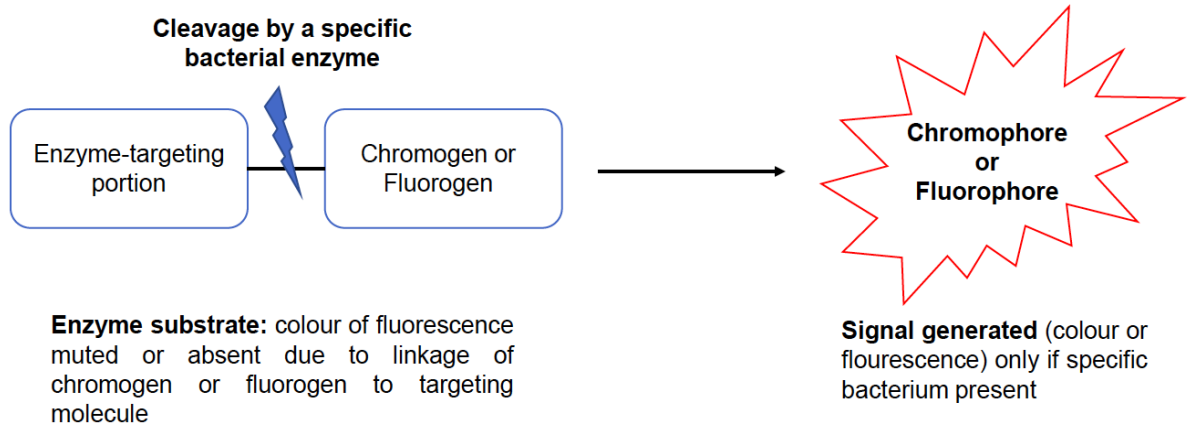



**Figure 2-1** Schematic illustration of phenotypic bacterial identification using chromogenic/fluorogenic substrates in culture media.

**Table 2-2** Examples of bacterial pathogens targeted by chromogenic media. *Adapted by Varadi, L.*[6]

| Pathogen | Enzyme targeted |
|---|---|
| **Gram-positive pathogens** | |
| *Staphylococcus aureus* (including MRSA) | Phosphatase or α-glucosidase |
| *Clostridium difficile* | β-glucosidase |
| Group B *Streptococcus* | Phosphatase |
| | |
| **Gram-negative pathogens** | |
| *Salmonella* spp. | C8-esterase or α-galactosidase |
| *Shigella* spp. | β-ribosidase |
| *Escherichia coli* | β-glucosidase or β-galactosidase |
| *Pseudomonas æruginosa* | β-alanyl aminopeptidase |

Chromogenic media are also used to screen for colonization of patients with antibiotic resistant bacteria, such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and *Enterobacteriaceæ* with production of extended-spectrum β-lactamases (ESBL) or carbapenemases.[20]

### 2.1.2 Biochemical testing

Many of today's clinical, microbiological laboratories still rely on biochemical testing of bacteria for their identification. The determination of nutritional and metabolic capabilities of a bacterial isolate is the most common approach used to determinate the genus and species of the tested strain. The available methods use a combination of tests to establish the enzymatic capabilities as well as the isolates ability to grow or survive the presence of specific inhibitors, e.g., salts, bile, toxins or antibiotics. Biochemical panel tests typically include sugars to detect acidification (via fermentation or oxidation) using a pH indicator. Other tests included in such panels may target enzymes participated in amino acid metabolism or detect hydrolases, such as urease or β-galactosidase.[6] These methods are usually technically easy and cheap, the generated data are simple to score and interpret, and all tests can be performed, even in the smallest clinical laboratories on a large number of samples.[17]

Biochemical testing can be processed manually as single/rack-tube tests or strip tests as well as panel tests, such as well-known BioMèrieux's API system. These panels are always designed for a particular group of bacteria, e.g., *Enterobacteriaceæ*, Gram-positives, or non-fermenting Gram-negatives, but their drawback is time consumption. Increasingly, such testing is automated, and there is a diversity of commercially available devices, such as BD Phoenix or Vitek 2 instruments using ID cards. In this case, the result for some species is usually available within few hours with the possibility to automatically perform antimicrobial susceptibility testing.[21]

### 2.1.3 Antibiogram-based typing

Antimicrobial resistance (AMR) has emerged as a significant public health problem all over the world. Infections caused by resistant microbes do not respond to treatment, resulting in prolonged illness and a greater risk of death. Treatment failures also lead to more extended periods of contagiousness, with increased numbers of infected people moving in the community.[22] For these reasons, it is desirable to monitor the natural sensitivity of individual bacterial strains, which is species-specific, for the rapid differentiation of resistant strains. Changes in susceptibility may reflect spontaneous point mutations or plasmid-born type of resistance.[5]

An antibiogram is identifying antibiotics' susceptibility based on the susceptibility of individual strain to each antibiotic. Patterns of the susceptibility to antimicrobial agents are used for typing because they are readily available, easy to determine, and relatively cheap.[23, 24] Susceptibility profiles expressed as diameters of inhibition zones combined with cluster analysis can provide useful typing data as an addition to data gained by other methods.[17] There exist international databases (e.g., EARS-Net – European Antimicrobial Resistance Surveillance Network) monitoring antibiotic profiles of isolates, including data on the geographical origin and clinical nature of these isolates. Nevertheless, these are primarily used to estimate frequencies of resistance, of course, they could be used for epidemiological

aims concerning the spread of specific resistance markers.[25,26] Antibiograms are also often used to assess local susceptibility rates, as a tool in selecting empiric antibiotic therapy, and in monitoring resistance trends over time within an institution.

### 2.1.4 Serotyping

Serotyping is using a commercial latex agglutination by series of polyclonal and monoclonal antibodies to detect different antigenic determinants on the surface of the bacterial cell.[5] The use for epidemiological typing is based on the fact that microorganisms frequently show variations in the antigenic constitution, not only between distantly related or unrelated organisms but even within groups of closely related organisms.[27] These approaches are widely used in healthcare-associated or food-associated microbiology laboratories.[17] Typeability and discrimination are variable, complicated by cross-reaction dependent on a type of detected protein antigen and a type of antibodies.[27,28] But with adequate quality control of both reagent and method, serotyping can be a reproducible method with broad application.[17] In practice, serotyping for the differentiation of intestinal pathogens, most commonly *Salmonella enterica*, *Shigella* spp. and *Escherichia coli*, is used in our latitudes. It is also used for respiratory pathogens, especially pneumococci and *Hæmophilus influenzæ* strains.

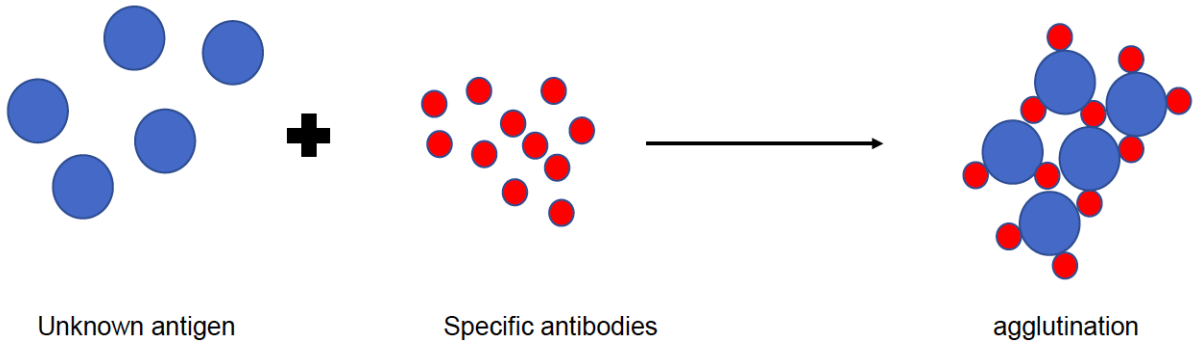



**Figure 2-2** Principle of serological identification. An unknown microbe is mixed with serum containing specific antibodies. Microscopically or macroscopically observable aggregates indicate a correct match between antibody and antigen confirming microbe identification.

The genera of the family *Enterobacteriaceæ* are most often typed by O and H surface antigens. The O (somatic) antigen is determined by the outer part of the lipopolysaccharide (LPS), and the H antigen is based on the flagellar protein antigens. Each O and H antigen has a unique code number. Determination of the serotype is based on the distinct combination of O and H antigens. Additionally, K antigens, the components of the polysaccharide capsules, can also be used.[29] The most diverse Gram-negative bacterial species is *Salmonella enterica* containing more than 2 600 different serovars differentiated by their antigenic presentation.[30] Serotyping is also used to distinguish strains of remarkable virulence or public health importance, such as *Vibrio choleræ* (*O1* serotype is the pandemic strain) or invasive types of *E. coli*, e.g., enterotoxigenic, enteroinvasive (the most common is *O124*), enterohemorrhagic (the most common is *O157:H7*), and enteropathogenic (e.g., *O55*, *O111*, *O126* or *O86*) serotypes.[31]

In 1933 Rebecca Lancefield[28] published schema for serotyping of β-hemolytic streptococci, except *Streptococcus pneumoniæ*, into one of 20 groups (marked with letters) based on the presence of C (carbohydrate) polysaccharide and teichoic acid antigens in the bacterial cell wall. In contrast, *S. pneumoniæ* is typed by capsular polysaccharide antigens using Quellung reaction.[32] Currently, over 90 pneumococcal serotypes have been described, and a large part of these serotypes is responsible for the majority of cases of invasive pneumococcal disease.[33] Another pathogen differentiated by capsular polysaccharide antigens is *H. influenzæ*. It can be encapsulated with one of six types of antigenically diverse capsules which can be serotyped using antisera to each capsule (serotypes *a-f*). The most virulent and invasive type of *H. influenzæ* is type *b* (*Hib*) causing epiglottitis most often in children under five years of age.[34]

### 2.1.5 Phage and bacteriocin typing

These typing approaches allow evaluating the lytic properties of test isolates that have been exposed to a defined set of bacteriophages, or bactericidal toxins (bacteriocins). These conventional methods are specified for a limited number of species for which such agents have been identified.[17] The effect of the phage results in the killing of the given bacterium, this is visible and therefore measurable. The bacteriophage typing is most often used for the two common *Salmonella* subtypes, *S.* Enteritidis and *S.* Typhimurium. But these systems also exist for some other *Salmonella* serotypes and a few other bacteria, especially *S. aureus* including methicillin-resistant *S. aureus*.[35-37] Bacteriocins are bacterial protein products which are lethal for other members of the same species and, occasionally, for other species. In general, they are active in very low concentrations against specific strains. Bacteriocins are used for some Gram-negative bacteria, especially *Klebsiella* and *Pseudomonas*, and for some Gram-positives, such as *Clostridium perfringens* or *C. difficile*.[38-40] Both of those methods represented an important tool in epidemiological studies of some bacterial strains, however, in recent years they have been replaced by faster and more precise approaches.[17]

### 2.1.6 SDS-PAGE protein analysis

The electrophoresis with the use of sodium dodecyl sulfate (SDS) was first described by Laemmli[41] as a method for the separation of polypeptides in complex mixtures and the determination of their molecular weights. In the 1980s, this approach was used to analyze a variety of bacteria, but since 1990s it has been replaced by DNA-based methods. The SDS-PAGE method can be used both for analysis of cellular or extracellular components of a bacterial cell.[17] To analyze bacterial cells, it is possible to process whole cells, or cell lysed either by sonication and subsequent centrifugation or by proteolytic enzymes, e.g., lysozyme or Proteinase K. The cells or lysate are solubilized in buffer with denaturing SDS and separated by polyacrylamide electrophoresis (PAGE). Subsequently, the gel is stained with Coomassie blue or silver and analyzed. The gel can be digitized, and the image subjected to cluster analysis (**Figure 2-3**).[42-44]

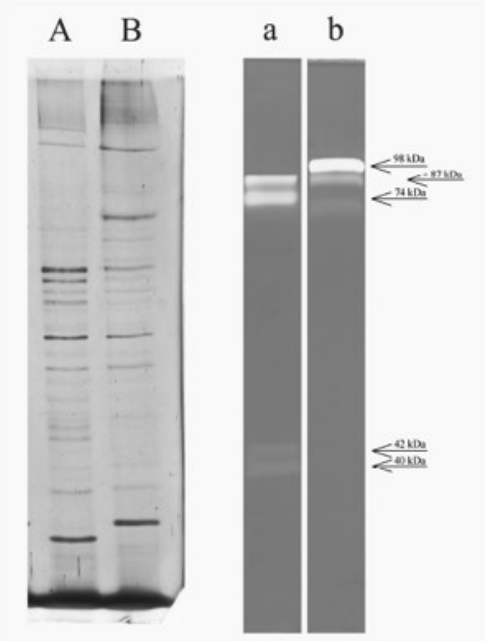



**Figure 2-3** Profiles of secretory products of two different strains of *Pœnibacillus larvæ* (A, B). A, B – SDS-PAGE analysis (10% polyacrylamide gel stained by silver; a, b – protease detection on zymograph gel (10% polyacrylamide gel with 0.2% gelatine stained by Coomassie blue R-250).[45]

### 2.1.7 MALDI-TOF MS

The idea of using mass spectrometry for identification of bacteria was proposed in 1975, but there was no way how to analyze intact proteins without degradation during the process.[46] In 1985 Koichi Tanaka described ionization of macromolecules by laser method using a combination of ultrafine metal powder (the particle diameters measuring a few tens of nanometers) and glycerol, for which was awarded the Nobel Prize in Chemistry in 2002.[47] Three years later, Franz Hillenkamp and Michael Karas published soft desorption ionization using nicotinic acid as the absorbing matrix, enabling ionization of proteins with molecular masses exceeding 10 000 Daltons.[48] Since 2010, the identification of bacterial and fungal pathogens by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is the method of choice in most advanced clinical laboratories.[6]

**Principle and methodology**

Single colonies are selected and spotted on the "spots" of the stainless steel MALDI-TOF target. After drying all spots are overlapped by an organic compound called matrix which works as an energy-absorbent. As a matrix, α-cyano-4-hydroxy-cinnamic acid is used in the identification of bacteria and yeasts (10 mg of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). The overlapping of a sample by 1 µl of 70% formic acid solution can be used to improve the quality of mass spectra, which may be particularly helpful for correct identification of a microorganism, such as yeasts and other difficult measurable strains. After drying, the target plate is placed in the mass spectrometer (**Figure 2-4**).[10, 46, 49]

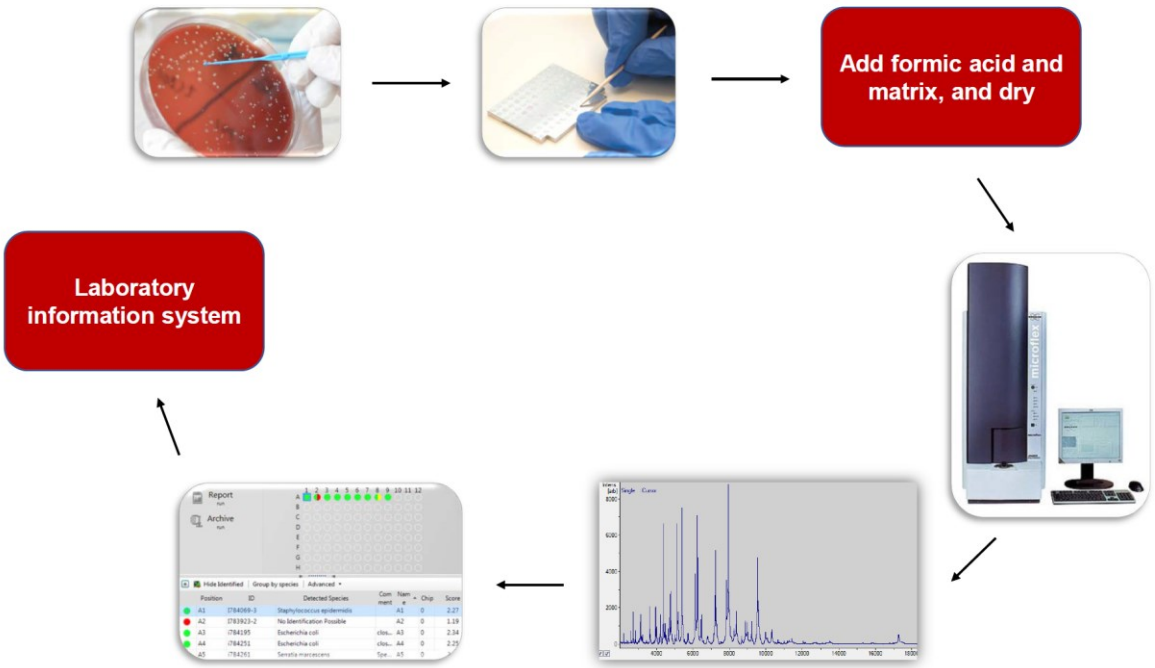



**Figure 2-4** MALDI-TOF MS process.

The matrix isolates analyzed samples, absorbs energy from a laser thus protects them from fragmentation by the heating energy. The result is desorption of molecules of the sample and the matrix and subsequent their ionization through random collision in the gas phase, where the charge is transferred from the matrix to the microbial macromolecules. The ionized molecules are then separated on the basis of the time they reach the detector, based on mass-to-charge ($m/z$) ratio into TOF mass analyzer, a tube under vacuum. In practice, the smaller ions reach the detector first, following by larger analytes (**Figure 2-5**). Subsequently, the mass spectrum is automatically generated. MALDI-TOF identification of microorganisms is primarily based on detection of ribosomal proteins, but mitochondrial proteins may also be isolated by extraction, as well as cold shock or heat shock proteins, or DNA binding proteins. All those proteins are giving "fingerprint" mass spectra which are unique for an individual organism in genera, species, and strain. The acquired protein spectra are automatically compared to an online database of reference spectra. The microorganisms may be identified at the family, genus or species level with some probability (**Figure 2-4**). For example, in MALDI Bruker Daltonic's BioTyper system identification score above 2.30 represents a high probability of species identification, score 2.00 till 2.29 represents secure genus identification and probable species identification, the score below 1.99 probable genus identification and score under 1.69 shows no reliable identification.[10, 46, 49-52]

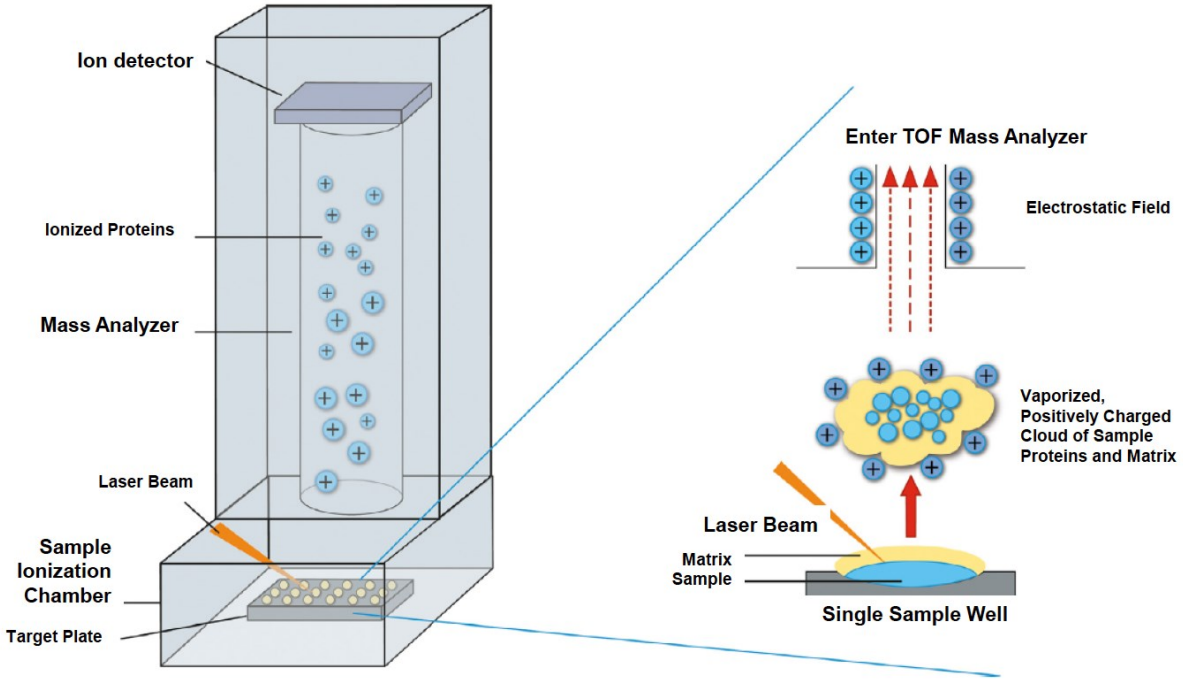



**Figure 2-5** A brief illustration of MALDI-TOF MS. *Adapted by Patel, R.*[46]

## Application and limits of MALDI-TOF MS in microbiology

### *Bacteria*

In a routine laboratory, MALDI-TOF is commonly used for identification of aerobic and anaerobic bacteria, fungi and mycobacteria. The use of biochemical and metabolic identification of bacteria requires a 24-hour incubation with not always the exact result. In the meantime, patients are administered empirical antibiotics, which are sometimes inappropriate. MALDI-TOF identification, on the other hand, takes only 5 minutes including preparation.[50] Overall >90% of all isolates are identified at the species level, 98% are identified at the genus level and, only <1% are either identified incorrectly or not at all. The most common reason is that the isolate is not included in the database.[49] Misidentification is most commonly observed with taxonomically closely related bacteria, such as *E. coli* with the highly pathogenic *Shigella*, or *S. pneumoniæ* with some commensal species of oral streptococci, e.g., *S. mitis*.[6, 46, 49] MALDI-TOF does not precisely differentiate all species within some groups of microbes, such as the *Enterobacter cloacæ* complex,[53] *Burkholderia cepacia* complex[54] or *Acinetobacter calcoaceticus-A. baumannii* complex.[55]

### *Anaerobes*

MALDI-TOF identification of anaerobes has become the method of choice, replacing 16S ribosomal ribonucleic acid (rRNA) gene sequencing which is considered to be the "gold standard" method for detecting of fastidious and uncultivable bacteria.[56] The easiest way to identify anaerobes by MALDI-TOF is a direct smear of the sample on the MALDI target without any extraction, but this approach often

shows a low identification score <2.00 with high possibility of incorrect or no reliable identification. The better way is pre-extraction of proteins either on-plate by semi-extraction using 70% formic acid or off-plate extraction by 70% ethanol and 70% formic acid, to improve the quality of mass spectra.[57-59]

***Yeasts and filamentous fungi***

In clinical laboratories, yeast identification mainly relies on conventional methods based on microscopy and morphological features, latex agglutination, and biochemical assays. But for identification of clinically relevant yeasts, MALDI-TOF can also be used. MALDI-TOF MS-based identification of yeast requires a pretreatment of the sample before the acquisition of the spectra. The most commonly used is on-plate protein extraction by 70% formic acid or complex procedures can be used more such as ethanol/formic acid extraction or formic acid/acetonitrile extraction.[60, 61] In the case of identification of filamentous fungi, the gold standard is based on morphologic features and DNA sequencing. However, DNA sequencing is expensive, labor-intensive, and is subjected to a high risk of environmental contamination due to its multistep workflow.[62] Identification of filamentous fungi by MALDI-TOF is complicated. Generally, fungal cultures are heterogenic. Not only do they consist of different cell types such as mycelium, spores, and fructification organs, but there is also a significant difference between the center of a colony and the periphery, and it also depends on the age of the culture. The solution should be the use of protein isolation from the culture suspension rather than from their own colonies grown on plates. Also, on-plate extraction with organic acids such as formic acid can be used.[63, 64]

***Mycobacteria***

Identification of tuberculous or even non-tuberculous mycobacteria and other acid-fast organisms such as *Nocardia* by MALDI-TOF posed a particular challenge. Historically, the identification has been done using time-consuming cultivation, biochemical testing, DNA probes, gas-liquid chromatography or DNA sequencing.[46] An important step before MS processing is to inactivate live cells for safety reasons and subsequent cell lysis to obtain of protein components for analysis.[49] To kill the cells, the heat inactivation at 95°C for 30 min can be used, followed by resuspension in ethanol to a final concentration of 75%. There are several ways of protein extraction. Often 0.5 mm zirconia/silica beads combined with sonication is used as well as by extraction by 70% formic acid and acetonitrile, also combination of both methods is possible. The entire process takes 90 minutes including MALDI-TOF measurement.[65] Nevertheless, MALDI-TOF identification of mycobacteria has some limits, for example, is not able to differentiate members of the *Mycobacterium tuberculosis* complex and some closely related species such as *M. chimæra* and *M. intracellulare* and others.[46, 66, 67]

***Antimicrobial susceptibility testing***

For antimicrobial susceptibility testing (AST) and resistance detection a variety of approaches are used, for example disk diffusion method which was first time published in 1966 by Bauer *et al*.[68] Another is the broth microdilution (BMD) method, it is a reference method for AST testing with which other methods are compared during development, standardization, and clinical testing. Nowadays, genetic methods

such as PCR, DNA probes, DNA sequencing etc. are used to detect antibiotic resistance.[69] Because some resistance-associated markers are proteins (e.g., β-lactamases) MALDI-TOF MS may be used for their detection.[46] For example, in July 2011 the assay for detection of carbapenemases by Hrabak *et al.*[70] was published. This assay is based on detection of degradation products of meropenem. The buffered solution of meropenem is mixed with overnight culture to concentration 3 McF and it is incubated for 2–4 hours at 37°C. Afterwards, degradation products *m/z* 358.5 (decarboxylated degradation product of meropenem), and *m/z* 380.5 (decarboxylated sodium salt of the degradation product of meropenem) are detected using MALDI-TOF MS (**Figure 2-6**).[71] This assay can also be used for detection of OXA-like carbapenemases adding bicarbonates to reaction buffer.[72] Moreover, approaches to differentiate methicillin-susceptible and methicillin-resistant *S. aureus* (MRSA) have been described, based on a different protein mass spectrum.[73, 74] Also Bruker Daltonics introduced a subtyping module for detection of MRSA by identification phenol-soluble moduline (PSM-mec, *m/z* 2413), which is a staphylococcal toxin encoded by class *A mec* gene complex.[75] MALDI-TOF might prospectively also be used to detect vancomycin-resistant enterococci (VRE), resistance to colistin or fosfomycin.

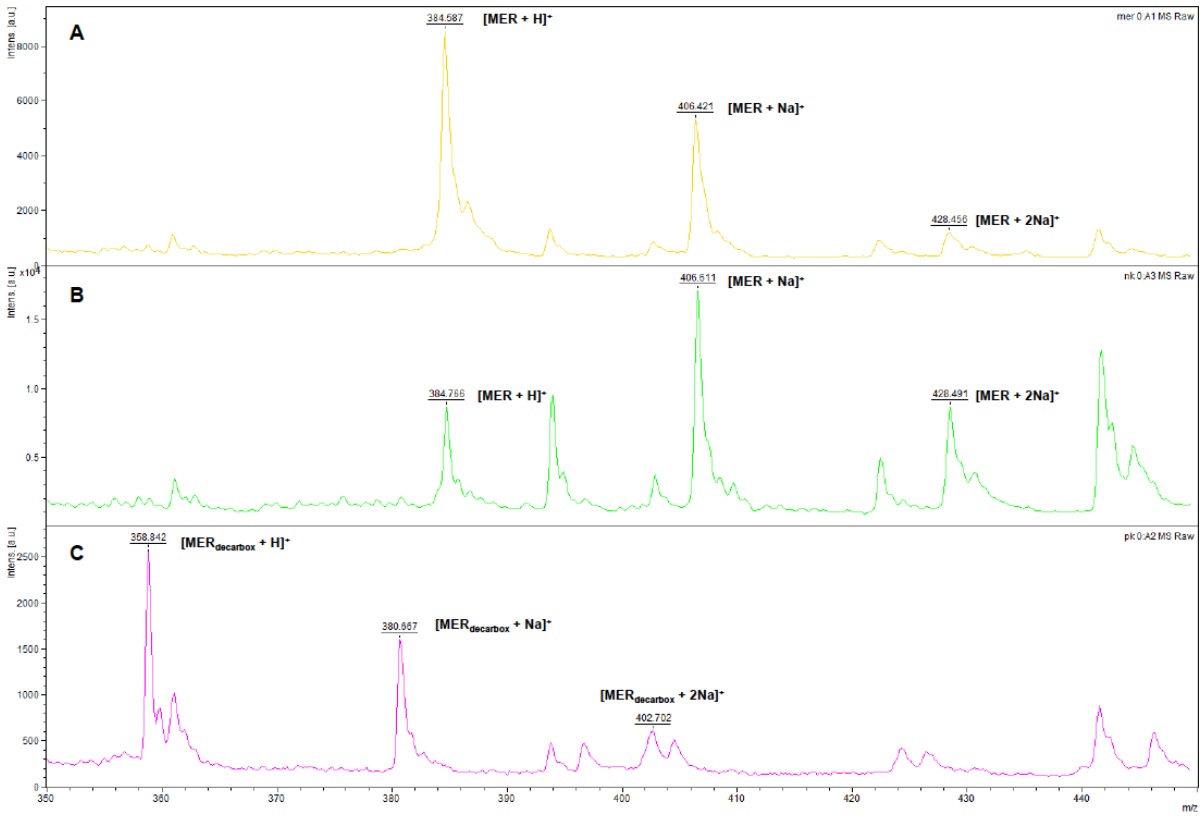



**Figure 2-6** MALDI-TOF MS spectra of meropenem and its sodium salt variants and degradation products. (**A**) Spectrum of the meropenem solution; (**B**) spectrum of the negative control (non-carbapenemase-producing isolate of *Klebsiella pneumoniæ*; (**C**) spectrum of an KPC-2-producing *Klebsiella pneumoniæ* isolate; [Meropenem + H]$^+$ – meropenem molecule (*m/z* 384.6); [Meropenem + Na]$^+$ – meropenem sodium salt (*m/z* 406.6); [Meropenem + 2Na]$^+$ – meropenem disodium salt (*m/z* 428.5); [Meropenem$_{decarbox}$ + H]$^+$ – decarboxylated degradation product of meropenem after carbapenemase hydrolysis (*m/z* 358.8); [Meropenem$_{decarbox}$ + Na]$^+$ – decarboxylated sodium salt of the degradation product of meropenem after carbapenemase hydrolysis (*m/z* 380.7); Meropenem$_{decarbox}$ + 2Na]$^+$ – decarboxylated disodium salt of the degradation product of meropenem after carbapenemase hydrolysis (*m/z* 402.7).

***Direct testing of clinical samples***

In case of need of a rapid detection, the direct detection from a clinical specimen without required cultivation is an advantage. In recent years a number of works focused on detecting a pathogen in positive blood culture or urine have been described. Identification of a pathogen in blood is complex procedure because the nonmicrobial cells, serum proteins, and broth components must be removed before analysis.[49] For the preparation of the specimen, simple blood centrifugation can be used to remove blood cells, this can be combined with on-plate formic acid extraction.[76] Other published works mention the use of detergents such as sodium dodecyl sulfate or saponin, to remove blood cells.[77, 78] On the use of lysis buffer is also based commercial kit MALDI Sepsityper™ of Bruker Daltonics enabling isolation and identification of a pathogen in 30 minutes.[79] In the case of detection of a pathogen directly from urine, Zboromyrska *et al*.[80] described a protocol for extraction of bacterial cells by centrifugation of urine and subsequent on-plate extraction of proteins by 70% formic acid. On the other hand, Huang *et al*.[81] published an approach for pre-extraction of proteins from urine pellet by formic acid/acetonitrile. The drawback of direct detection of a pathogen from a specimen is burdened with high identification failure, >20% in case of blood culture and around 15% when is it urine. It depends on a number of bacterial cells, other components of specimen or cultivation medium (blood) and extraction procedure.[80-82]

***Bacterial strain typing and taxonomy***

Genetic methods such as PFGE, MLST, or WGS are commonly used for bacterial typing, which I will mention below. In the last decade, mass spectrometry has also proved to be useful for bacterial strain typing and taxonomy investigation. As mentioned before, MALDI-TOF detects bacterial proteins, and the result is a protein fingerprint typical for individual bacterial species. But it is sometimes necessary for epidemiological reasons to distinguish strains on the subspecies level or distinguish virulent strain (e.g., *E. coli* O157:H7) from non-pathogenic (e.g., a common *E. coli*). The problem is that phenotypic expression (protein production) is not an absolute reflection of the genetic background. Mostly it is the formation of bacterial proteins closely related to the culture conditions, the type of medium, the added supplements, and other factors. Depending on those factors, the mass spectrum may vary. For general identification is necessary to have a database in which one can compare the acquired spectrum to the reference spectrum. The main disadvantage even though the microbial identification is carried out with a high percentage of correct identification, only little spectral information is provided for use in further studies. Therefore, for typing, private databases are created, often only of a regional character.[51, 83-85] In recent years many approaches for typing of bacteria have been described, e.g., for differentiation of the main clonal lineages of *S. aureus*,[86] for fast detection of STEC *E. coli*,[87] for typization of *A. baumannii*,[88] *Salmonella* spp.,[89] and last but not least for *Mycobacterium* spp.[90]

### 2.1.8 Others spectrophotometric methods

**Infrared spectroscopy**

In the field of microbiology either infrared (IR) spectroscopy or its more advanced version IR with Fourier transformation (FTIR) can also be used, which are based on absorption spectroscopy. IR spectra of intact microbial cells are highly specific, fingerprint-like signatures that can be used to differentiate, identify, and classify diverse microbial species and strains. IR spectroscopy may also be used for detection *in situ* intracellular compounds, for tracking of metabolic pathways or cell-drug interaction.[91] The research group around Shapaval published several works on fungal[92] and yeast[93] identification, such as *Saccharomyces cerevisiæ*[94] or *Candida* spp.[95] using FTIR spectroscopy, over the period 2010-2017. There are even some works describing identification and taxonomy classification of bacteria. For example, Helm *et al.*[96] used FTIR spectroscopy for cluster analysis of some Gram-positive and Gram-negative bacteria. Bosh *et al.*[97] used FTIR spectroscopy for rapid identification of non-fermenting Gram-negative bacteria isolated from sputum samples from cystic fibrosis patients. Last but not least, FTIR spectroscopy was also used to quantify the bacterial population in pure culture.[98]

**Raman spectroscopy and Surface-enhanced Raman spectroscopy**

In the previous decades, non-invasive and non-destructive tools such as Raman spectroscopy or Surface-enhanced Raman spectroscopy (SERS), are being used to identify microorganisms. Raman spectroscopy is a vibrational spectroscopic technique for "molecular fingerprinting" of tissues, cells, proteins, nucleic acids, and other small organic and inorganic compounds. It provides information about the chemical structure of any kind of sample and relies on the changes in the polarizability of functional groups when atoms vibrate in a molecule. The method has the advantage of providing information on both chemical composition and the structure of biological molecules. Nevertheless, SERS is more sensitive, with higher intensity of the signal and is more suitable for analyzing biological material.[99-101] In 2004, Jarvis *et al.*[102] were among the first researchers to perform SERS experiments for discrimination among *E. coli*, *Klebsiella oxytoca*, *K. pneumoniæ*, *Citrobacter freundii*, *Enterococcus* spp., and *Proteus mirabilis*. From that days, at least 400 publications have been published on this topic presenting application and limits of this method. SERS was also used to identify a biological agent in case of a possible bioterrorist attack, in this case, the detection of anthrax spores.[103]

### 2.1.9 Use of "omics" in microbiology

"Omics" is a shorthand term for a group of recent technologies used to examine the molecular composition of a cell, tissue, or organism at a particular point in time or under specific conditions. There are methods that analyze, for example, protein contents of a bacterial cell (proteomics), RNA transcripts (transcriptomics), bacterial genes (genomics), cell metabolism (metabolomics) or diversity of polysaccharides, glycans, and lipopolysaccharides (glycomics).[17] These methods, together with bioinformatics, allow the understanding of how bacterial cells work either individually or in the community of other cells. Very important tools for bioinformatics are online databases for collecting and comparing data. The most well-known are PubMed and Bacteriome.org. These complex approaches,

suitable for automation, can aid the search for new ways of result interpretation different from the culture-based methods. Recently, human microbiome has been the subject of intensive research. Omics methods help understand the interactions between bacterial species and the host (human).[104-106]

## 2.2 Genotypic typing methods

The ability to discriminate among individual genomes is essential for several issues of microbiology research including, for example, taxonomy, population genetics of microbes, microbial epidemiology and spread of resistance genes. The increase in virulence and transmissibility, antibiotic resistance, and possibility of genetic manipulation led to the need to have these tools on your side.[107, 108] Genotypic methods provide more sensitive strain differentiation and higher levels of standardization, reproducibility, typeability, and discriminatory power when compared with phenotypic typing methods.[14] Over the years a number of methods of choice for typing bacterial isolates have been emerged. They can be based on *(i)* the direct characterization of genetic section using restriction endonucleases for analysis of plasmids or chromosomal DNA in combination with conventional electrophoresis, and hybridization by DNA/RNA probes, *(ii)* PCR amplification of the genes and subsequent characterization of the amplicon, or *(iii)* complete sequencing of individual genes, plasmids, chromosomes, or whole genome.[5, 6] However, genetic typing assays have some drawbacks. In general, these approaches mostly require a relatively large amount of high-quality DNA/RNA, a high degree of technical instrumentation, are labor-intensive and are often financially demanding compared to some phenotypic methods.[107]

### 2.2.1 Plasmid profile typing and fingerprinting

Plasmids are circular or linear extrachromosomal DNA elements that are found typically in prokaryotic microorganisms and are non-essential for vital functions of a cell. They are capable of replicating autonomously, independently from the chromosome. Naturally occurring plasmids differ in size from one to several hundred kilobase pairs, and in a copy number from one to hundred copies per cell. Copy number is characteristic for any plasmid under a specific condition and is controlled by plasmid-coded system, a replicon, that determines an initialization of replication. Many plasmids contain even other genes that are not useful only to themselves, but also to their host, for example, genes for drug or detergent resistance, virulence factors, genes controlling conjugation, or production of toxins. These genes, along with the plasmid, can be transferred to another bacterial cell of the same or another genus by a horizontal gene transfer, most commonly by conjugation, less often by transformation, or transduction (**Figure 2-7**). Plasmids are also important genetic tools used to manipulate and research microorganisms through the introduction, modification or removal of target genes.[109-114]

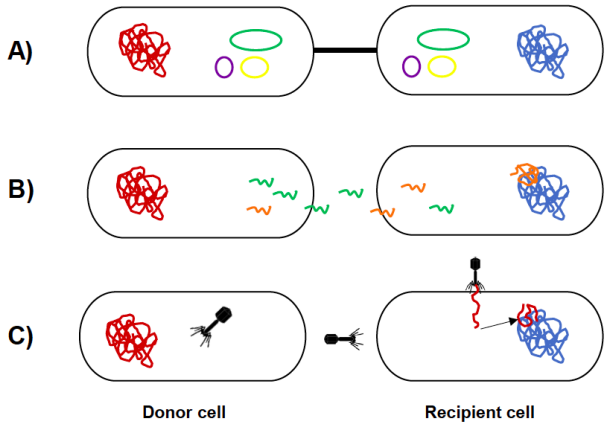



**Figure 2-7** Horizontal gene transfer mechanisms in bacteria. **A) Bacterial conjugation** – transfer of genes between cells that are in physical contact with one another by sex-pili; **B) Bacterial transformation** – transfer of cell-free or "naked" DNA from one cell to another; **C) Bacterial transduction** – transfer of genes from one cell to another by a bacteriophage.

### Replicon typing

Classification of plasmids became important at the end of the 1950s after the discovery of R plasmids (R stands for resistance) and the recognition of their ability to transfer several drug resistance genes by conjugation using F plasmids (F stands for fertility) that provide the own conjugation. Based on the presence/absence of the F plasmid and sex-pili, the strains are then classified as conjugative, respectively non-conjugative. However, later on it had been found that some types of plasmids are unable to conjugate, so other procedures had to be developed to distinguish them.[111] During the 1970s and 1980s detailed procedures based on replicons incompatibility (Inc) were developed. These approaches for plasmid classification are based on the insertion of plasmid with an "unknown" Inc group into a bacterial cell with a plasmid of known Inc group, based on the stability of plasmids during conjugation. The rule is that plasmids belonging to the same Inc group are "incompatible" therefore, the new plasmid is not accepted by the cell. While plasmids with different replication control are "compatible" and can coexist in one cell line.[110, 115, 116] In 1988 Couturier *et al.*[111] described a new method for identification of major replicons of plasmids circulating among the *Enterobacteriaceæ*. This method was based on conjugation and Southern blot hybridization with 19 DNA probes in size from 304 to 2 250 bp that recognize B/O, FIA-C, FII, HI1, HI2, I1, K, L/M, N, P, Q, T, U, W, X, Y, and rep9 replicon families. Unfortunately, all these methods cannot be used to analyze a large number of samples, as it would be very laborious and time-consuming. Following PCR implementation into practice, PCR-based replicon typing (PBRT) methods were developed. In 1996 Götz *et al.*[117] introduced PCR-based detection of plasmids on the bases of previously published sequences, but it was limited only to the IncP, IncN, IncW and IncQ family plasmids. A complete PCR scheme for identifying and tracing major plasmids occurring in *Enterobacteriaceæ* was presented by Carattoli *et al.*[109] in 2005. They used 18 pairs of primers to perform multiplex PCRs, recognizing FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons. At present, the most accurate method for mapping plasmids is complete sequenation of plasmid allowing detection of any point mutations as well.

**Restriction Fragment Length Polymorphism (RFLP) plasmid profiling**

RFLP method for plasmid profiling by simple electrophoresis of Gram-negative and Gram-positive bacteria was one of the first molecular methods to be used as a bacterial typing tool.[118, 119] Since the normal size of plasmids is ranging from one to several hundred kilobase pairs, the gel electrophoresis alone cannot be used (size limitation from 100 bp to 25-30 kb).[120] In particular, is challenging to differentiate plasmids that vary only by 10 kb to 15 kb. Therefore, fragmentation by restriction endonucleases to increase the discriminatory power had begun to be used.[5] Restriction endonucleases (RE) are enzymes cleaving both strands of DNA on a phosphodiester linkage in a specific sequence site (so called restriction site). The recognition sequence of a restriction enzyme is most commonly a palindromic sequence, i.e., a sequence that is the same for front and back reading. Two types of palindrome sequences are distinguished: *(i)* a mirror palindrome where the sequence is read in a single chain (e.g., CTAATC), *(ii)* an inverted palindrome where the sequence is read within both complementary strands (e.g., GAATTC, the complementary sequence is CTTAAG). Part of the reaction may also be methylase that protects the host organism/cell by methylation of the DNA. REs were primarily found in bacteria, where they serve as a defense mechanism against bacteriophages or undesirable plasmids, however they also exist in archæa, viruses, and eukaryotes. At present, >3500 restriction enzymes that recognize >259 different DNA sequences are known. Although many similarities exist among REs, there remain important differences such as *in vivo* function, organism origins or site-specific DNA recognition. Restriction endonucleases are divided into four groups (I-IV) and some subgroups according to their specificity of cleavage, structure and other properties (**Table 2-3**). REs are used in restriction methods, e.g., RFLP, AFLP (described below), furthermore for DNA fragmentation or cloning.[121, 122]

**Table 2-3** Basic classification of restriction endonucleases. *Adapted by Williams, R.*[121]

| Type of REs | Examples[1] | Cofactors and Activators | Recognition site | Cleavage site |
|---|---|---|---|---|
| **I** | *Eco*KI, *Eco*AI, *Eco*BI, *Cfr*AI | $Mg^{2+}$, ATP | Interrupted bipartite | Distant and variable from recognition site |
| **II** | *Eco*RI, *Bam*HI, *Hin*dIII, *Sma*I | $Mg^{2+}$ | Palindromic or Interrupted palindrome | Defined, within recognition site |
| **III** | *Eco*P15I, *Sty*LTI *Eco*PI, *Hin*fIII | $Mg^{2+}$, ATP | Nonpalindromic | Cuts approx. 25 bases away from recognition site |
| **IV** | *Bcg*I, *Bsp*24I, *Bae*I, *Cje*I | $Mg^{2+}$ | Interrupted bipartite | Cuts both strands on both sides of recognition site a defined |

[1] Restriction enzymes are named based on the organism in which they were discovered. For example, the enzyme *Hin*dIII was isolated from *Hæmophilus influenzæ*, strain Rd. The first three letters of the name are abbreviated from a genus and species names of an organism. The fourth letter typically comes from the bacterial strain designation. The Roman numerals are used to identify specific enzymes from bacteria that contain multiple restriction enzymes. Typically, the Roman numeral indicates the order in which restriction enzymes were discovered in a particular strain.

Before electrophoresis, extracted plasmids are digested with suitable "frequent-cutting" RE which recognizes specific DNA sequences, thereby cutting the plasmid DNA into a number of fragments depends on the number of enzyme recognition sites. Subsequently, the thus prepared samples are applied to the electrophoretic gel using a loading buffer. Agarose gel electrophoresis (AGE) is based on the fact that DNA is negatively charged, and it migrates to a positive pole in an electric field. An agarose gel acts as a molecular sieve under the influence of an electric field, therefore plasmid fragments of different molecular weights are separated into clearly defined bands in the gel forming specific plasmid fingerprint. Then, individual fragments are either visualized using ethidium bromide, and the plasmids are compared to each other, or probe hybridization can be used to identify individual genes (described below). At present, a software designed for these purposes can also be used for faster, easier fragment analysis. In practice, the digested plasmids produce around 5-10 linear fragments, but large plasmids can produce many restriction fragments which can make interpretation more difficult. Therefore, this method is not suitable for analysis of Gram-negative rods, but it can still be used for performing of Gram-positives such as staphylococci, where plasmids are typically <50 kb and produce only a few fragments after digestion by REs. AGE is most often used for epidemiological or resistance surveillance studies to identify the source of infection or to compare strains from one outbreak limited both temporally and geographically.[5, 123-125]

**PFGE plasmid size profiling**

For analysis of plasmids can also be used Pulsed-Field Gel Electrophoresis (PFGE) which has better discriminatory power and reproducibility than conventional gel electrophoresis and allows to separate large plasmids. Their closed-circular form moves very slowly through the pulsed-field gel and can often be undetected therefore they should be linearized. The most commonly used protocol for PFGE plasmid size profiling is using S1 nuclease linearization of plasmid DNA in cells embedded in agarose plugs allowing detection of large plasmids up 600 kb. Only the linear forms migrate at rates that allow accurate size determination.[17, 126, 127] Shortly, bacterial cells are mixed with melted agarose and poured into a plug mold. After solidification plugs are treated by lysis solutions, enzymes (e.g., Proteinase K, RNase) and subsequently by S1 nuclease which causes linearization of plasmids. As a result, agarose plugs contain pure plasmid DNA and the plasmids breakdown in electrophoresis is prevented. Digested plugs are applied to wells in an agarose gel and subjected to pulsed-field electrophoresis. The voltage here does not proceed constantly in one direction but is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 60 degrees either side. Then DNA does not migrate in a straight path through the gel, but zig-zag and the size-dependent separation of fragments takes more time, mostly around 15-30 hours. (**Figure 2-8**) The resulting band patterns can then be visualized with ethidium bromide staining and compared with linear marker manually or normalized by a suitable software such as BioNumerics (Applied Maths, Austin TX, USA) and compared against a local or networked database (**Figure 2-9**). Again, the method may be associated with Southern blotting and hybridization.[127, 128]

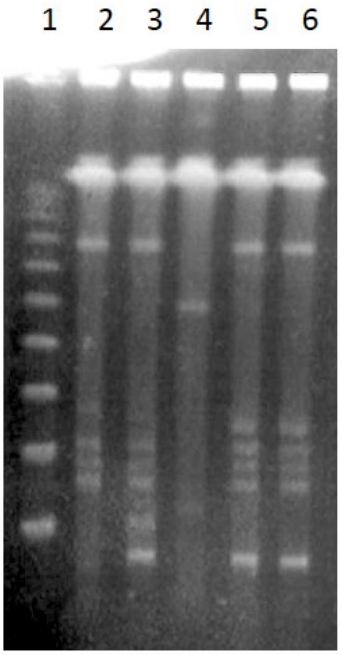



**Figure 2-8** Example of pulsed-field gel electrophoresis of plasmid DNA from five isolates (lanes 2-6) of *Citrobacter freundii* after digestion with S1 nuclease. Lanes 1 contain molecular weight markers (*Hin*dIII digest of phage lambda DNA, range 48.5-1000 kb). (*unpublished data*)

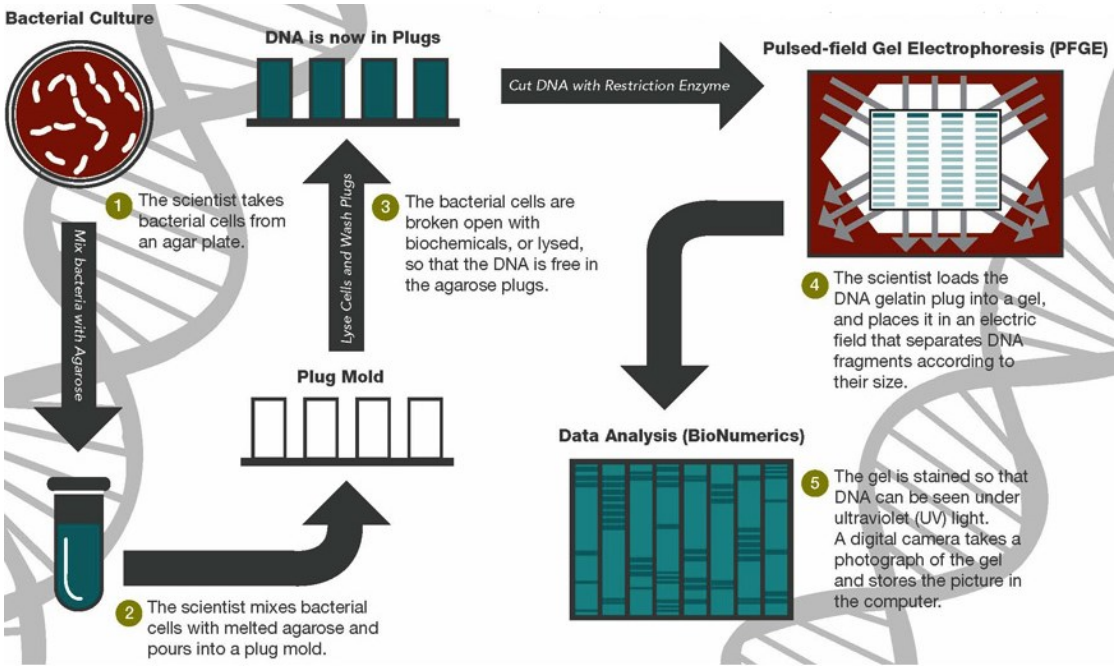



**Figure 2-9** The pulsed-field gel electrophoresis process using agarose plugs.[129]

**Southern blotting and direct hybridization**

In 1975, Southern *et al.*[130] described a method that allows to transfer double-stranded DNA fragments from an electrophoretic gel to a nitrocellulose membrane by fluid raising fluid raising followed by fixation of the attached DNA on a membrane and subsequent identification of specific sequences by hybridization probes. Labeled hybridization probes are incubated with a membrane where they are specifically bound to complementary sequences. Unbound probes are then washed off, and the result is visualized (**Figure 2-10**). Previously, radioisotope-labeled probes, typically $^{32}P$ was used, but there was a risk of handling and waste disposal.[131] Currently non-radioactive detection approaches are mostly used:

*(i)* ***chemiluminescence***, a process in which light is generated by enzymatic modification of suitable substrates, e.g., alkaline phosphatase-luminol, peroxidase-luminol;

*(ii)* ***chromogenic (enzymatic) detection***, in which an enzymatic activity causes a color precipitate at the site of the reaction, e.g., avidin-biotin, streptavidin-biotin, alkaline phosphatase (AP)-biotin, horseradish peroxidase (HRP)-biotin, AP-digoxigenin, HRP-digoxigenin;

*(iii)* ***fluorescence***, in which molecular excitation of certain compounds at one wavelength results in an emission of light at a different, characteristic wavelength. The more commonly used fluorophores for probe labeling are cyanine dyes such as Cy3 or Cy5;

*(iv)* ***chemifluorescence***, in which a fluorogenic substrate is converted enzymatically into a fluorescent product which, upon laser excitation, may be captured using a fluorescence imaging system.[132]

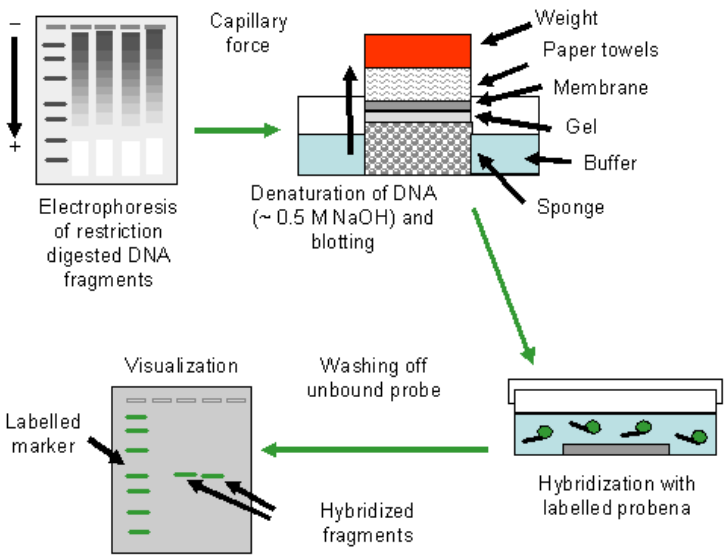



**Figure 2-10** Southern blotting and hybridization process.[133]

Hybridization has broad application, it can be used for binary typing of bacteria, there is a number of patterns for various bacterial species that have been developed, e.g., *E. coli*,[134] *S. aureus*,[135] or it can also be used for identification of uncultivable bacteria while using 16S rRNA gene detection.[136] Direct hybridization can be as well used to define the nature of mobile elements involved in antibiotic resistance,[137] for ribotyping (mentioned below) or for typing DNA amplified by PCR. In addition, this approach is used in commercial kits for identifying strains of *Mycobacterium tuberculosis* complex using detection of the DNA insertion element IS*6110* or detecting their resistance to conventional antituberculotics such as rifampicin and isoniazid.[138]

### 2.2.2 Analysis of chromosomal DNA

**Analysis of RFLPs fragments by conventional gel electrophoresis**

Restriction Fragment Length Polymorphism (RFLP) analysis of bacterial chromosome is one of the first techniques to be widely used for profiling and comparison of bacteria. RFLP measures the size of restriction fragments by conventional gel electrophoresis after enzymatic restriction. The number of obtained restriction fragments depends on the frequency and distribution of cutting sites especially recognized by REs in the nucleotide sequence of the bacterial chromosome. Most often "frequent cutters" are used cleaving chromosome into hundreds of pieces ranging approximately from 0.5 to 50 kb in length. During the electrophoresis fragments between 0.5 to 25 kb are divided into distinct bands, although a single band may contain more fragments of a similar size. Larger fragments remain on the top of the gel and do not migrate through. The fragment pattern provides a typical banding profile or "fingerprint" of chromosomal DNA that can be easily visualized with ethidium bromide staining. Two clonally related strains contain almost identical sequences with conserved restriction sites generating identical electrophoretic profiles. These profiles can be processed by suitable software and compared with a local or online database. RFLP profile can also be simplified by subjecting to Southern blot and subsequent hybridization with labeled probes.[5, 11, 139]

**Ribotyping**

Ribotyping is a special modification of RFLP analysis of the number and location of the ribosomal RNA (rRNA) gene sequences in a bacterial genome. After RFLP fragment are processed by Southern hybridization using probes targeting 16S rRNA, 23S rRNA, 5S rRNA in the bacterial genome, or 18S rRNA in case of fungi. The use of ribosomal RNA gene restriction patterns has been applied to the analysis of taxonomic and epidemiological relationships among strains of different groups of organisms. Ribotyping is generally a robust method with good reproducibility and stability, but the discriminatory power is usually lower than that of PFGE restriction typing. It is also time-consuming method dependent on skilled personnel. Fully automated robots for ribotyping have been developed to make it easier, for example, The RiboPrinter™ Microbial Characterization System (DuPont Qualicon, Wilmington DE, USA) using computer analysis to compare ribotype profiles. It provides much faster results, speeding up the ribotyping process from approximately five days to only eight hours.[140-143] But for example, Dalsgaard *et al.*[144] described the lower discriminatory power of RiboPrinter, which is not able to distinguish similar

heavy and weak fragments, compared to traditional ribotyping. In this case in *Vibrio cholerae* O1 restricted by *Eco*RI routinely used for automated ribotyping.

In routine practice, ribotyping is most commonly used to distinguish between different genotypes of *Clostridium difficile*. The method is based on the amplification of intergenic spacer region (ISR) between 16S and 23S rRNA genes. The ISRs differ in length and in combination with variable number of alleles of the ribosomal operon present in different *C. difficile* strains, PCR amplification of ISRs with only a single primer pair results in a banding pattern, specific for a given PCR-ribotype. This technique is considered to be highly discriminative, reproducible and can be performed relatively easily and rapidly. It can be used for interlaboratory comparison and a reference library has also been created.[145-147]

**RFLP followed by Pulsed-Field Gel Electrophoresis (PFGE)**

PFGE was first described in 1984 by Schwartz *et al.*,[148] and for many bacteria it remains the gold standard strain typing method for outbreak investigation and surveillance application. Until then, most techniques used the digestion of the chromosomal DNA into smaller fragments followed by electrophoretic analysis. PFGE is using the DNA embedded into agarose plugs as described above (**2.2.1**), preventing possible breakage of DNA during electrophoresis. In this case, the bacterial genome's size is typically from 2000 to 5000 kb pairs and it is digested by restriction endonucleases with six or more base pair recognition sites, also called "rare cutters" (e.g., *Xba*I, *Avr*II, *Sma*I, *Spe*I), usually generating fewer than 30 large restriction fragments in size between 20 and 600 kb pairs. The choice of RE is one of the most important aspects in determining the PFGE banding patterns because the restriction site of each RE is unique. As described above, DNA is going zig-zag through pores of electrophoretic gel, separating in an approximate size-dependent manner resulting in the higher level of fragment resolution. After separation the pattern is visualized with ethidium bromide/UV light (**Figure 2-11**) and normalized with specialized imagine analysis software, such as above mentioned BioNumerics (Applied Maths, Austin TX, USA), and compared against a local or networked database of PFGE patterns. [5, 9, 11, 17, 139] The best-known database is PulseNet, established by the Centers for Disease Control and Prevention (CDC, Atlanta GA, USA) in 1996 for surveillance and outbreak detection of bacterial foodborne pathogens.[149] PulseNet has two parts – PulseNet USA and PulseNet International which currently consists of 88 countries from around the world. It also publishes highly standardized protocols for PFGE, MLVA, and WGS (**2.2.4**).[150]

As proposed by Tenover *et al.*,[151] two compared isolates by PFGE without differences in bands, should be termed "indistinguishable" belonging to the same type (e.g., A). Isolates differing by one up to four bands are "closely related" and should be assigned to subtypes of the same type (e.g., A1). When two strains are differing by five up to eight bands then they are "possibly related" and should be assigned to distinct type (e.g., B). If two isolates differ by more than eight bands then those are termed "unrelated".

PFGE has remarkable discriminatory power and reproducibility, therefore has become a widely applicable method for comparative typing of many bacterial species, e.g., *Streptococcus pyogenes*,[152] Gram-negative bacteria,[153] or *Listeria monocytogenes*.[154] The main disadvantage of this technique is

time-consumption, usually the whole process takes around 5-6 days. But over the years, a number of modifications have been made to shorten method to 1-3 days.[153, 155, 156]

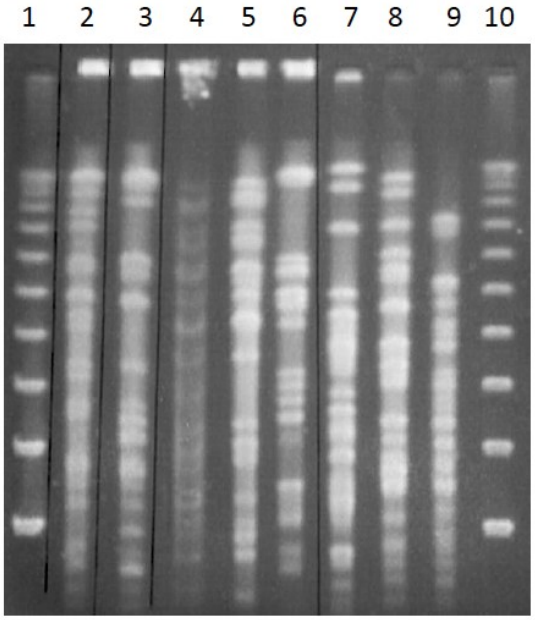



**Figure 2-11** Example of PFGE protein profile. As restriction endonuclease has been used *Xba*I. Pattern comparison of *Enterobacteriaceæ* strains, visualized with ethidium bromide and short-wave UV light. Lanes **2-6** contain *Escherichia coli*, lanes **7-9** contain *Citrobacter freundii*, and lanes 1 and 10 contain molecular weight markers (*Hin*dIII digest of phage lambda DNA, range 48.5-1000 kb). (*unpublished data*)

### 2.2.3 Typing approaches using PCR amplification

In 1987, Kary B. Mullis[157] introduced a revolutionary method for nucleic acid analysis, The Polymerase Chain Reaction (PCR), for which he and his colleague Michael Smith were awarded the Nobel Prize in Chemistry in 1993. PCR is a powerful amplification technique that can generate amplicons of a specific segment of DNA from only a small amount of starting material (i.e., DNA template or target sequence). During the PCR reaction in a thermocycler the cycles are repeated (denaturation, annealing, elongation), those involve heating and cooling for DNA melting and enzymatic replication of the DNA using thermostable DNA polymerase, primer sequence (oligonucleotides complementary to target region to be amplified) and deoxynucleotide triphosphates (dNTPs) in an appropriate buffer (Tris-HCl, $MgCl_2$, KCl, bovine serum, PCR-clear water). Most PCR methods can amplify DNA templates of up to ~10 kb pairs, although templates up to 40 kb can be amplified by using newer polymerases. This process of denaturation, annealing, and polymerase elongation is repeated cyclically (30-40 cycles). During each cycle, the number of copies increases exponentially (**Figure 2-12**). Classic PCR has an end-point detection using conventional gel electrophoresis with ethidium bromide staining/UV light or SYBR Green.[158, 159]

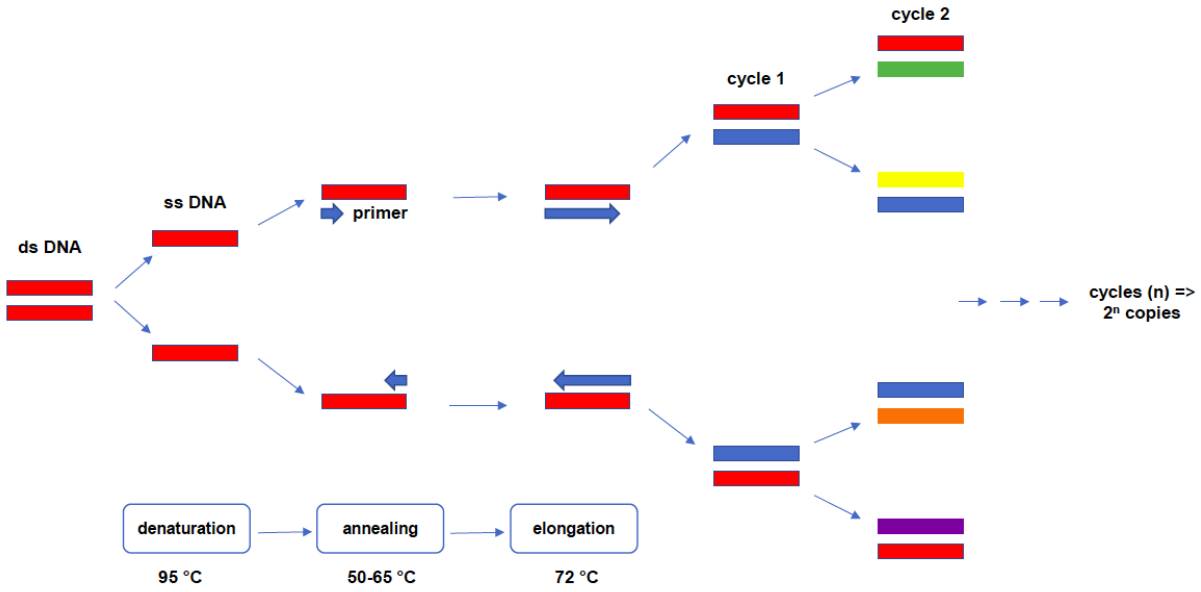



**Figure 2-12** Principle of the polymerase chain reaction (PCR).

PCR techniques are widely used in microbiology for pathogen identification, they are especially powerful for the detection of slow growing and fastidious microorganisms. But are also used for epidemiological investigations such as restriction analysis of chromosomal or plasmid DNA, detection of mutations or modifications of DNA, allelic variations, genetic fingerprinting, DNA cloning and last but not least for a nucleotide sequencing.[160]

**PCR-based DNA fingerprinting**

DNA fingerprinting is a set of methods based on the detection of polymorphisms of selected parts of genomic DNA. These techniques have been applied for typing of bacterial isolates to clonally related lineages (clones, strains) within a single species (intraspecies mapping), for identification of isolates of different species (interspecies mapping), for chromosome mapping, and also for taxonomic studies. Most genomic variations may be given by Single nucleotide polymorphisms (SNPs) which confers a single nucleotide base – point polymorphism. But it can also change the larger sections of the genome, the so-called repetitive sequences – Short tandem repeats (STR, also microsatellites) and Variable number tandem repeats (VNTR, also minisatellites). Often, these methods are using either only PCR or a combination of restriction endonucleases and PCR and can be associated with capillary electrophoresis, conventional gel electrophoresis, Southern hybridization or gene sequencing. The significant advantages of these typing methods are their discriminatory power and their universal applicability. On the contrary, they are laborious, they require a trained and skilled technician, and often a specific protocol must be used for each pathogen. the most suitable techniques for comparison of polymorphism and genetic variability will be discussed below (**2.2.4**).[161-164]

***PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)***

PCR-RFLP involves restriction analysis of PCR amplicons obtained by amplification by primers for specific sequences of interest. The technique exploits that SNPs, multi-nucleotide polymorphisms (MNPs) and microindels (deletions, duplications or their combinations involving the gain or loss of 1 to 50 nucleotides) are often associated with the creation or loss of a restriction enzyme recognition site resulting in a change in the length of the fragments. Amplicons are digested with an appropriate restriction enzyme. Resulting fragments are then electrophoretically separated and most often evaluated by patterns comparison which can be associated with Southern hybridization. It is an inexpensive method that does not require advanced instruments. Selection of REs can be difficult because several SNPs affect the same restriction enzyme recognition site which may result in a problematic identification of the exact genetic variation. Overall, this method is not suitable for processing a large number of samples with multiple types of SNPs.[11, 165, 166] **T-RFLP** (terminal restriction fragment length polymorphism) is a modification of PCR-RFLP that uses fluorescent-labeled primers for amplification of DNA under investigation. Resulting PCR products are digested by one or more REs, and later on the labeled fragments are separated by capillary electrophoresis or subjected to sequencing (**Figure 2-13**). In capillary electrophoresis, location and a number of peaks on the sample spectrum are recorded, including fluorescent intensity. This modification is more powerful with higher resolution, suitable for a larger number of samples than standard PCR-RFLP.[167, 168] Both methods are used for 16S rRNA genes mapping in microbial communities or for intraspecies differentiation.[169, 170]

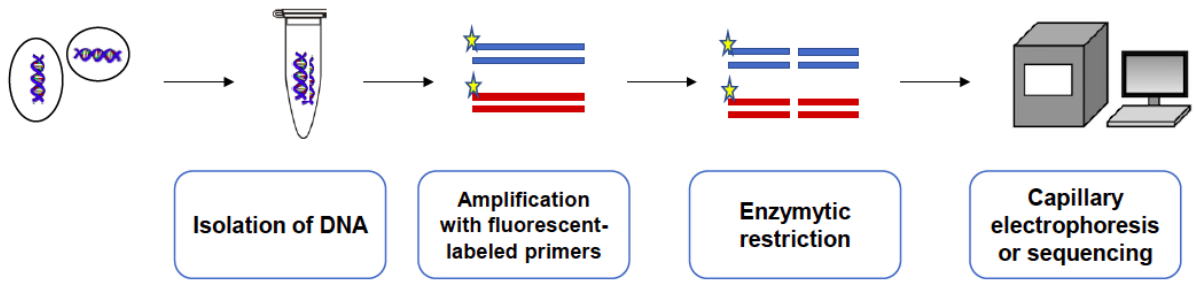



**Figure 2-13** T-RFLP principle.

***Amplified Fragment Length Polymorphism (AFLP)***

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique includes five following steps:

1) **Restriction** of the DNA by one or two specific restriction enzymes, most often *Eco*RI and *Mse*I endonucleases are used. A result is a large number of fragments having a cleavage site at one end of *Mse*I and second of *Eco*RI.

2) **Ligation of** oligonucleotide **adapters** complementary to the "cohesive ends" of cleaved fragments. T4 DNA ligase is used for ligation.

3) **Pre-selective amplification** of ligated fragments by primers complementary to adapters. The primer is always one nucleotide longer than the adapter, therefore only the fragments with complementary nucleotide are amplified resulting in amplification of about ¼ fragments.

4) Further reduction of the number of fragments occurs by **selective amplification** using primers longer by three nucleotides than adapters. It amplified about 1/256 of all fragments.

5) For the **evaluation** of band patterns, conventional gel electrophoresis may be employed or capillary electrophoresis if fluorescent-labeled primers are used (**Figure 2-14**).

The AFLP technique can detect various polymorphisms (mostly SNPs or microindels) in different genomic regions simultaneously. It is also highly sensitive, reproducible, with high discriminatory power and cannot be contaminated by various DNAs because of the use of specific restriction sides. AFLP is very applicable for investigation of a taxonomy of many bacterial strains allowing clustering into a dendrogram, and for some bacterial species, intra-center databases have been developed.[171–174]

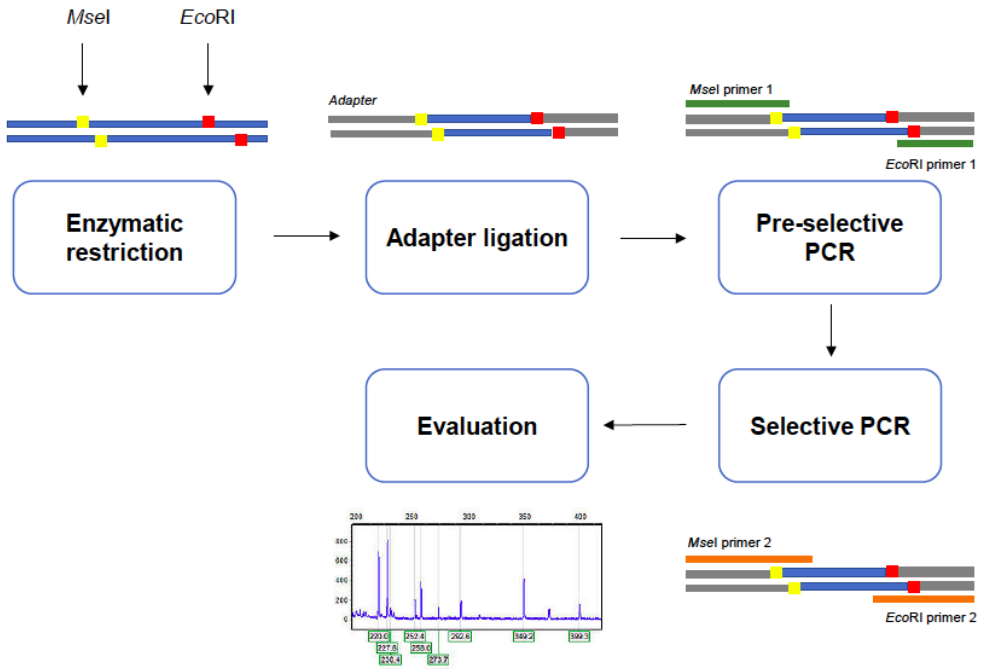



**Figure 2-14** AFLP principle.

***Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA)***

MLVA is genotyping method searching for **Variable Number of Tandem Repeats** (**VNTRs**, also **minisatellites**) in the genome. VNTRs are tandem repeat nucleotide units, most of them with a length from several bases to more than 100 base pairs. They occur more often in the non-coding part of the genome, but they can also be in the coding part, always in several copies. The number of repeats in a

tandem can be highly variable, even among strains of the same species. VNTRs tend to a high degree of mutation during replication, so the amplified fragments will vary in length depending on the number of repeats and given locus. MLVA is usually performed after performing of PFGE to get more details about a type of bacteria. After isolation of the DNA are VNTRs detected by PCR with specific primers that are complementary to the well conserved regions flanking the tandem repeats, subsequently are PCR products divided by size. The different sizes will tell how related the bacterial strains are to each other. Then the segments are loaded on capillary electrophoresis producing electropherogram (**Figure 2-15**).[11, 17, 19, 108, 175] The PCR products sizes are converted into allele types using specialized software, this determines how closely they are related. However, in case of large repeat units, the conventional gel electrophoresis can be even used. Several web-based MLVA databases and analysis platforms are currently in use, including MLVA-Net (Pasteur Institute, France), MLVA bank (University of Orsay, France), and PulseNet as a part of PulseNet International network (CDC, Atlanta GA, USA). MLVA allows to observe differences between bacteria with very similar PFGE pattern in more details during outbreaks. But it requires trained and skilled personnel and specific protocol for each microorganism.[175, 176]

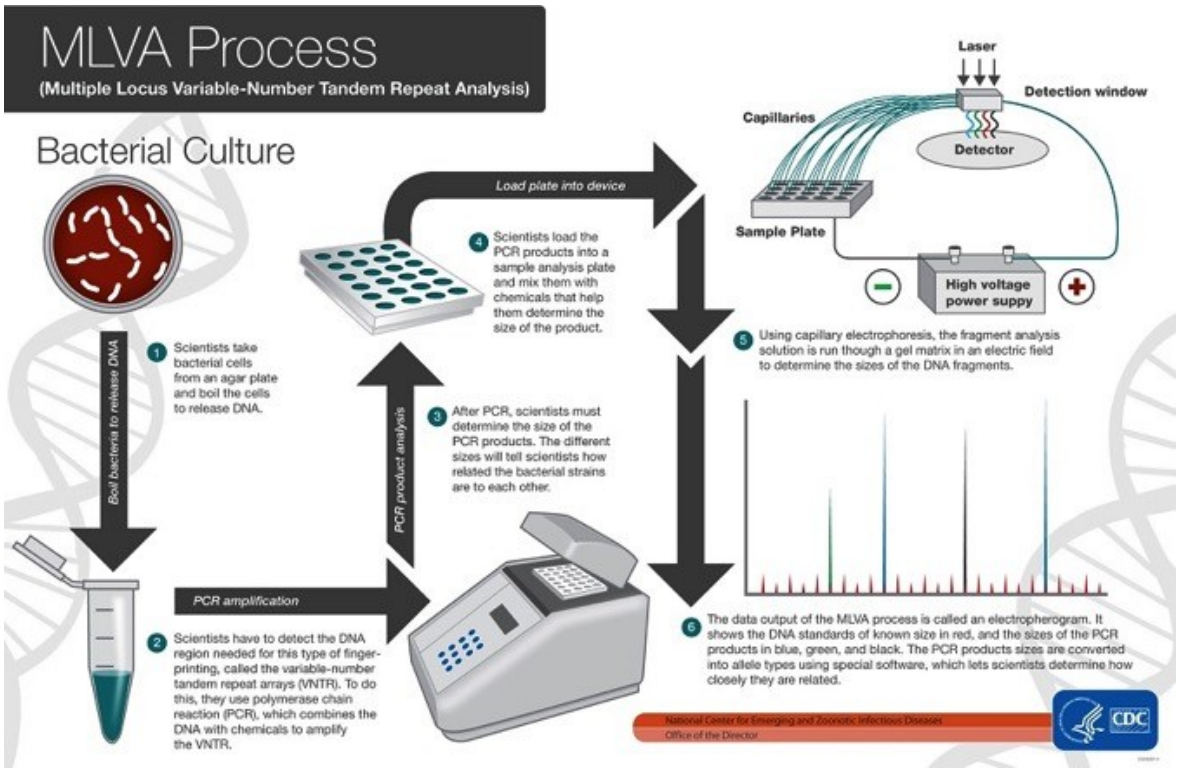



**Figure 2-15** MLVA process.[175]

In practice, for example, MLVA is used to distinguish individual isolates in the *Mycobacterium tuberculosis* complex. The method is based on PCR analysis of variable number of tandem repeated sequences (VNTRs) in the bacterial genome, where specific primers target the flanking sequences of

these tandem repeat regions (each around 15 bp). Variations in the number of repeats in each of the VNTR loci generate PCR amplicons of varying fragment length, and this size polymorphism forms the basis for the generation of the various allele. It is both robust and highly discriminatory method, used to characterize the circulating *M. tuberculosis* complex clones in various populations.[177-179]

In addition to these most commonly used methods, there are many others that are for detection of DNA polymorphisms. For example, **Random Amplified Polymorphic DNA (RAPD)** utilizes random 10-base oligonucleotides as primers to amplify fragments of genomic DNA by PCR. RAPD has been extensively used in the study of genetic diversity, taxonomy investigation, or identification of serotypes. Its disadvantage is the inability to distinguish clonally related and unrelated strains, dependence on specific conditions and high quality of the DNA, and the need to optimize the protocol depending on the strain.[180, 181] Similar method is **Arbitrarily Primed PCR (AP-PCR)** using arbitrary primers for amplification in three parts, each with its own stringency, temperature, and concentration of components. Like the RAPD, it is challenging to optimize conditions depending on the strain tested. The resulting band pattern is related to used primers.[182, 183] And last but not least, **Short Tandem Repeat (STR),** so-called **microsatellites** with length from one to six nucleotides, analyzes how many times base pairs repeat themselves on a particular location on a strand of DNA. These tandem repeats are highly polymorphic and mainly contribute to genetic variability.[184]

### Sequence-based approaches

#### *Single-Locus Sequence Typing (SLST)*

SLST is sequence-based typing method used to determine the relationships among bacterial isolates based on the comparison of sequence variations in a single target gene. It is essential to select gene sequences that are (highly) variable.[185] A typical example is the typing of strains among Group A β-hemolytic streptococci using M protein, the so-called *emm* gene, which encodes an important surface antigen and virulent factor. It is mostly used for typing *S. pyogenes*, and sequenation is able to distinguish more than 225 different serovars.[186, 187] Evaluation of *emm* type can be processed using specific databases, such as StrepTyping Database established by the Centers for Disease Control and Prevention (CDC, Atlanta GA, USA) or using BLAST.[188] For typing of methicillin-resistant *Staphylococcus aureus* (MRSA) *spa* gene coding of a cell wall component Protein A is widely used, which exhibits a high degree of polymorphism.[189] Likewise, the *fla*B gene encoding the flagellin B subunit may be used in the *Campylobacter* epidemiology or the *por*B gene of an outer membrane protein for *Neisseria gonorrhoeæ* typing.[190, 191]

#### *Multi-Locus Sequence Typing (MLST)*

MLST is a technique using DNA sequencing to uncover allelic variants in several conserved genes. Since genes are subject to varying degrees of genetic drift, housekeeping genes are most often sequenced because they are present in all isolates within a species and they are not under too strong selective pressure. Therefore, MLST employs the sequencing and analysis of 400 bp to 500 bp fragments of

multiple housekeeping genes, typically seven, that encode essential function and metabolic pathways in bacterial cell.[9] For example, the MLST typing scheme for *Klebsiella pneumoniæ* includes seven genes which are essential to energetic metabolism, transmembrane transport and synthesis of new nucleotides: *rpo*B (β-subunit of RNA polymerase), *gap*A (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *pho*E (phosphorine E), *inf*B (translation initiation factor 2), *ton*B (periplasmic energy transducer).[192] These genes are amplified by PCR and specifically designed primers, and subsequently sequenced, typically using capillary Sanger sequencing. The data are then assembled and compared with online database of known sequence variants. Based on this, each allele is assigned an allelic group number based on the perfect match against the database. By combining all allelic groups of housekeeping genes, the sequence type (ST) of the given strain is established. There are three basic databases, the PubMLST.org (Oxford University, UK), the Pasteur MLST Institute (Pasteur Institute, France) and the MLST.net (Imperial College, UK), enabling worldwide comparison and sharing of STs and support submission of the raw sequence data files from the laboratory when a new allele type is propounded. Analogous schemes have been developed for many clinically important bacterial species. MLST is highly portable and reproducible global standard for many bacterial pathogens, nevertheless it has drawbacks. MLST is not very suitable for most outbreak investigations and routine surveillance application. Moreover, it is a rather laborious, expensive and time-consuming method.[11, 17, 193, 194]

### 2.2.4 Sequencing methods

The development of DNA sequencing technologies has a rich history, with multiple shifts occurring within a few decades (**Figure 2-16**). The effort to know the composition of DNA, whether ours or foreign, has been here since its discovery and first isolation in 1870 by Friedrich Miescher. The spatial arrangement of the DNA molecule was first revealed by Watson and Crick[195] in 1953 when they described its double-helical structure. However, the ability to "read" or sequence DNA did not follow for some time. During the 1950s and 1960s, scientists attempted to sequence a ribosomal or transport RNA, the genomes of single-stranded RNA bacteriophages, and also proteins. RNA species were first fragmented with RNases, subsequently were separated by chromatography and electrophoresis, then all fragments were decoded by sequential exonuclease digestion, and finally, the sequence was deduced from overlapping.[196-198] In 1965 Robert Holley *et al.*[199] gained the first whole nucleic acid sequence of alanine transfer RNA from *Saccharomyces cerevisiæ*. The first two widely-known methods for DNA sequencing appeared in 1977. One, based on chemical degradation of DNA, was published by Maxam and Gilbert[200]. And the second, known as "Sanger sequencing" using DNA synthesis and chain-terminating inhibitors, was published half a year later by Sanger and colleagues.[201] Another major milestone in the development of sequencing methods was the discovery of "pyrosequencing" by Ronaghi *et al.*[202] in 1996 using an enzymatic reaction producing luminescence, which is then detected. In the last ten years, development was very fast, gradually completing the process of automatization. The whole-genome sequencing and new techniques were developed, and overall, the method became more accessible, cheaper and quicker.

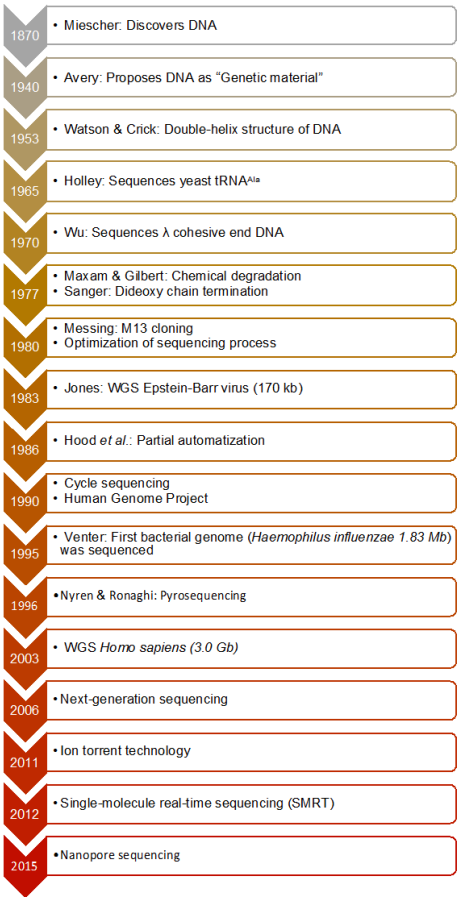



**Figure 2-16** Timeline of development of sequencing methods.

### 2.2.5 An overview of the most important sequencing methods

#### First generation sequencing

***Sanger method***

As mentioned above, after publishing of Maxam and Gilbert sequencing method[200] was developed faster, a more efficient technique to sequence DNA by Frederick Sanger and his colleagues.[201] Indeed, Sanger's work in this area was so groundbreaking that it led to his receipt of the Nobel Prize in Chemistry in 1980. First Sanger team's version of this sequencing method was based on "plus and minus" sequencing[203] and was eventually used for almost complete sequenation of the genome of bacteriophage ΦX174.[204] Later that year, simpler and more rapid "chain terminator" sequencing method was published, and, shortly after, the complete sequence of 5.368 bp long ΦX174 genome was determined, representing the first DNA genome ever completely sequenced. The method is useful for sequencing a short single-stranded DNA sequence, and it basically uses the biological process of DNA replication. The selected sequence is inserted into a reaction mixture containing a suitable radiolabeled primer (α-P$^{32}$-dATP), a DNA polymerase (Taq or T7 polymerase), a supply of four essential deoxyribonucleotides (dATP, dGTP, dCTP, dTTP) and one of four dideoxynucleotides (ddATP, ddGTP, ddCTP, ddTTP). The

mixture is first heated to denature the template DNA, then cooled so that the primer can bind to the single-stranded template. Subsequently, the temperature is raised again, allowing DNA polymerase to synthesize new DNA starting from the primer. DNA polymerase will continue adding nucleotides to the strand until adding a dideoxynucleotide instead of a normal one. At that point, no further nucleotides can be added, since the dideoxynucleotide has no OH-group for further binding. This process is repeated in a number of cycles, and it gives rise to a mixture of different lengths of dideoxynucleotide-terminated fragments. Earlier, the sequencing was then evaluated by gel electrophoresis. At present, the entire process is automatized, and the reaction does not take place in separate tubes for individual dideoxynucleotides, but in one mixture in very small volumes (about 10-20 µl). Labeling also does not use radioisotopes, but most commonly fluorescents, which may indicate either dideoxynucleotide or primer. The mixture after sequenation is subjected to capillary electrophoresis in which the fragments pass through a very thin capillary (in µm), from the smallest to the largest fragments. During this time, the capillary is illuminated by a laser, thereby the fluorescents are exciting, and the resulting light is captured by a detector. The data recorded by the detector consist of a series of peaks in fluorescence intensity creating a sequence chromatogram (**Figure 2-17**). Sanger sequencing gives high-quality sequence for relatively long chains of DNA (up to about 900 bp). It's typically used to sequence individual pieces of DNA, such as bacterial plasmids or DNA copied in PCR. However, this technique is expensive and inefficient for larger-scale projects, such as the sequencing of an entire genome. At present, the most prominent and most advanced devices operating on Sanger's principle are Applied Biosystems (ABI, Foster City CA, USA) machines.[196-198, 205-207]

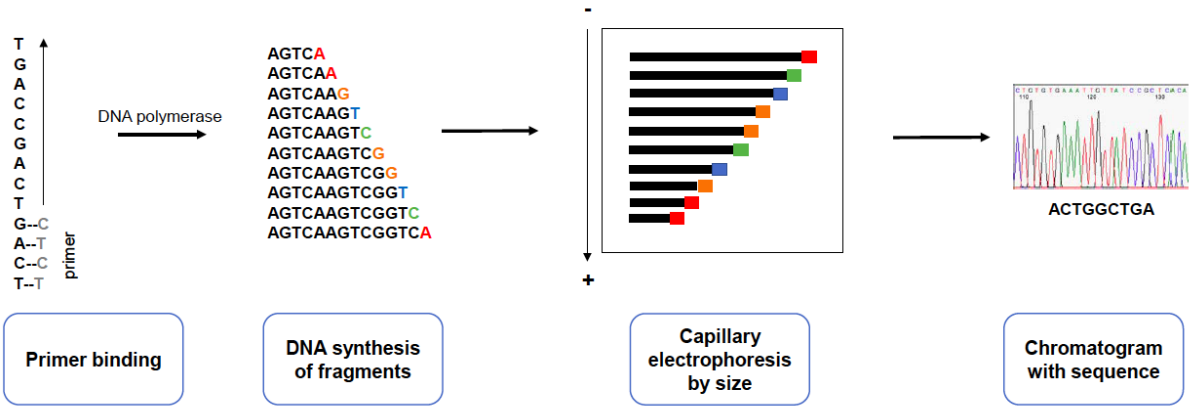



**Figure 2-17** Sanger sequencing principle. *Adapted by Mayhall, G.*[208]

## Second generation sequencing

### *Pyrosequencing*

Probably the most successful non-Sanger method developed is pyrosequencing, first described in the literature by Hyman in 1988.[209] But it was put into practice a few years later by Nyrèn and his student

Ronaghi in Stockholm in 1996.[202] Pyrosequencing is a non-fluorescence technique that measures the release of inorganic pyrophosphate, which is proportionally converted into visible light by a series of enzymatic reactions. The process is based on the work of four enzymes – DNA polymerase, ATP-sulfurylase, luciferase, and apyrase. The reaction mixture further comprises enzyme substrates – adenosine phosphosulfate (APS), luciferin, and a sequenced template with appropriate primers on which the DNA polymerase can begin synthesis and of course, one of the deoxyribonucleotides (dATP, dGTP, dCTP, dTTP). During incorporation of deoxyribonucleotide (dNTP) releases pyrophosphate (PPi) which is in the presence of ATP-sulfurylase and adenosine 5'-phosphosulfate (APS) converted to ATP. Luciferase with ATP mediates conversion of luciferin to oxyluciferin, which generates a light signal (**Figure 2-18**). The intensity of the light determines if zero, one or more deoxyribonucleotides have been incorporated, thus showing how many complementary nucleotides are present on the template strand. The resulting light was detected by the detector (CCD camera), and the signal was transferred to the computer generating pyrogram. After that, the nucleotide mixture had removed before the next nucleotide mixture was added. This process was repeated with each of the four nucleotides until the DNA sequence of the single stranded template is determined. But this procedure was laborious, time-consuming and unsuitable for automation. That is why the same team two years later added the enzyme apyrase to the process. Apyrase removed all deoxyribonucleotides that were not incorporated by the DNA polymerase into the chain (**Figure 2-18**).[197, 198] Thanks to this step, the whole process could be automated. This technique is widely applicable, alternative approach for the detailed characterization of nucleic acids. Pyrosequencing has potential advantages, such as accuracy, flexibility, and parallel processing. Furthermore, the technique avoids the need for labeled primers, labeled nucleotides, and gel electrophoresis. Pyrosequencing has been successful for both confirmatory sequencing and *de novo* sequencing.[210]

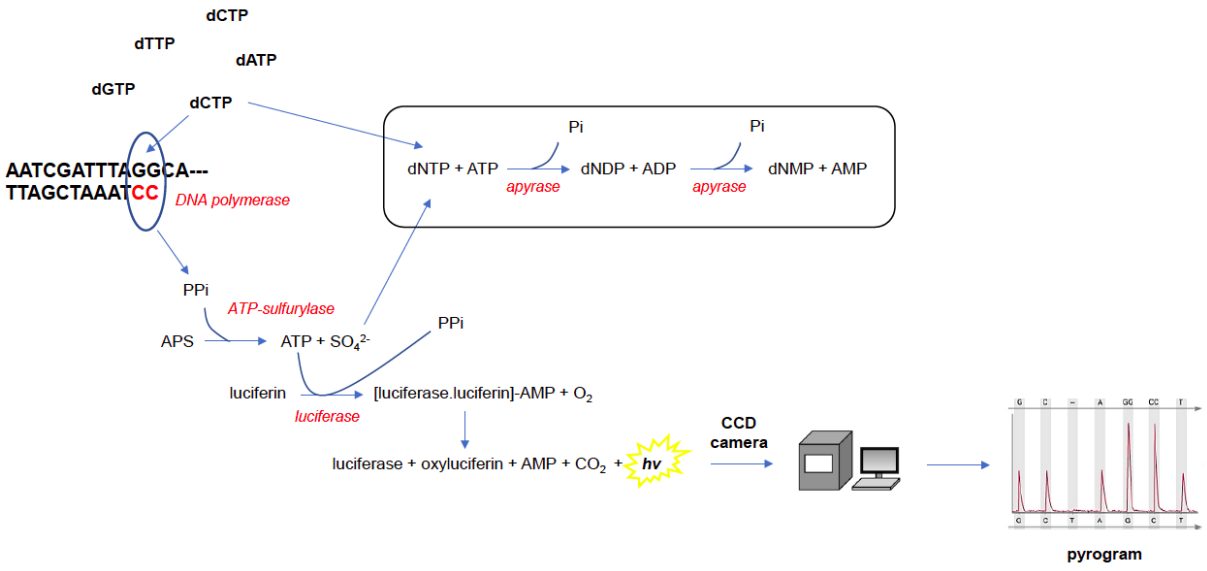



**Figure 2-18** Pyrosequencing principle. *Adapted by Fakruddin, M. et al.*[210]

Pyrosequencing was in 2005 licensed to **454 Life Sciences** (Branford CT, USA), a biotechnology company founded by Jonathan Rothberg, and later in 2007 purchased to Roche (Basel, Switzerland). This platform uses a method of pyrosequencing of fragments that are prepared by emulsion PCR (emPCR). In the first step, DNA is fragmented into 300-800 bp fragments. At the ends of the generated fragments, two types of adapters are attached, with the aim that each fragment carries one adapter at one end and a second adapter at the other end. Fragments that do not carry both adapters are removed by washing. Using an adapter sequence, the fragments are immobilized on special beads in such a way that each bead carries one fragment. Then, beads are mixed with water/oil emulsion, containing PCR reagents and primers complementary to adapters. This mixture is then subjected to thermal cycling, which leads to clonal amplification of the fragments bound to the beads. After PCR run, the beads are removed from the emulsion and individually placed into the wells of the PCR plate (PicoTiterPlate) together with the DNA polymerase, ATP-sulfurylase, and luciferase. Subsequently, all is done by common pyrosequencing with luminescence detection by CCD camera as was described above. Because the platform became uncompetitive, it was canceled by Roche in 2013.[206, 210, 211]

The **Illumina Genome Analyzer** (Illumina, San Diego CA, USA), introduced in 2006, is based on the concept of "sequencing by synthesis" (SBS) to produce sequence reads of ~32-40 bp from tens of millions of surface-amplified DNA fragments simultaneously. In the first step, DNA is fragmented into 200 bp sections, their ends aligned, phosphorylated, and adenylated at the 3'end. At the ends of the generated fragments, two types of adapters are attached so that each fragment carries one adapter at one end and a second adapter at the other end. After that are fragments denatured and, as single-chain molecules, are attached to single-stranded oligonucleotides that are bound to an optically transparent surface of the reaction cell, so-called "flow cell". These oligonucleotides are complementary to the adapters used and serve as primers in the subsequent amplification. The next step is solid-phase bridge amplification producing up to 1000 identical copies of every single template arranged in the clusters (**Figure 2-19**). Then DNA polymerase and four differentially labeled fluorescent nucleotides are added those have their 3'-hydroxyl group chemically inactivated to ensure that only a single base is incorporated per cycle. Each base incorporation cycle is followed by an imaging step to identify the incorporated nucleotide by a chemical step that removes the fluorescent group and deblocks the 3'-end for the next base incorporation cycle. After finishing the sequencing run, the sequence of each cluster is subjected to quality filtering to eliminate low-quality reads and is analyzed.[212-214]

At present, Illumina offers several devices with different performance. MiSeq and MiSeqDx are low-capacity sequencers designed for sequencing of small genomes, amplicon sequencing and targeted sequencing of selected genome regions. Because of its reliability, MiSeqDx is recommended for in vitro molecular diagnostics. Lines NextSeq and NovaSeq are high capacity table sequencers suitable for analysis of whole genomes, exons, and transcripts.[215]

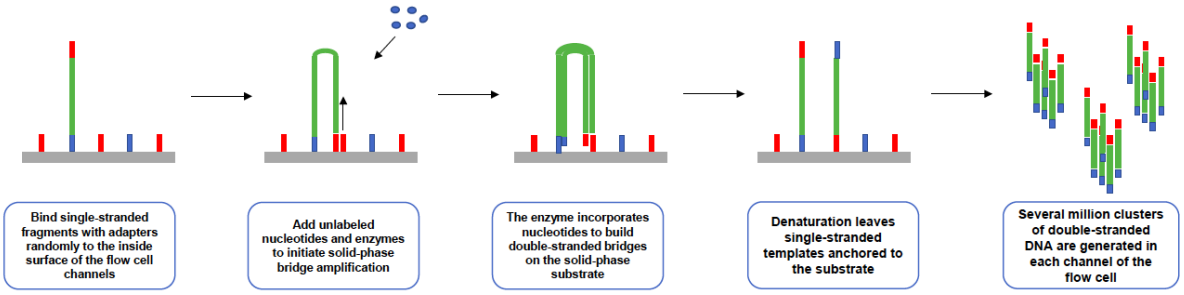



**Figure 2-19** Principle of bridge (cluster) PCR. *Adapted by Illumina Inc.*[212]

## Next (third)-generation sequencing (NGS)

### *Applied Biosystems SOLiD sequencer*

This instrument that achieved commercial release by Life Technologies (Carlsbad CA, USA) in October 2007 is based on the principle of sequencing-by-ligation (SBL) using DNA ligase. In the first step, the DNA is fragmented into 60-90 bp long sections, then a 25 bp adapter P1 is attached to the 5'-end and, a P2 adapter of the same length is attached at the 3'-end. The adapter-attached molecules are then bound to 1 µm magnetic beads on which the emulsion PCR is performed as described above at 454 sequencing. After amplification, the beads are covalently attached to the surface of a specially treated glass slide or FlowChip(s) that is placed into a fluidics cassette within the sequencer. Later on the primer is hybridized to the adapter, and a mixture of oligonucleotide octamers is bound. These octamers include pairs of fourth and fifth bases that are labeled by one of four fluorescent dyes at the end of the octamer. A set of fluorescently labeled two-phase probes competes for ligation to the sequencing primer. After fluorescence detection of labeled bases, the octamers are cleaved at the fifth base, and the fluorescence is removed. Then the ligation process can be repeated. In the second round, sequences nine and ten are determined, in the third, fourteenth and fifteenth sequences, etc. The number of ligation cycles, detection of fluorescence and cleavage of ligated probes depends on the required length of the sequence to be read. Because each position in the sequence is characterized by two fluorescence signals, the reliability of base determination is enhanced. Once the required length is reached, the newly created chain is removed, and the whole process is repeated with a new sequential primer always shorter by one dNTP (n-2, n-3 and n-4). Five rounds of primer reset are completed for each sequence tag. Through the primer reset process, virtually every base is interrogated in two independent ligation reactions by two different primers. According to ABI, the newest SOLiD® 3plus platform yields 60 Gb of usable DNA data per run. Due to the two base encoding system, an inherent accuracy check is built into the technology and offers 99.94% accuracy.[213, 214, 216, 217]

### *Ion Torrent Sequencing*

Ion Torrent technology is another product of Life Technologies (Carlsbad CA, USA) released in 2010. This method is based on "sequencing by synthesis" (SBS), but it does not detect fluorescence or luminescence, but it is based on the detection of hydrogen ions that are released during the

polymerization of DNA. Sequencing DNA is, as with 454 Roche, fragmented and subsequently amplified with emulsion PCR. Own sequencing does not take place in the flow cell, but it utilizes a semiconductor chip with microwells. A clonally amplified target molecules are bind on microbeads that are individually located in the wells of the chip. The chip is then situated within the flow cell and individual dNTPs are sequentially added. Integration of nucleotide releases $H^+$ that changes the pH of the surrounding solution proportional to the number of incorporated nucleotides. The latest version of the instrument (Ion Chef System) is claimed to have throughput more than 20 Gb pairs, with reads of 400 bp, and runtime up 4 hours. This technology may be best suited to small-scale applications such as microbial genome sequencing, microbial transcriptome sequencing, targeted sequencing, amplicon sequencing, or for quality testing of sequencing libraries.[218, 219]

***Nanopore sequencing***

When using the nanopore sequencing, a single molecule of DNA or RNA can be sequenced without the need for PCR amplification or chemical labeling of the sample. Nanopores are formed by pore-forming proteins, such as different kinds of biological channels, e.g., α-hemolysin of *Staphylococcus aureus*, or MspA of *Mycobacterium smegmatis*. Solid-state nanopore technology can be as well used with various metal or metal alloy substrates with nanometer-sized pores ($10^{-9}$ nm) that allow DNA or RNA to pass through. These substrates most often play integral roles in the sequence recognition of nucleic acids as they translocate through the channels along the substrates. Single-stranded DNA or RNA passes through the pore by electrophoresis blocking ion flow, decreasing the current for a length of time proportional to the length of the nucleic acid. Solid-state nanopores can also be used for double-stranded DNA molecules.[220, 221] The first platform working on this principle was introduced in 2014 by Oxford Nanopore Technologies (Oxford, UK) – GridION™ and the latest mobile phone sized USB device MinION™. In the MinION™, the manufacturer provides less than 10 minutes of preparation before proper sequencing without the need of PCR. It also provides a variable reading length dependent on the previous library preparation and throughput 10 to 20 Gb pairs per 48 hours.[222] It is intended for pathogen analysis, environmental research, *de novo* reads, now it is expanding for larger genomes and datasets. Also, it have already been used for human genome sequencing.[223]

***Single Molecule Real-Time Sequencing (SMRT)***

Currently, one of the most widely used third-generation technology is probably the single molecule real-time (SMRT) platform from Pacific Biosciences (Menlo Park CA, USA). At present, PacBio offers two devices – PacBio RS II, and newer Sequel – working on this platform, designed for long-read sequencing. PacBio machines are capable of producing incredibly long reads, 10-15 kb in length, which are useful for de novo genome assemblies. With high throughput up to 10 Gb pairs per cell, runtime flexibility from 30 minutes to 20 hours per SMRT cell, and accuracy 99.99%.[224] SMRT technology utilizes zero-mode waveguides (ZMWs), microfabricated nanostructures which are essentially tiny holes (~70 nm in diameter and ~100 nm in depth) in a metallic film covering a chip. Inside each ZMW, a single active DNA polymerase with a single molecule of single-stranded DNA template is immobilized to the

bottom of the well through which light can penetrate and create a visualization chamber that allows monitoring of the activity of the DNA polymerase and incorporating of nucleotides at a single molecule level. Each of the four DNA bases is labeled by one of four different fluorescent dyes. When a nucleotide is incorporated by the DNA polymerase, the fluorescent tag is cleaved off and diffuses out of the observation area of the ZMW where its fluorescence is no longer observable. A detector detects the fluorescent signal of the nucleotide incorporation, and the base call is made according to the corresponding fluorescence of the dye.[219, 225, 226] The data is generated by so-called "contigs" – different lengths of DNA fragments containing only of A, C, T and G bases without gaps. Ultra-log contigs provide complete and uninterrupted sequence information across full genes or chromosomes. These contigs can be chained together using additional information about the relative position and orientation of the contigs in the genome, which creates "scaffold".[227]

SMRT technology may be applicable for a broad range of genomics research. Primarily, it is intended for long reads, especially for *de novo* assemblies of novel genomes. SMRT sequencing is also able to sequence genomic regions with extremely high GC contents with 100% accuracy because other techniques have poor quality of sequencing in this case.[228] Another advantage of SMRT sequencing is the direct detection of DNA modifications, such as methylation.[229]

The development of Sanger sequencing 41 years ago has revolutionized biological research. The implications of this technique became even more far reaching with the introduction of NGS methods in 2008. These techniques have significantly increased their accessibility, the amount and quality of sequencing data produced per instrument and have dramatically decreased the costs of generating sequencing data. The following **Table 2-4** compares the most important sequencing techniques and platforms, ranging from Sanger to the latest technologies such as SMRT sequencing.

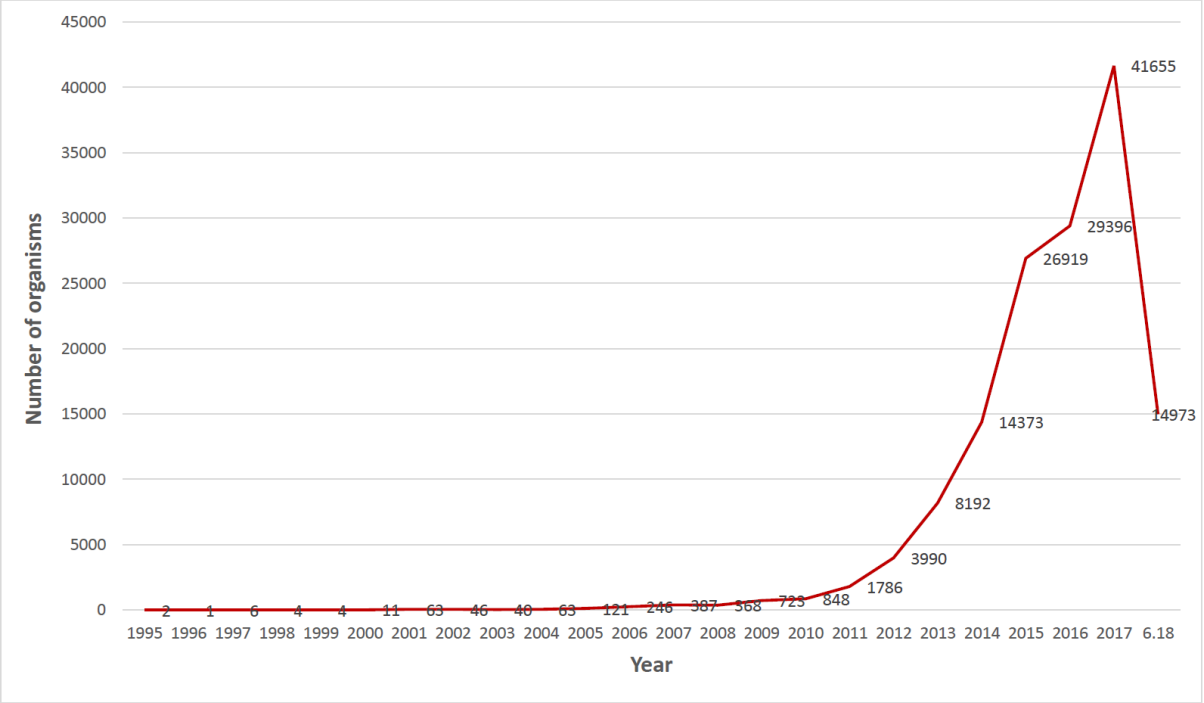
**Table 2-4** Comparison of available sequencing techniques. *Adapted by Prasad, M. et al.*[230] *and Mardis, E. et al.*[231]

| | Ion Torrent | 454 Roche | Illumina | SOLiD | PacBio | Helicos | Sanger method |
|---|---|---|---|---|---|---|---|
| **Sequencing chemistry** | detection of released H+ | pyrosequencing | SBS; reversible terminators | ligation | SMRT; fluorescently labeled dNTPs | reversible terminators | dideoxy-chain termination |
| **Adapter used** | adapters | adapters | adapters | adapters | hairpin adapters | poly(A) adapters | specific primers |
| **Amplification method** | emulsion PCR | emulsion PCR | bridge amplification *in situ* | emulsion PCR | linear amplification | no amplification | sequencing PCR |
| **Separation method** | ion spheres and high-density array | microbeads and "picotitre" plate | glass slide hybridization | beads on glass slide | captured by DNA polymerase in microcell | flow cell hybridization | capillary electrophoresis |
| **Read Length** | 200–400 bp | 700 bp | 50–250 bp | 50–75 bp | 1000 bp | 25 bp | 400–900 bp |
| **Reads per run** | up to 5 million | 1 million | up to 3 billion | 1.2–1.4 billion | 35–75 thousand | 1 billion | 35–75 thousand |
| **Max. output per run** | 1 Gb | 700 Mb | 600 Gb | 20 Gb | 20 Gb | 35 Gb | NA |
| **Accuracy** | 98% | 99.9% | 98% | 99.9% | 99.9% | 99% | 99.9% |
| **Time per run** | 2 hours | 24 hours | 1–10 days | 1–2 weeks | 30 min to 20 hours | 5–10 days | 2 hours |
| **Cost per 1 mil. bases (in US$)** | $1 | $10 | $0.05–0.15 | $0.13 | $2 | NA | $2400 |
| **Advantages** | relatively low-cost equipment; fast reaction | long read length; fast reaction | high sequence yield | low cost per base of sequencing | longest read length | PCR is not necessary, tolerates degraded samples | long individual reads |
| **Disadvantages** | homopolymer error | homopolymer error; relatively expensive run | needs high DNA concentration; very expensive equipment | slower than other sequencing methods | equipment very expensive | time to sequence single nucleotide is too high; high error rate | high cost per base; unsuitable for WGS |
| **Application** | Multiplex-PCR products, microbiology and infectious diseases, mutation detection, point mutation | bacterial and viral genomes, multiplex-PCR products, validation of point mutation, targeted mutation detection | WGS (prokaryotes, eukaryotes, human), RNA-seq, multiplex-PCR products, mutation detection, forensics, prenatal testing | WGS (prokaryotes, eukaryotes, human), RNA-seq, multiplex-PCR products, mutation detection | WGS (prokaryotes, eukaryotes, human), microbiology and infection disease genomes, methylation detection | Multiplex-PCR products, microbiology and infectious diseases, mutation detection, point mutation | gene sequenation, point mutation |

### Whole-Genome Sequencing (WGS)

For a few decades, a number of methods have been used for taxonomic purposes, comparison of strains in an outbreak or other epidemiological investigations. First, phenotypic typing methods and typing methods for genotype analysis were used, such as simpler electrophoretic and restriction methods, later on approaches based on PCR amplification were exploited. Sequencing procedures began to be involved from the 1980s, particularly for sequencing individual genes and revealing possible point mutations, and from 1995 whole genome sequencing became also available. Currently, a combination of all these approaches is largely used, thanks to rapid technology development, sequence throughput, data quality, and decreasing per-isolate cost. The genomic sequencing era may be divided into two periods. In the first decade, from 1995, when the first bacterial WGS of the *Hæmophilus influenzæ* genome[232] was performed to 2005 when the sequencing was relying on the classic Sanger method and was time- and money-consuming. Fewer than 300 bacterial genomes were sequenced during this period as shows **Graph 2-1**. Since 2005, the development of new and high output sequencing methods, and subsequently even NGS, together with a steep decrease of the cost of sequencing equipment and appropriate reagents enabling many laboratories to develop their own sequencing projects, led to a very sharp increase in the number of sequenced genomes. For example, the number of the whole genome sequenced prokaryotes is approaching 41 644 for the year 2017 alone (**Graph 2-1**). Today, major platforms in this sphere include Illumina, Life Technologies IonTorrent, and Pacific BioSciences as described above. The greatest challenge to the use of WGS for outbreak investigations lies in the massive volume that is generated, often around 50 to 100 Gb of raw sequence, which should be assembled, processed and evaluated

resulting in a higher need for bioinformatics both in research and in clinical microbiology laboratories.[233-235]




**Graph 2-1** Number of bacterial and archaeal genomes sequenced each year and submitted to NCBI in the period from 1995 to June 2018. Source: GenBank prokaryotes.txt file downloaded 21 June 2018.[236]

### 2.2.6 DNA Microarrays

The effort and the ability to immobilize thousands of DNA fragments on a surface, such as coated glass slide or membrane, have led to the development of DNA microarray technology. Simply defined, a microarray is a collection mostly of DNA or RNA which can be probed with target molecules to produce either quantitative (gene expression) or qualitative (diagnostic) data. Specially treated microscope slides or chips are carrying an order mosaic of single-stranded sequences representing most or all of the genes of an organism. A single-stranded fluorescent-labeled DNA/RNA sample obtained by isolation or PCR amplification is then added. If the tested DNA is complementary to the probe, it is established and then the result can be visualized (**Figure 2-20**). In contrast to Southern blotting, where a limited number of probes are attached onto nitrocellulose or nylon membrane, a DNA microarray includes tens of thousands of probes arrayed directly on the chip, and it therefore, represents a high-output genotyping method. There are two types of DNA microarrays which differ in the size of the probes used: *(i)* A cDNA microarray uses cDNA as probes and is often used to identify the presence or absence of a gene; *(ii)* An oligonucleotide microarray, on the other hand, uses short oligonucleotides as probes and is usually used to identify SNPs. Oligonucleotide microarrays are more discriminatory than cDNA microarray due to the use of shorter probes.[139, 237-239]

Currently, DNA microarray technology is being used to study many bacterial species ranging from standard identification of laboratory strains and pathogens to environmental isolates. Many companies also produce automated microarray tests in the form of panels for the detection of pathogens or resistance mechanisms from a number of clinical specimens. For example, FilmArray® Blood Culture Identification Panel (BioMèrieux, France) offers a potential tool for detection more than 25 pathogens and 4 antibiotic resistance genes in one hour with high specificity.[240] In other study have been compared this FilmArray® Blood Panel with an analogous panel from Verigene® Bloodstream Infection Tests (Luminex, The Netherlands). They processed 118 monobacterial positive blood cultures by both panels with 92% of accurately identified bacteria.[241]

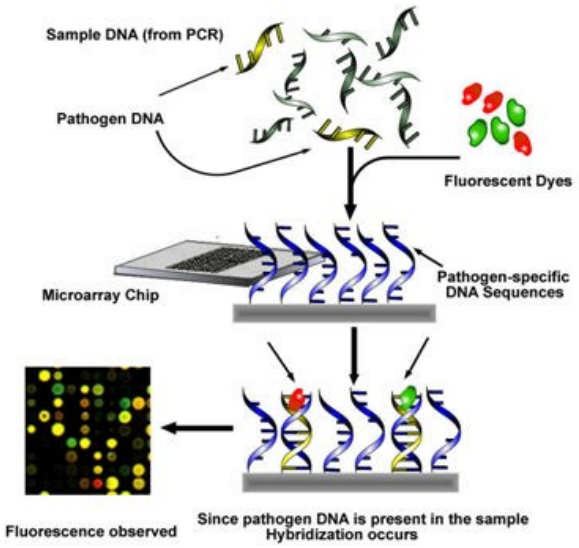



**Figure 2-20** Principle of DNA microarray process.[242]

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## 3 Hypothesis

- The development of antibiotic resistance of Gram-negative bacteria and their spread in healthcare facilities, where they can cause both short- and long-term outbreaks of varying degrees, is a major problem for hospitals in recent years.
- It is essential to detect these pathogens in a timely manner, to correctly identify them, to understand the relationships between the isolates and, in particular, to find out their source and the way of spreading.
- A wide variety of bacterial typing systems are currently in use that vary greatly with respect to the effort required, the cost, the reliability and the ability to discriminate among bacterial strains. No one technique is optimal for all forms of investigation.
- The choice of typing technique is crucial for increasing our understanding of the pathogenesis and transmission, and eventual disease prevention.

## 4 Aim of the study

- Development and validation of standardized method for a robotic deposition of bacterial or yeast samples on MALDI target.
- Epidemiological investigation of a series of outbreaks caused by carbapenemase-producing *Enterobacteriaceæ* in Czech hospitals, using a wide range of bacterial typing methods, ranging from basic phenotypic approaches to whole-genome sequencing.

# 5 Summary of the methodology

In the first study for the development and validation of a robot for automatic deposition several types of manual sample deposition were used, whether classic dry spotting or modified "wet deposition" into a droplet of 70% formic acid. Subsequently, spectra acquisition was performed on MicroFlex LT mass spectrometer (Bruker Daltonik, Bremen, Germany) using MALDI Biotyper software version 3.1. The following four publications focus on the epidemiology of several of the most common carbapenemases in the Czech Republic (KPC, OXA-48, NDM, and IMP), either within hospital outbreaks or their occurring across the country. In particular, we focused on detecting the possible clonal spread and tracking the path and source of their transmission. All isolates were typified by a number of methods. Not only common phenotyping procedures, such as the determination of minimal inhibitory concentration (MIC) of antibiotics, but also the MALDI-TOF mass spectrometry have been used either to identify pathogens or to determine their hydrolytic activity to meropenem. Additionally, PCR-based genotyping procedures, such as MLST, integron mapping, replicon typing, or detection of specific resistance genes have been used. Also, the ability to transfer plasmids via conjugation or transformation was also monitored. The obtained transconjugants/transformants were subjected to S1 plasmid size profiling by PFGE, followed by Southern blotting for the detection of resistance probes with labeled probes. Finally, whole-genome sequencing was performed to verify and complete the obtained data. The following **Table 5-1** is a summary of all methods used in this dissertation thesis, always referring to the relevant chapter where they are described in detail.

**Table 5-1** A summary of the methods used in this dissertation thesis.

| Method used | Number of the chapter |
|---|---|
| MALDI-TOF MS identification of bacteria/fungi[1] | 6, 7, 8, 9, 10 |
| MALDI Colonyst deposition of bacteria/fungi | 6 |
| On-plate semi-extraction of bacteria/fungi | 6 |
| Wet deposition of bacteria/fungi | 6 |
| MALDITOF MS meropenem hydrolysis assay | 7, 8, 9 |
| Phenotypic testing using inhibitors of carbapenemases[2] | 7, 8, 9 |
| PCR confirmation of carbapenemases using specific primers | 7, 8, 9, 10 |
| Sanger sequencing of individual genes | 7, 8, 9, 10 |
| Determination of minimal inhibitory concentration (MIC) of antibacterial agents by the broth dilution method[3] | 7, 8, 9, 10 |
| Multilocus sequence typing (MLST)[4-9] | 7, 8, 9, 10 |
| Conjugation of plasmids into *Escherichia coli* A15 cells | 7, 8, 9 |
| Transformation of plasmids into recipient *E. coli* DH5α cells | 7, 8, 9, 10 |
| Plasmid isolation | 7, 8, 9, 10 |
| S1 plasmid size profiling by PFGE | 7, 8, 9 |
| Southern blotting and hybridization with digoxigenin-labeled probe | 7, 8, 9 |
| PCR-based replicon typing (PBRT) method[10] | 7, 8, 9 |
| Preparing of a library for WGS | 7, 8, 9, 10 |
| Closing plasmids by PCR | 7, 8, 9, 10 |

| Method used | Number of the chapter |
|---|---|
| Sequence analysis by BLAST algorithm[11] | 7, 8, 9, 10 |
| Illumina WGS | 7, 8, 9, 10 |
| Detection of β-lactamases by isoelectric focusing (IEF) using nitrocefin | 8, 9 |
| PCR confirmation of β-lactamases using specific primers | 8, 9 |
| PCR differentiation of IncL and IncM plasmids[12] | 8 |
| MALDI-TOF MS imipenem hydrolysis assay[13] | 10 |
| PCR detection of virulence-associated genes (ExoS, ExoT, ExoU, and ExoY) | 10 |
| PCR integron analysis | 10 |

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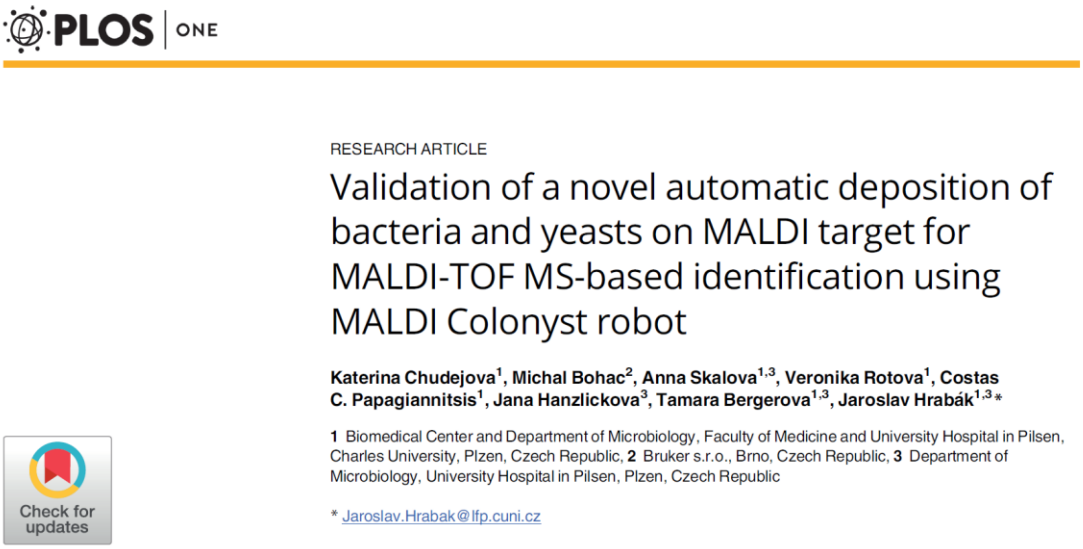
## 6 Validation of a novel automatic deposition of bacteria and yeasts on MALDI target for MALDI-TOF MS-based identification using MALDI Colonyst robot



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RESEARCH ARTICLE

# Validation of a novel automatic deposition of bacteria and yeasts on MALDI target for MALDI-TOF MS-based identification using MALDI Colonyst robot

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) -based identification of bacteria and fungi significantly changed the diagnostic process in clinical microbiology. We describe here a novel technique for bacterial and yeast deposition on MALDI target using an automated workflow resulting in an increase of the microbes' score of MALDI identification. We also provide a comparison of four different sample preparation methods. In the first step of the study, 100 Gram-negative bacteria, 100 Gram-positive bacteria, 20 anaerobic bacteria and 20 yeasts were spotted on the MALDI target using manual deposition, semi-extraction, wet deposition onto 70% formic acid and by automatic deposition using MALDI Colonyst. The lowest scores were obtained by manual toothpick spotting which significantly differ from other methods. Identification score of semi-extraction, wet deposition and automatic wet deposition did not significantly differ using calculated relative standard deviation (RSD). Nevertheless, the best results with low error rate have been observed using MALDI Colonyst robot. The second step of validation included processing of 542 clinical isolates in

routine microbiological laboratory by a toothpick direct spotting, on-plate formic acid extraction (for yeasts) and automatic deposition using MALDI Colonyst. Validation in routine laboratory process showed significantly higher identification scores obtained using automated process compared with standard manual deposition in all tested microbial groups (Gram-positive, Gram-negative, anaerobes, and yeasts). As shown by our data, automatic colony deposition on MALDI target results in an increase of MALDI-TOF MS identification scores and reproducibility.

## 6.1 Introduction

Fully automated clinical microbiology laboratory is currently a high priority development project of several commercial companies.[1] In contrast to biochemical or hematology laboratories, which use standard tubes and have minimal diversity of materials, microbiology must accept plenty of sorts of specimen or transport media. Therefore, there is a problem of standardization of diagnostic processes. The situation is, however, improving using new technologies such as mass spectrophotometry, molecular techniques or automatic lines.[2]

Automated process is expected to positively affect the quality of processed specimens and their standardization, including documentation of microbial cultures on plates. Automatic lines currently include automation of sample inoculation process, smart incubation with possible documentation of microbial growth on the plates and workbenches allowing plate reading via high-resolution imaging.[1, 3]

Most important aspect in clinical microbiology is a taxonomical identification of cultivated microbes. In the past, identification of bacteria and yeasts, was based mainly on biochemical detection of enzymes produced by microorganisms. In the last decade, introduction of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) revolutionary changed identification process by its accuracy and rapidness.[4-6]

MALDI-TOF MS identification increased workflow efficiency having turnaround time of 6-10 minutes per identification of one isolate and reduced the cost per identification.[3] This technique allows precise identification of most microbes comparing with biochemical tests. Only some species (e.g., *Streptococcus* spp., *Enterobacter* spp., some Gram-negative non-fermenting rods) are indistinguishable by MALDI-TOF MS identification.[6] *Mycobacterium* spp., *Nocardia* spp., *Actinomyces* spp. and filamentous fungi, however, can be identified with very high probability to a species level.[7, 8]

Except for taxonomical identification of bacteria, yeasts and filamentous fungi, MALDI-TOF MS is able to detect important resistance mechanisms (e.g., carbapenemases) and categorize microbes to susceptibility group based on their growth in presence of tested antibiotics or changes of protein profile after antibiotic exposure.[9-12] Direct detection of microbes from clinical specimen is another challenge of current development in application of that technique in clinical microbiology.[13-15] As well as bacterial toxins can be efficiently detected by MALDI-TOF MS.[16]

One of the main advantages of MALDI-TOF MS-based identification is the direct deposition of intact bacterial/yeast cells on the MALDI target. Then, cells are overlaid by a matrix solution. Thus, no further sample manipulation is needed. For better quality of spectra acquisition resulting in a higher score of species identification, specific extraction protocols can be used.[7] Simply, the spot target with the microbe can be overlaid by 1 microliter of 70% formic acid. In some microbes (e.g., yeasts and anaerobic bacteria), in tube extraction with ethanol and formic acid/acetonitrile may provide an advantage solution for spectra acquisition.[7] However, direct spotting, usually performed by a wooden toothpick, does not allow standardization of the amount of microbes deposited on the target spot. This process is highly dependent on the experience of technician and may significantly influence identification score.

Automation of colony picking for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) identification and antibiotic susceptibility testing is still under development. One of the first devices enabling automatic deposition of colonies on the MALDI target is Copan Colibri (COPAN Diagnostics Inc., CA, USA), which was introduced during 25th ECCMID in 2015 in Copenhagen.[17]

We describe here a novel technique for bacterial and yeast deposition on MALDI target using an automated workflow resulting in an increase of the score of microbe identification. We also provide a comparison of four different sample deposition methods.

## 6.2 Materials and Methods

### 6.2.1 Bacterial isolates

The microbes were cultivated on 5% sheep blood agar, Endo agar, Candi Select agar (Bio-Rad, Prague, Czech Republic), PVX agar, or SCS agar (BioMèrieux, Prague, Czech Republic) at 35°C overnight.

All samples were collected in routine diagnostic laboratory of University Hospital in Plzen (Czech Republic) which is a tertiary care hospital providing a complete medical service, including transplantology. Thus, community-acquired as well as hospital acquired pathogens are routinely identified in the laboratory. Mostly, clinical materials of upper and lower respiratory tract, gastrointestinal tract, urogenital tract, wounds and exudates, or blood cultures were included in the study.

### 6.2.2 Methods for manual deposition of bacteria/yeasts on MALDI-TOF MS target

For direct spotting on the MALDI target (MSP 96 Target, Catalog No. 224989, Bruker Daltonics GmbH, Bremen, Germany), bacterial culture was transferred by a toothpick forming a thin film on the spot. After drying, the spot was covered by 1 microliter of a matrix solution [10 mg/ml of alfa-cyano-4-hydrocinnamic acid (CHCA) (Bruker Daltonik, Bremen, Germany) in 50% acetonitrile (Sigma-Aldrich, Prague Czech Republic) and 2.5% trifluoroacetic acid (Sigma-Aldrich, Prague Czech Republic)].

On-plate extraction (semi-extraction) was performed by spotting of bacteria as described above. After drying, the spot was covered by 1 microliter of 70% formic acid and allow to dry. Then, 1 microliter of matrix was applied.

In the wet deposition, 1 microliter of 70% formic acid was pipetted on the spot. Instantly, bacteria were collected manually with a platinum inoculation loop and resuspended, in the formic acid droplet, on the spot. After the spot was dried, 1 microliter of the matrix was applied.

### 6.2.3 Automatic deposition using MALDI Colonyst

MALDI Colonyst (Biovendor Instruments, Brno, Czech Republic) was used for automatic sample preparation according to the manufacturer instructions. Shortly, three vials were loaded into the machine (70% formic acid, MALDI matrix solution and 70% acetonitrile as Washing solution

respectively) and the system has been automatically washed prior spotting of bacteria. Additionally, MALDI target was inserted into the holder and automatic picking process has been started. Within each round, the Petri dish was inserted into the holder and colonies were selected by the simple mouse click within the high-resolution pictures in the MALDI Colonyst software tool and automatically picked by the platinum tips of the robot. Colony deposition was performed using "wet deposition" into the automatic pre-spotted formic acid droplet as described above. Colony picking and their deposition as well as MALDI matrix deposition were carried out automatically. The quality of deposition process has been automatically documented using screen shots of each step of the deposition process. The photos are automatically stored in the computer.

### 6.2.4 MALDI-TOF MS measurement

Spectra acquisition was performed on MicroFlex LT mass spectrometer (Bruker Daltonik, Bremen, Germany) using MALDI Biotyper software version 3.1. Bruker Bacterial Test Standard was used for a calibration.

### 6.2.5 Statistical analysis

To determine statistically significant differences, repeatability of automatic deposition method was determined. Thirty different microbial isolates (8 Gram-positive microbes, 8 Gram-negative microbes, 6 anaerobes, and 8 yeasts) were measured and processed by MALDI Colonyst robot. Each isolate was spotted ten times from the same culture plate. For each microbial group (Gram-negative bacteria, Gram-positive bacteria, anaerobe bacteria and yeasts), arithmetic mean of identification score ($\bar{x}$), standard deviation (SD) and relative standard deviation (RSD%) were determined. Based on those parameters, the accuracy of the method and the reproducibility of the parallel measurements was evaluated.[18]

## 6.3 Results

An average relative standard deviation (RSD%) of automatic method was below 5.00% for each microbial group. Higher RSD% were identified in *Candida pararugosa* and *Peptostreptococcus micros* only (RSD% 8.21 and 5.56 respectively). Excellent repeatability was detected in Gram-negative bacteria (RSD% 1.81), followed by Gram-positive microbes. The lowest RSD were identified in yeasts and anaerobic bacteria (**Table 6-1**).

**Table 6-1** Repeatibility of automatic bacterial deposition using MALDI Colonyst determined by standard deviation (SD) and relative standard deviation (RSD%). Each strain was spotted ten times from the same culture plate and identified using MALDI-TOF MS.

| Species | x̄ | SD | RSD% | Species | x̄ | SD | RSD% | Species | x̄ | SD | RSD% | Species | x̄ | SD | RSD% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *K. pneumoniæ* | 2.39 | 0.03 | 1.21 | *S. agalactiæ* | 2.41 | 0.06 | 2.33 | *C. albicans* | 2.13 | 0.07 | 3.27 | *F. nucleatum* | 2.21 | 0.04 | 1.69 |
| *K. oxytoca* | 2.26 | 0.05 | 2.19 | *S. pyogenes* | 2.30 | 0.08 | 3.66 | *C. parapsilosis* | 1.97 | 0.04 | 2.11 | *F. magna* | 2.16 | 0.05 | 2.25 |
| *E. coli* | 2.31 | 0.04 | 1.69 | *S. pneumoniæ* | 2.25 | 0.06 | 2.89 | *C. tropicalis* | 2.04 | 0.06 | 3.01 | *P. micros* | 1.72 | 0.10 | 5.56 |
| *P. æruginosa* | 2.48 | 0.07 | 2.67 | *S. epidermidis* | 2.17 | 0.04 | 1.81 | *C. pararugosa* | 1.79 | 0.15 | 8.21 | *B. thetaiotaomicron* | 2.35 | 0.06 | 2.51 |
| *A. bereziniæ* | 2.27 | 0.05 | 2.13 | *S. aureus* | 2.49 | 0.04 | 1.41 | *C. glabrata* | 2.06 | 0.06 | 2.90 | *C. innocuum* | 2.02 | 0.03 | 1.70 |
| *E. cloacæ* | 2.34 | 0.03 | 1.34 | *E. fæcalis* | 2.44 | 0.04 | 1.77 | *S. cerevisiæ* | 2.15 | 0.03 | 1.62 | *C. difficile* | 2.18 | 0.06 | 2.65 |
| *P. mirabilis* | 2.42 | 0.03 | 1.32 | *S. warneri* | 2.23 | 0.04 | 1.93 | *C. lusitaniæ* | 2.27 | 0.05 | 2.17 | | | | |
| *C. freundii* | 2.31 | 0.04 | 1.90 | *C. amycolatum* | 2.26 | 0.03 | 1.18 | *C. guilliermondii* | 2.07 | 0.09 | 4.18 | | | | |
| Gram-negative microbe | 2.35 | 0.04 | 1.81 | Gram-positive microbe | 2.32 | 0.05 | 2.12 | Yeasts | 2.06 | 0.07 | 3.34 | Anaerobes | 2.11 | 0.06 | 2.63 |

In the first step of validation study 240 clinical isolates were used. The collection was composed of 100 Gram-positive bacteria, 100 Gram-negative bacteria, 20 anaerobes and 20 yeasts. In this step, identifications were performed by all four methods (direct spotting, semi-extraction, wet deposition and automatic deposition). All samples were measured in triplicates.**Table 6-2** shows an average identification score and error rate for each group of microbes and spotting methods. Average identification scores are calculated with and/or without errors (error is defined as an identification with a score below 1.70 or the spectra were no peaks were found). The spectra with no peaks found were excluded from the calculation of the average score. The lowest scores were obtained by manual toothpick spotting which significantly differ from other methods. Identification score of other methods did not significantly differ using calculated RSD. Nevertheless, the best results with low error rate have been observed using MALDI Colonyst robot (see **Figure 6-1**)

**Table 6-2** Identification score of microbes processed by four different methods of deposition on MALDI target.

| | | Gram-positive | Gram-negative | Anaerobes | Yeasts |
|---|---|---|---|---|---|
| | Nr. of strains identified | 100 | 100 | 20 | 20 |
| | Total nr. of identifications | 300 | 300 | 60 | 60 |
| **Direct spotting** | Nr. of "score below 1.70" | 46 | 5 | 14 | 40 |
| | Nr. of "no peaks found" | 3 | 1 | 0 | 7 |
| | Nr. of total errors | 49 | 6 | 14 | 47 |
| | Total errors [%] | 16.33% | 2.00% | 23.33% | 78.33% |
| | Average identification score w/o errors* | 2.09 | 2.24 | 2.17 | 1.87 |
| | Average identification score with errors** | 1.98 | 2.23 | 2.01 | 1.49 |
| **Semi-extraction** | Nr. of "score below 1.70" | 5 | 4 | 3 | 1 |
| | Nr. of "no peaks found" | 3 | 0 | 0 | 1 |
| | Nr. of total errors | 8 | 4 | 3 | 2 |
| | Total errors [%] | 2.67% | 1.33% | 5.00% | 3.33% |
| | Average identification score w/o errors* | 2.21 | 2.27 | 2.21 | 2.00 |
| | Average identification score with errors** | 2.21 | 2.27 | 2.18 | 1.99 |
| **Manual wet deposition** | Nr. of "score below 1.70" | 6 | 0 | 1 | 7 |
| | Nr. of "no peaks found" | 2 | 0 | 0 | 1 |
| | Nr. of total errors | 8 | 0 | 1 | 8 |
| | Total errors [%] | 2.67% | 0.00% | 1.67% | 13.33% |
| | Average identification score w/o errors* | 2.24 | 2.33 | 2.25 | 1.97 |
| | Average identification score with errors** | 2.24 | 2.33 | 2.23 | 1.93 |
| **Automatic deposition** | Nr. of "score below 1.70" | 5 | 0 | 1 | 1 |
| | Nr. of "no peaks found" | 2 | 0 | 0 | 0 |
| | Nr. of total errors | 7 | 0 | 1 | 1 |
| | Total errors [%] | 2.33% | 0.00% | 1.67% | 1.67% |
| | Average identification score w/o errors* | 2.28 | 2.38 | 2.26 | 2.03 |
| | Average identification score with errors** | 2.26 | 2.38 | 2.25 | 2.02 |

*/** Error is defined as the score with a value below 1.70, or spectra with no peaks ("no peaks found"). The spectra characterized as "no peaks found" were excluded from the calculation of the average identification score.

In general, the lowest error rate (no acquisition of spectra or no identification possible) was identified in Gram-negative bacteria in all tested groups. The highest error rate has been observed in yeasts deposited by a manual method using a toothpick (78.33%).

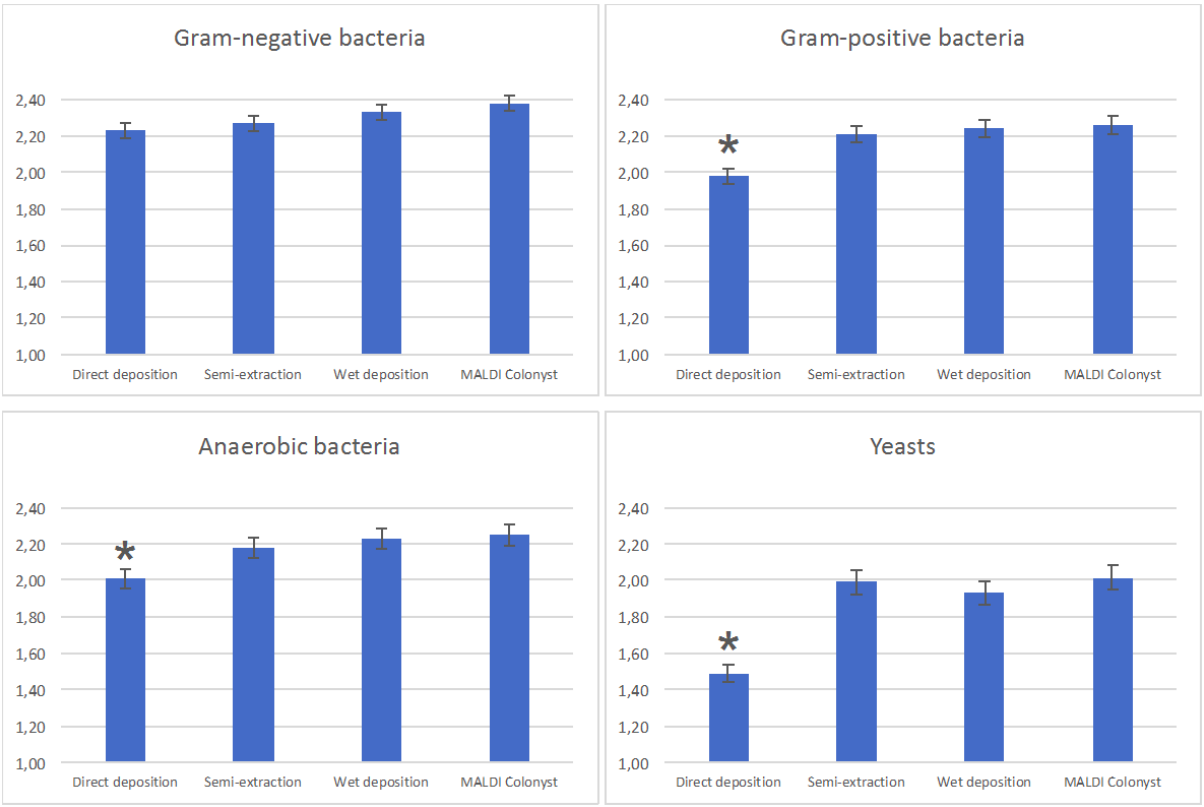


**Figure 6-1** Comparison of different deposition methods with plotted RSD. Statistically significant differences are indicated by asterisks.

In the second step of the study, 542 clinical isolates were included. Those isolates consisted of 244 Gram-negative aerobic/facultative bacteria, 213 Gram-positive bacteria, 46 anaerobic bacteria and 39 yeasts. The isolates were simultaneously spotted by manual method (direct spotting for bacteria, semi-extraction for yeasts) and by automatic deposition using MALDI Colonyst. Colonies for both methods were selected from the same culture plate.

Automatic deposition showed better identification score in all groups (in Gram-positive, Gram-negative, anaerobes and yeasts) – see **Table 6-3**. The highest difference was observed in yeasts (2.02 and 2.14 respectively), anaerobes (2.04 and 2.22 respectively) and in some Gram-positive bacteria. On the other hand, the lowest difference in Gram-negative bacteria, where both methods differ by 0.07 only. All differences, however, were higher than the reproducibility of the method.

**Table 6-3** Comparison of identification scores in the second stage of the study. Routine clinical samples were identified by direct spotting or semi extraction (yeasts) and by automatic deposition using MALDI Colonyst from the same culture.

| Bacteria | Nr. of isolates | Manual deposition average score | Deposition by Colonyst average score |
|---|---|---|---|
| **Gram-positive microbes (total number)** | 213 | 2.20 | 2.31 |
| *Staphylococcus* sp. | 71 | 2.17 | 2.25 |
| *Streptococcus* sp. | 52 | 2.27 | 2.34 |
| *Enterococcus* sp. | 63 | 2.25 | 2.39 |
| Other Gram-positive microbes | 27 | 2.01 | 2.18 |
| **Gram-negative microbes (total number)** | 244 | 2.30 | 2.37 |
| *Enterobacteriaceæ* | 171 | 2.33 | 2.40 |
| Non-fermenting bacteria | 44 | 2.25 | 2.34 |
| Other Gram-negative microbes | 29 | 2.22 | 2.30 |
| **Anaerobes** | 46 | 2.04 | 2.22 |
| **Yeasts** | 39 | 2.02 | 2.14 |

## 6.4 Discussion

The main focus of clinical microbiology is the isolation and identification of a pathogenic bacteria and subsequent determination of their susceptibility to antimicrobial agents. Currently, the MALDI-TOF MS method is the most common technique used for taxonomic identification in many states.

The aim of the study was to compare results of average identification score for routinely used manual deposition (semi-extraction for yeasts) with results of automatic deposition using MALDI Colonyst robot. Spotting by MALDI Colonyst significantly increased identification score of bacteria comparing with routine diagnostic process. The lowest difference was found in *Enterobacteriaceæ* isolates which usually possess high identification score comparing with other microbes. Their cells can be easily disrupted during drying on the spot and after covering by matrix solution. Similar results were also observed in yeasts, because of the fact, that semi-extraction method is routinely used in those microbes. In this study, we used only commonly processed isolates in routine practice of our laboratory. Therefore, identification score and thus efficiency of automatic colony deposition may differ in laboratories with different patient spectra and isolated yeast's species.

Results from routine testing were in concordance with the comparison of four spotting methods tested on 240 clinical isolates. Semi-extraction, wet deposition and automatic spotting using MALDI Colonyst provided similar identification score, significantly higher the scores obtained after direct deposition. No difference was observed in Gram-negative bacteria.

One of the main advantages of automatic deposition on a target is a semi-standardization of biomass amount in the spot. After picking up by the platinum loop, only a part of microbes is resuspended into the droplet of 70% formic acid. A part of biomass remains attached on the loop and is removed during

sterilization process. During drying of the spot, the lyses is enhanced, and final spot is homogenously covered by bacterial lysate. Homogenous surface of the spot may also explain higher identification score in both wet-deposition techniques. Our results are in agreement with the publication of Theel *et al.*[19] demonstrating that direct deposition of *Corynebacterium* spp. to a formic acid drop may enhance identification score of these bacteria.

Another advantage of automatic colony deposition using MALDI Colonyst is the significant decrease of consumable cost, since there is no need of pipette tips for formic acid deposition (in case of semi-extraction) and matrix deposition. Based on standard MALDI preparation procedure, the tip should be replaced after each spot. Such a decrease of the running costs shall lead to increase of the number of selected colonies from each of the Petri dish for MALDI identification while not extending the preparation time and manual workload. Deposition of one 96 MALDI target takes approximately 45 minutes using MALDI Colonyst. This time includes loading plates into the instrument, selecting colonies from images on the screen, drying of the spot, pipetting and drying of the matrix. Manual deposition of one MALDI plate takes the similar time (ca. 40 minutes) dependent on the technician's skills. The reported cost of the robot, however, is comparable with other automats used in clinical microbiology or biochemistry (catalogue price ca. 60 000 EURO). Thus, the initial investment to the machine may limit its use especially in small laboratories, because the cost of one spotting is therefore dependent on number of identifications processed daily. Last but not least, the whole automated deposition process is well monitored and recorder for further validation and retrospective quality control.

Described wet-deposition method allows integrating of MALDI-TOF mass spectrometry into automatic microbiology lines. Another possibility for automatic deposition of microbes on the MALDI target may be performed by pipetting of prepared inocula (e.g., for antibiotic susceptibility testing) on the spot. This procedure, however, may be more expensive, because not all microbes taxonomically identified in routine laboratory are subjected for antibiotic susceptibility testing and therefore, preparing of inocula is redundant.

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**Competing interests**

The authors declare the following interests: Patent applications for automatic depositions using MALDI-TOF MS target have been submitted (Industrial Property Office of the Czech Republic, Nr. PV2015-847). The study was partially supported by the company BioVendor – Laboratorní medicína, a.s. (Brno, Czech Republic) as a contractual

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**Writing – original draft:** Jaroslav Hrabak.

**Writing – review & editing:** Katerina Chudejova, Michal Bohac, Costas C. Papagiannitsis, Tamara Bergerova.

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# 7 Characterization of KPC-encoding plasmids from *Enterobacteriaceæ* isolated in a Czech hospital

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MECHANISMS OF RESISTANCE

## Characterization of KPC-Encoding Plasmids from *Enterobacteriaceae* Isolated in a Czech Hospital

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Ten *Enterobacteriaceæ* isolates collected in a Czech hospital carried *bla*$_{KPC}$-positive plasmids of different sizes (~30, ~45, and ~80 kb). Sequencing revealed three types of plasmids (A to C) with the Tn*4401a* transposon. Type A plasmids comprised an IncR backbone and a KPC-2-encoding multidrug resistant (MDR) region. Type B plasmids were derivatives of type A plasmids carrying an IncN3-like segment, while type C were IncP6 plasmids sharing the same KPC-2-encoding MDR region with type A and B plasmids.

**Keywords:** *Citrobacter freundii*, *Tn4401a*, IncR, ST18, Illumina sequencing

KPC-type β-lactamases comprise a distinct group of plasmid-borne enzymes with carbapenemase activity mainly occurring in *Klebsiella pneumoniæ*. KPC-producing *Enterobacteriaceæ* have emerged as challenging pathogens causing healthcare-associated infections, due to their extremely drug-resistant phenotypes and ability to cause infections associated with high mortality.[1] KPC producers have disseminated worldwide and currently constitute an important public health problem.[2] In Europe, Greece and Italy are the most affected countries, with high proportions of KPC-producing *K. pneumoniæ*.[3] In the Czech Republic, however, the occurrence of KPC producers has been rare. A sporadic case of KPC-2-producing *K. pneumoniæ* recovered from a patient, who had been previously hospitalized in Greece, was detected in the Czech Republic in 2009.[4] Additionally, in 2011, an outbreak of KPC-3-producing *K. pneumoniæ* was observed in another Czech hospital,[5] with the index case being a patient repatriated from Italy.

In the present study, we describe the molecular characterization of KPC-2-producing *Enterobacteriaceæ* isolates, mainly of the species *Citrobacter freundii*, recovered in the University Hospital of Hradec Kralove (Czech Republic).

From 2014 until 2016, a total of 10 nonrepetitive *Enterobacteriaceæ* isolates showing carbapenemase activity on matrix-associated laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) meropenem hydrolysis assay[6] were detected. Among them, 7 of the isolates were identified to be *C. freundii*, 1 was identified to be *K. pneumoniæ*, 1 was identified to be *Escherichia coli*, and 1 was identified to be *Morganella morganii* (**Table 7-1**). Phenotypic testing, PCR screening, and sequencing[7] showed that all isolates were positive for the presence of $bla_{\text{KPC-2}}$ gene. The 10 KPC-2-producing isolates were recovered from 7 patients, 6 of which were hospitalized in the same unit (**Table 7-1**). In addition, 5 of the patients had overlapping stays in several combinations, suggesting transmission of KPC-2 producers.

Susceptibility to various antimicrobial agents was determined by the broth dilution method.[8] MICs, interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org/), showed that all KPC-2 producers exhibited resistance to aminopenicillins, aminopenicillin-inhibitor combinations (data not shown), cephalosporins and aztreonam, and were nonsusceptible to carbapenems. Additionally, KPC-2 producers also exhibited resistance to several non-β-lactam antibiotics, whereas all isolates remained susceptible to tigecycline (**Table 7-1**).

The population structure of KPC-2-producing isolates studied by multilocus sequence typing (MLST)[9-11] is shown in **Table 7-1**. The *C. freundii* isolates comprised three sequence types (STs). ST18 was the most prevalent, accounting for five isolates. ST18 was previously found among NDM-1-producing isolates from Denmark and VIM-1-producing isolates from Spain.[12, 13] The *K. pneumoniæ* isolate was assigned to the high-risk clone ST11, previously associated with the production of several carbapenemases,[14] while the *E. coli* isolate belonged to ST216.

None of the clinical isolates was capable of transferring the *bla*$_{KPC-2}$ gene to the *E. coli* A15 laboratory strain by conjugation. Plasmid DNAs from clinical isolates were extracted using a Qiagen maxikit (Qiagen, Hilden, Germany) and used to transform *E. coli* DH5α cells. Transformants were selected on Luria-Bertani agar plates with ampicillin (50 μg/ml), confirmed to be KPC-2 producers by PCR[7] and MALDI-TOF MS meropenem hydrolysis assay,[6] and tested for antimicrobial susceptibility (see **Table S1** in the supplemental material). The plasmid location of the *bla*$_{KPC-2}$ genes was demonstrated by the S1 nuclease analysis of clinical and recombinant strains,[15] followed by hybridization with a digoxigenin-labeled *bla*$_{KPC}$ probe. Plasmid analysis revealed the transfer of plasmids, most of which (*n*=6) were sized ~45 kb. The remaining plasmids were ~80 kb (*n*=2) or ~30 kb (*n*=2). Replicon typing showed that eight of the plasmids, including those ~45 kb and ~80 kb in size, were positive for the IncR replicon[16] (**Table 7-1**), whereas the two remaining plasmids were nontypeable by the PCR-based replicon typing (PBRT) method.[17, 18]

**Table 7-1** Characteristics of KPC-2-producing *Enterobacteriaceæ*.

| Species and isolate | Hospital department[a] | Isolation date | Clinical material | ST[b] | Size of *bla*$_{KPC}$ plasmid (kb) | Type of plasmid sequence (replicon) | MIC (μg/ml) of:[c] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Ctx | Caz | Fep | Atm | Imp | Mem | Etp | Gen | Amk | Tob | Sxt | Cip | Col | Tgc | Fos |
| *C. freundii* | | | | | | | | | | | | | | | | | | | | | |
| Cfr-27569 | ICU | 11/2014 | Urine | 18 | 46.826 | A (IncR) | >8 | >16 | >16 | >16 | 16 | >16 | >4 | >32 | 16 | >8 | >64 | >8 | 0.25 | 0.25 | ≤2 |
| Cfr-31260 | ICU | 09/2015 | Urine | 18 | 46.826 | A (IncR) | >8 | >16 | >16 | >16 | >32 | >16 | >4 | >32 | 32 | >8 | >64 | >8 | 0.12 | 0.25 | ≤2 |
| Cfr-31816 | ICU | 10/2015 | Urine | 18 | 46.826 | A (IncR) | >8 | >16 | >16 | >16 | >32 | 16 | 4 | >32 | >32 | >8 | >64 | >8 | 0.12 | 0.12 | ≤2 |
| Cfr-33038[d] | ICU | 11/2015 | Rectal swab | 18 | 46.826 | A (IncR) | >8 | >16 | >16 | >16 | >32 | >16 | >4 | >32 | 16 | >8 | >64 | >8 | 4 | 1 | 8 |
| Cfr-33795 | ICU | 04/2016 | Urine | 142 | 30.051 | C (IncP6) | >8 | >16 | >16 | >16 | 4 | 8 | 4 | 4 | 32 | >8 | >64 | 4 | 0.12 | 0.5 | ≤2 |
| Cfr-36049 | ICU | 09/2016 | Wounds | 87 | 81.348 | B (IncR and IncN3) | >8 | >16 | >16 | >16 | 8 | 8 | >4 | 1 | 16 | >8 | 1 | ≤0.06 | 1 | 0.06 | ≤2 |
| Cfr-36808 | HD | 11/2016 | Rectal swab | 18 | 46.826 | A (IncR) | >8 | 16 | 16 | >16 | 12 | 16 | >4 | >32 | >32 | >8 | >64 | >8 | 0.25 | 0.12 | ≤2 |
| *K. pneumoniæ* | | | | | | | | | | | | | | | | | | | | | |
| Kpn-35786[d] | ICU | 09/2016 | Catheter | 11 | 46.826 | A (IncR) | >8 | >16 | >16 | >16 | >32 | >16 | 2 | >32 | 16 | >8 | >64 | >8 | >16 | 1 | >128 |
| *E. coli* | | | | | | | | | | | | | | | | | | | | | |
| Eco-36682[d] | ICU | 11/2016 | Catheter | 216 | 81.348 | B (IncR and IncN3) | 4 | 4 | 2 | >16 | 32 | 4 | 2 | 2 | >32 | >8 | 0.06 | ≤0.06 | ≤0.06 | 0.25 | ≤2 |
| *M. morganii* | | | | | | | | | | | | | | | | | | | | | |
| Mmo-37590[d] | ICU | 12/2016 | Urine | NA | 30.051 | C (IncP6) | >8 | 8 | >16 | >16 | 16 | 8 | 1 | 2 | 32 | 8 | >64 | 8 | >16 | 0.5 | >128 |

[a] ICU, internal care unit; HD, hematology department.
[b] ST, sequence type; NA, not applicable.
[c] Ctx, cefotaxime; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Imp, imipenem; Mem, meropenem; Etp, ertapenem; Gen, gentamicin; Amk, amikacin; Tob, tobramycin; Sxt, trimethoprim-sulfamethoxazole; Cip, ciprofloxacin; Col, colistin; Tgc, tigecycline; Fos, fosfomycin.
[d] KPC-2-like-producing isolates recovered from the same patient.

Plasmid DNAs from all KPC-2-producing transformants were extracted using a Qiagen large-construct kit (Qiagen, Hilden, Germany). Multiplexed plasmid 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[19] with the sliding window size of 4 bp, required average base quality ≥17 and minimum read length of 48 bases. For assembly of the

plasmids, reads were mapped to the reference *E. coli* K-12 substrain MG 1655 genome (GenBank accession no. U00096) using the BWA-MEM algorithm,[20] in order to filter out the chromosomal DNA. Then, all the unmapped reads were assembled by use of the de Bruijn graph-based *de novo* assembler SPAdes v3.9.1,[21] using k-mer sizes 21, 33, 55 and 77. *De novo* assembly resulted in sets of contigs with length-weighted average k-mer coverage ranging from 23x to 95x. The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (www-is.biotoul.fr/), and the open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using the Mauve (version 2.3.1) program.[22]

Plasmid analysis revealed three types of *bla*$_{KPC-2}$-carrying plasmid sequences (types A to C; **Table 7-1**), with type A being the most prevalent. All plasmids contained the Tn*4401a* isoform of the Tn*4401* transposon, which is similar to that described in plasmid pNYC lacking 100 bp upstream of *bla*$_{KPC-2}$.[23]

All *bla*$_{KPC-2}$-carrying plasmids that were ~45 kb in size belonged to type A and showed high degrees of similarity to each other. The plasmids included a contiguous segment of 12,036 bp (nucleotide [nt] 1 to 10294 and 45085 to 46826; GenBank accession number MF497780) sharing extensive similarity with the backbone of the recently described IncR plasmids.[24] This segment was composed of regions responsible for the replication (*repB* gene and iteron region), maintenance (*resD* gene) and stability (*parAB*, *vagCD* and *umuDC* operons) of the plasmids (**Figure 7-1**).

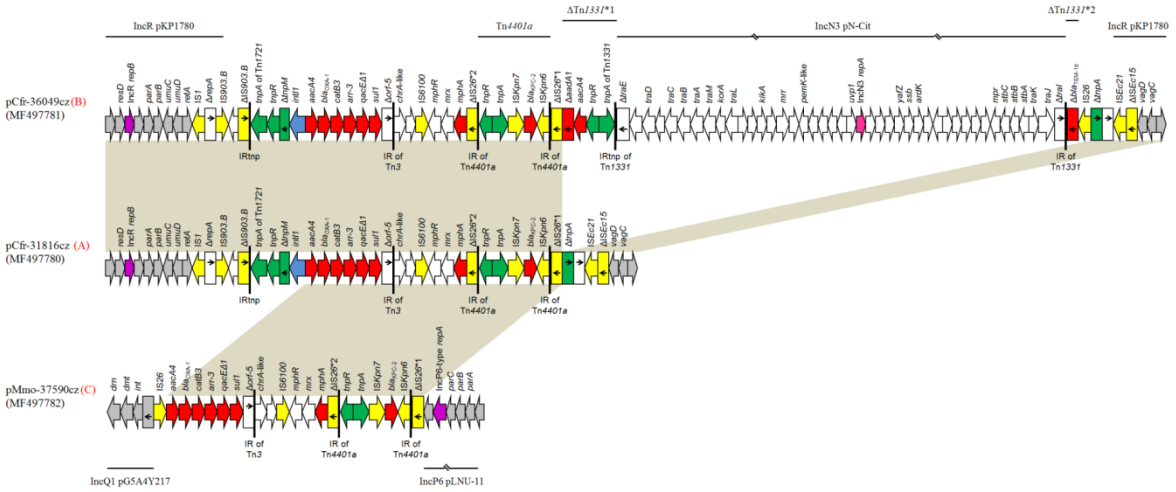


**Figure 7-1** Linear maps of the *bla*$_{KPC-2}$-carrying plasmids. For each plasmid, the type of plasmid sequence is indicated in red next to the plasmid name. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements and transposases are shown in red, yellow and green, respectively. intI1 genes are shaded blue. Gray arrows or rectangles indicate plasmid scaffold regions; the replication genes are shown in purple. The remaining genes are shown in white. Homologous segments (representing ≥99% sequence identity) are indicated by light gray shading. Thin lines above and below the maps correspond to highly similar sequences from other plasmids.

In the remaining 34,790 bp sequence (nt 10295 to 45084; GenBank accession number MF497780) adjoining the boundaries of the IncR backbone, a multidrug resistance (MDR) region containing the KPC-2-encoding transposon Tn*4401a* was identified. The Tn*4401a* was localized within a copy of insertion sequence IS*26* (ΔIS*26*). Target site duplications of 5 bp (ATGCA) at the boundaries of the Tn*4401a* indicated insertion by transposition. Upstream from ΔIS*26**1, an IS*Ec21*-like element and a 916 bp fragment of an IS*Ec15*-like element (ΔIS*Ec15*) were found. The IS*Ec21*-ΔIS*Ec15* structure was at the boundary of the plasmid backbone, downstream of *vagCD*, in the same configuration previously described in the IncR MDR plasmid pKP1780.[24]

In the remaining part of the MDR region, an IS*1* that was followed by a 674 bp segment of IncN replication region (Δ*repA*)[25] was found at the boundary of the plasmid backbone, downstream of *retA*. The IS*1*-Δ*repA* structure was also identified in pKP1780 at a similar position.[24] Next to this sequence, an intact *IS903.B*-like element and a second copy of *IS903.B*-like truncated at the 3' end (ΔIS*903.B*) were found. The deleted part of IS*903.B* was occupied by a Tn*1721*-like fragment (Δ Tn*1721*-like) consisting of the 38-bp inverted repeat (IRtnp) of the transposon, *tnpA*, *tnpR* and *tnpM*. The Tn*1721*-like sequence also included an integron similar to In37 from pHSH2, whose variable region comprised the *aacA4*, *bla*$_{\text{OXA-1}}$, *catB3*, and *arr-3* cassettes.[26] The IRi of In37 was located within the *tnpM* gene of the Tn*1721*-like transposon, while the 3'CS of In37 was truncated 174 bp after the start codon of *orf5* (Δ*orf5*). Immediately downstream of Δ*orf5*, a Tn*501*-like sequence including the 38-bp inverted repeat (IR) of the transposon, a *chrA*-like gene encoding a chromate ion transporter, an IS*6100*, a macrolide resistance operon [*mph(A)*] and the remaining part of IS*26* (ΔIS*26**2) was found. A similar structure has also been observed in plasmid pLEW517, conferring resistance to ampicillin, streptomycin, sulfonamides and mercury, has also been observed in plasmid pLEW517 from the primate intestinal *E. coli* strain 517-2H1.[27]

Type B plasmids, pCfr-36049cz and pEco-36682cz, appeared to be derivatives of type A IncR KPC-2-encoding plasmids, characterized during the present study. Type B plasmids differed from type A plasmids by the presence of an additional 43,522 bp sequence (nt 41399 to 75920; GenBank accession number MF497781), upstream of ΔIS*26**1. This sequence comprised two fragments of the Tn*1331* transposon flanking a central sequence (**Figure 7-1**). The central sequence (nt 46232 to 74780; GenBank accession number MF497781) shared extensive similarity with the sequence of pN-Cit (96% coverage and 95% identity), an IncN3-type plasmid originally described from *C. freundii* STE strain collected in France from a patient who had been transferred from India.[28] The IncN3-derived sequence possessed genes encoding transfer locus, and *repA* gene that was 98% similar to the respective region of pN-Cit. However, a part of the IncN3 transfer system was missing, explaining the inability of pCfr-36049cz and pEco-36682cz to transfer via conjugation.

Plasmids pCfr-33795cz and pMmo-37590cz, which were assigned to type C, included a contiguous segment of 4,062 bp (nt 261 to 4322; GenBank accession number MF497782) containing the partitioning genes, *parA*, *parB* and *parC*, and the replication gene *repA* (**Figure 7-1**). The *parABC* operon of pCfr-33795cz and pMmo-37590cz was identical to that of IncP6-type plasmids like pCOL-1 described from the

KPC-2-producing *Pseudomonas æruginosa* COL-1 strain isolated in Colombia,[29] and to pLNU-11 (GenBank accession number KX863568), which was identified from a *C. freundii* ATetA strain captured from the sediments of an urban coastal wetland. The putative *repA* product of pCfr-33795cz and pMmo-37590cz showed high amino acid sequence similarity (99%) with the replication initiation protein of pLNU-11. Additionally, type C plasmids included a 3,835 bp segment (nt 1 to 260 and 26477 to 30051; GenBank accession number MF497782) consisting of genes encoding a DNA invertase/recombinase (*int*), a deoxymethyltransferase (*dmt*) and a DNase (*drn*) of type II restriction module. The *int-dmt-drn* region has also been observed in IncQ1 *bla*$_{GES-5}$-carrying plasmids isolated from *E. coli* and *Serratia marcescens* persisting in Canada.[30] The remaining 22,154 bp sequence of pCfr-33795cz and pMmo-37590cz (nt 4323 to 26476; GenBank accession number MF497782), which contained the KPC-2-encoding transposon Tn*4401a*, was identical to the MDR region of type A and B plasmids (**Figure 7-1**). Contrary, in plasmid pCOL-1, the *bla*$_{KPC-2}$ gene was part of the Tn*4401b* isoform of the transposon and was located in a different insertion site.

In conclusion, the present study reports the "hidden outbreak" of ST18 KPC-2-producing *C. freundii* isolates in a Czech hospital. However, the *bla*$_{KPC-2}$ gene was also identified in other STs of *C. freundii* and other species of *Enterobacteriaceæ*. In one of the patients, four different KPC-2 producers were identified during the hospitalization, implying *in vivo* horizontal transfer of the *bla*$_{KPC-2}$-carrying plasmid. Sequencing data confirmed the presence of the same *bla*$_{KPC-2}$-carrying plasmid in two of these isolates (**Table 7-1**), further supporting this hypothesis. Of note was that, in the remaining two isolates recovered from the same patient, two different types of *bla*$_{KPC-2}$-carrying plasmids were identified indicating the ability of enterobacterial plasmids to further evolve through reshuffling.

Illumina analysis results showed that, in 6 out of the 10 isolates, the KPC-2-encoding transposon Tn*4401a* was localized on an IncR-type plasmid (type A). To our knowledge, this is first report on complete sequences of IncR plasmids carrying Tn*4401a* transposon. However, previous studies have reported the presence of multireplicon IncFII$_{K2}$-IncR KPC-2-encoding plasmids from ST101 *K. pneumoniæ* isolated in Italian hospitals.[31, 32] In addition, type B plasmids were derivatives of type A IncR *bla*$_{KPC-2}$-positive plasmids carrying an IncN3-derived segment. Type C plasmids belonged to IncP6 group and shared the same KPC-2-encoding MDR region with type A and B plasmids. Therefore, *en bloc* acquisition of the KPC-2-encoding MDR region by an InpP6-type replicon from type A or B plasmids is a plausible hypothesis regarding the formation of type C *bla*$_{KPC-2}$-carrying plasmids. All three types of plasmids were noncapable of transferring the *bla*$_{KPC-2}$ gene via conjugation, due to partial deletion or absence of the transfer system genes. Thus, the hypothesis of mobilization *in trans* of the *bla*$_{KPC-2}$-carrying plasmids by a co-resident plasmid cannot be excluded.

The data presented here contribute to the current knowledge of KPC-2-producing *Enterobacteriaceæ*. In agreement with the results of previous studies,[16,24,31,32] our findings underline the increasing clinical importance of the IncR plasmid family as well as the spreading potential of large MDR segments through reshuffling of enterobacterial plasmids.

**Accession number(s)**

The nucleotide sequences of pCfr-31816cz, pCfr-36049cz, pMMO-37590cz, pCfr-27569cz, pCfr-31260cz, pCfr-33038cz, pCfr-36808cz, pKpn-35786cz, pEco-36682cz and pCfr-33795cz have been deposited in GenBank under accession numbers MF497780, MF497781, MF497782, MG557994, MG557995, MG557996, MG557997, MG557998, MG557999 and MG558000, respectively.

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We have no conflicts to declare.

**Supplemental material**

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02152-17

**Supplemental File 1**, PDF file, 0.1 MB.

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## 8 Molecular characterization of OXA-48-like-producing *Enterobacteriaceæ* in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids


Molecular Characterization of OXA-48-Like-Producing *Enterobacteriaceae* in the Czech Republic and Evidence for Horizontal Transfer of pOXA-48-Like Plasmids

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The aim of this study was to characterize the first cases and outbreaks of OXA-48-like-producing *Enterobacteriaceæ* recovered from hospital settings in the Czech Republic. From 2013 to 2015, 22 *Klebsiella pneumoniæ* isolates, 3 *Escherichia coli* isolates, and 1 *Enterobacter cloacæ* isolate producing OXA-48-like carbapenemases were isolated from 20 patients. Four of the patients were colonized or infected by two or three different OXA-48-like producers. The *K. pneumoniæ* isolates were classified into nine sequence types (STs), with ST101 being predominant (n=8). The *E. coli* isolates were of different STs, while the *E. cloacæ* isolate belonged to ST109. Twenty-four isolates

carried *bla*$_{OXA-48}$, while two isolates carried *bla*$_{OXA-181}$ or *bla*$_{OXA-232}$. Almost all isolates (n=22) carried *bla*$_{OXA-48}$-positive plasmids of a similar size (~60kb), except the two isolates producing OXA-181 or OXA-232. In an ST45 *K. pneumoniæ* isolate and an ST38 *E. coli* isolate, S1 nuclease profiling plus hybridization indicated a chromosomal location of the *bla*$_{OXA-48}$. Sequencing showed that the majority of *bla*$_{OXA-48}$-carrying plasmids exhibited high degrees of identity with the pOXA-48-like plasmid pE71T. Additionally, two novel pE71T derivatives, pOXA-48_30715 and pOXA-48_30891, were observed. The *bla*$_{OXA-181}$–carrying plasmid was identical to the IncX3 plasmid pOXA181_EC14828, while the *bla*$_{OXA-232}$–carrying plasmid was a ColE2-type plasmid, being a novel derivative of pOXA-232. Finally, sequencing data showed that the ST45 *K. pneumoniæ* and ST38 *E. coli* isolates harbored the IS*1R*-based composite transposon Tn*6237* containing *bla*$_{OXA-48}$ integrated into their chromosomes. These findings underlined that the horizontal transfer of pOXA-48-like plasmids has played a major role in the dissemination of *bla*$_{OXA-48}$ in the Czech Republic. In combination with the difficulties with their detection, OXA-48 producers constitute an important public threat.

**Keywords:** *Klebsiella pneumoniæ,* Tn*1999.2*, IncL, OXA-181, OXA-232, ColE2-like, IncX3, Tn*1999.5*

## 8.1 Introduction

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

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

In the Czech Republic, the occurrence of carbapenemase-producing *Enterobacteriaceæ* (CPE) was rare, with only a total of two cases detected between 2009 and 2010.[12] In 2011, occurrence of CPE increased, and this was mainly due to two hospital outbreaks.[13] To contain this increase, in 2012, the Ministry of Health issued national guidelines for the management of patients infected and colonized with CPE.[13] In 2012 and 2013, only an outbreak of VIM-producing isolates and four sporadic cases were reported.[14] The sporadic cases included two NDM-producing *Enterobacteriaceæ*[15, 16] and the first two OXA-48-producing *K. pneumoniæ* isolates identified in the Czech Republic. These data supposed the success of the national guidelines. However, an increase in the occurrence of CPE was observed during 2014 and 2015, and this was mainly due to the spread of OXA-48-like-producing *Enterobacteriaceæ* in Czech hospitals.

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

## 8.2 Materials and methods

### 8.2.1 Bacterial isolates and confirmation of carbapenemase production

In 2014 and 2015, Czech hospitals referred a total of 630 *Enterobacteriaceæ* isolates with a meropenem MIC >0.125 µg/ml[17] to the National Reference Laboratory for Antibiotics. 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; MALDI Biotyper). All isolates were tested for carbapenemase production by the MALDI-TOF MS meropenem hydrolysis assay.[18] Isolates that were positive by the MALDI-TOF MS meropenem hydrolysis assay were subjected to metalo-β-lactamese, KPC, and OXA-48 detection using thedouble-disc synergy test with EDTA, the phenylboronic acid disc test, and the temocillin disc,[9, 19, 20] respectively. Additionally, carbapenemase genes (*bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$-like) were detected by PCR amplification.[1, 21-23] PCR products were sequenced as described below. Isolates positive for *bla*$_{OXA-48}$-like genes were further studied. Moreover, the two OXA-48-producing *K. pneumoniæ* isolates, recovered at the University Hospital Pilsen (Pilsen, Czech Republic) during 2013 were included in this study for comparative epidemiological purposes.

### 8.2.2 Susceptibility testing

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

### 8.2.3 Typing

All *bla*$_{OXA-48}$-like-positive isolates were typed by multilocus sequence typing (MLST).[25-27] The databases at http://pubmlst.org/ecloacae, http://mlst.warwick.ac.uk/mlst/dbs/Ecoli and http://bigsdb.web.pasteur.fr/klebsiella, were used to assign STs.

### 8.2.4 Detection of β-lactamases

The β-lactamase content of all *bla*$_{OXA-48}$-like-positive isolates was determined by isoelectric focusing (IEF). Bacterial extracts were obtained by sonication of bacterial cells suspended in 1% glycine buffer and clarified by centrifugation. Sonicated cell extracts were analyzed by IEF in polyacrylamide gels containing ampholytes (pH 3.5 to 9.5; APBiotech, Piscataway, NJ). The separated β-lactamases were visualized by covering the gel with the chromogenic cephalosporin nitrocefin (0.2 mg/ml; Oxoid Ltd., Basingstoke, United Kingdom).[28]

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

### 8.2.5 Transfer of bla$_{OXA-48}$-like genes

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

### 8.2.6 Plasmid analysis

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

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

### 8.2.7 Plasmid and chromosome sequencing

Plasmid DNAs from transconjugants and transformants were extracted using the Qiagen large-construct kit (Qiagen, Hilden, Germany). Additionally, the genomic DNA of *K. pneumoniæ* Kpn-82929/13 and *E. coli* Eco-32005/15 were extracted using the DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy). Plasmids and chromosomes were sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Initial paired-end reads were quality trimmed using Trimmomatic tool.[38] For assembling of the plasmids, reads were mapped to the reference *E. coli* K-12 substr. MG 1655 genome (GenBank accession no. U00096) using the BWA-MEM algorithm,[39] in order to filter out the chromosomal DNA. Then, all the unmapped paired-end reads were assembled via de Bruijn graph-based *de novo* assembler SPAdes.[40] The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISFinder database (www-is.biotoul.fr/), and the open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using Mauve (version 2.3.1) program.[41]

### 8.2.8 Accession number(s)

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

## 8.3 Results and Discussion

### 8.3.1 Carbapenemase-producing *Enterobacteriaceæ*

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

### 8.3.2 OXA-48-like-producing isolates

Altogether, 24 nonrepetitive isolates producing OXA-48-like carbapenemases were isolated from 18 patients in 2014 and 2015. Among them, 20 of the isolates were identified to be *K. pneumoniæ*, 3 were identified to be *Escherichia coli*, and 1 was identified to be *Enterobacter cloacæ*. Four of the patients were colonized or infected by two or three different OXA-48-like producers (**Table 8-1**). Additionally, the two OXA-48-like-producing *K. pneumoniæ* isolates identified in 2013 were studied.

OXA-48-like producers were collected from seven Czech hospitals located throughout the Czech Republic. Hospital B was the setting with the highest occurrence of OXA-48 producers. In June 2013, the first OXA-48 producer (Kpn-82929) identified in the Czech Republic was isolated from a newborn. The second OXA-48-producing isolate (Kpn-63870) was recovered from a patient who was directly repatriated from Romania. From April 2014 to March 2015, three further patients colonized/ or infected with OXA-48-producing *K. pneumoniæ* were identified. Additionally, in hospital B, an outbreak that included six patients diagnosed with OXA-48-producing *K. pneumoniæ* lasted from August to December of 2015. Only two cases of OXA-48-producing *K. pneumoniæ* isolates were reported in hospital A1. The first case, a 1-year-old child, who was directly repatriated from a Russian hospital, was colonized or infected by three OXA-48-producing isolates: *K. pneumoniæ* Kpn-04976 and Kpn-04963 and *E. cloacæ* Ecl-04292. One month later, the transmission of an OXA-48-producing *K. pneumoniæ* isolate (Kpn-05159) to an infant who stayed in the same department was found. An OXA-48 outbreak restricted to three patients occurred in hospital D. A patient that had recently travelled to Ukraine was diagnosed with two OXA-48-producing isolates of *K. pneumoniæ* (Kpn-30715 and Kpn-30891) in August of 2015. Two further patients colonized or infected with OXA-48-producing *K. pneumoniæ* were identified until September. The remaining four cases were detected in

four different hospitals. Two of those cases had recently traveled abroad (in India [Kpn-30929] and Tunisia [Kpn-31569]), while no data on whether the other two patients had traveled abroad or had previously been hospitalized were available.

All, 26 OXA-48-like producers exhibited resistance to piperacillin and piperacillin-tazobactam (data not shown), while the variations observed in the MICs of cephalosporins and carbapenems were observed (**Table 8-1**) might reflect the presence of additional resistance mechanisms in some of the isolates. Seventeen of the OXA-48-like producers also exhibited resistance to ciprofloxacin; 16 were resistant to gentamicin, 6 were resistant to tigecycline, and 5 were resistant to amikacin, whereas 2 isolates were resistant to colistin.

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

Sequencing of PCR products revealed three *bla*$_{OXA-48}$-type genes encoding OXA-48, OXA-181 and OXA-232 enzymes (**Table 8-1**).[1, 48, 49] Twenty-four of the isolates were found to produce the OXA-48 β-lactamase, while the ST18 *K. pneumoniæ* isolate produced the OXA-181 enzyme. The remaining *K. pneumoniæ* isolate, which belonged to ST15, coproduced the OXA-232 and NDM-1 carbapenemases. Additionally, most of *bla*$_{OXA-48}$-like-positive isolates were confirmed to coproduce the extended-spectrum β-lactamase CTX-M-15 (n=17), either alone or along with TEM-1 (n=16) and/or OXA-1 (n=5), whereas the ST45 OXA-48-producing *K. pneumoniæ* isolate coproduced the CTX-M-14 β-lactamase.

**Table 8-1** Characteristics of OXA-48-like-producing *Enterobacteriaceæ*.

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

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

### 8.3.3 *bla*$_{OXA-48}$-like-carrying plasmids

The *bla*$_{OXA-48}$-like genes from 24 out of 26 clinical strains were transferred by conjugation (n=23) or transformation (n=1) (**Table 8-1**). Neither the ST45 *K. pneumoniæ* isolate nor the ST38 *E. coli* isolate was capable of transferring the *bla*$_{OXA-48}$ gene by either conjugation or transformation. All *bla*$_{OXA-48}$-like-positive recombinants exhibited similar resistance phenotypes, showing resistance to piperacillin and piperacillin-tazobactam and decreased susceptibility or resistance to imipenem and ertapenem, while they remained susceptible to cephalosporins and meropenem. Additionally, all *bla*$_{OXA-48}$-like-positive recombinants were susceptible to non-β-lactam antibiotics.

Plasmid analysis of OXA-48-producing donor and transconjugant strains revealed the transfer of plasmids, all of which were ~60 kb (**Table 8-1**). The OXA-181-producing transconjugant carried *bla*$_{OXA-48}$-like-positive plasmid with a size of ~50 kb, while the OXA-232-producing transformant harbored a plasmid of ~10 kb that hybridized with a *bla*$_{OXA-48}$-like probe. Moreover, in the S1 nuclease profiles of the OXA-48-producing ST45 *K. pneumoniæ* and ST38 *E. coli* isolates, the *bla*$_{OXA-48}$-like probe hybridized only with largest DNA bands, corresponding to the chromosomal material.

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

### 8.3.4 Structure of OXA-48-like-encoding plasmids

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

All *bla*$_{OXA-48}$-carrying plasmids belonged to type A and were derivatives of the archetypal IncL *bla*$_{OXA-48}$-carrying plasmid pOXA-48 (**Figure 8-1**), originally described in the *K. pneumoniæ* 11978 isolate recovered in Turkey in 2001 and then reported worldwide.[50] Nineteen out of the 22 sequenced *bla*$_{OXA-48}$-carrying plasmids (type A0; **Table 8-1**) showed high degrees of similarity to each other and to pE71T (100% coverage, 99% identity), previously characterized from *K. pneumoniæ* E71T isolated in Ireland.[51] Plasmid pE71T differed from pOXA-48 by the insertion of two copies of IS*1R* element. The carbapenemase gene was part of the Tn*1999.2* transposon, which included IS*1R* integrated in IS*1999* located upstream of the *bla*$_{OXA-48}$ gene.[2] The second IS*1R* was inserted into *orf25*. Plasmids pOXA-48_30715 and pOXA-48_30890 (type A1), both of which were isolated from ST11 *K. pneumoniæ* isolates, differed from pE71T by the insertion of a 1,911 bp fragment encoding a reverse transcriptase (RetA) upstream of the *mucAB* operon. Plasmid pOXA-48_30891 (type A2) was a pE71T derivative carrying a novel variant of the Tn*1999.2* transposon (designated as Tn*1999.5*) in which the *lysR* gene was truncated by the IS*Kpn19* element. Interestingly, plasmids pOXA-48_30715 and pOXA-48_30891 were characterized from two different *K. pneumoniæ* isolates recovered from the same patient (**Table 8-1**). Among all type A *bla*$_{OXA-48}$-carrying plasmids, no resistance genes other than *bla*$_{OXA-48}$ were identified, as previously described for the pOXA-48 and its relatives.[37, 50, 51]

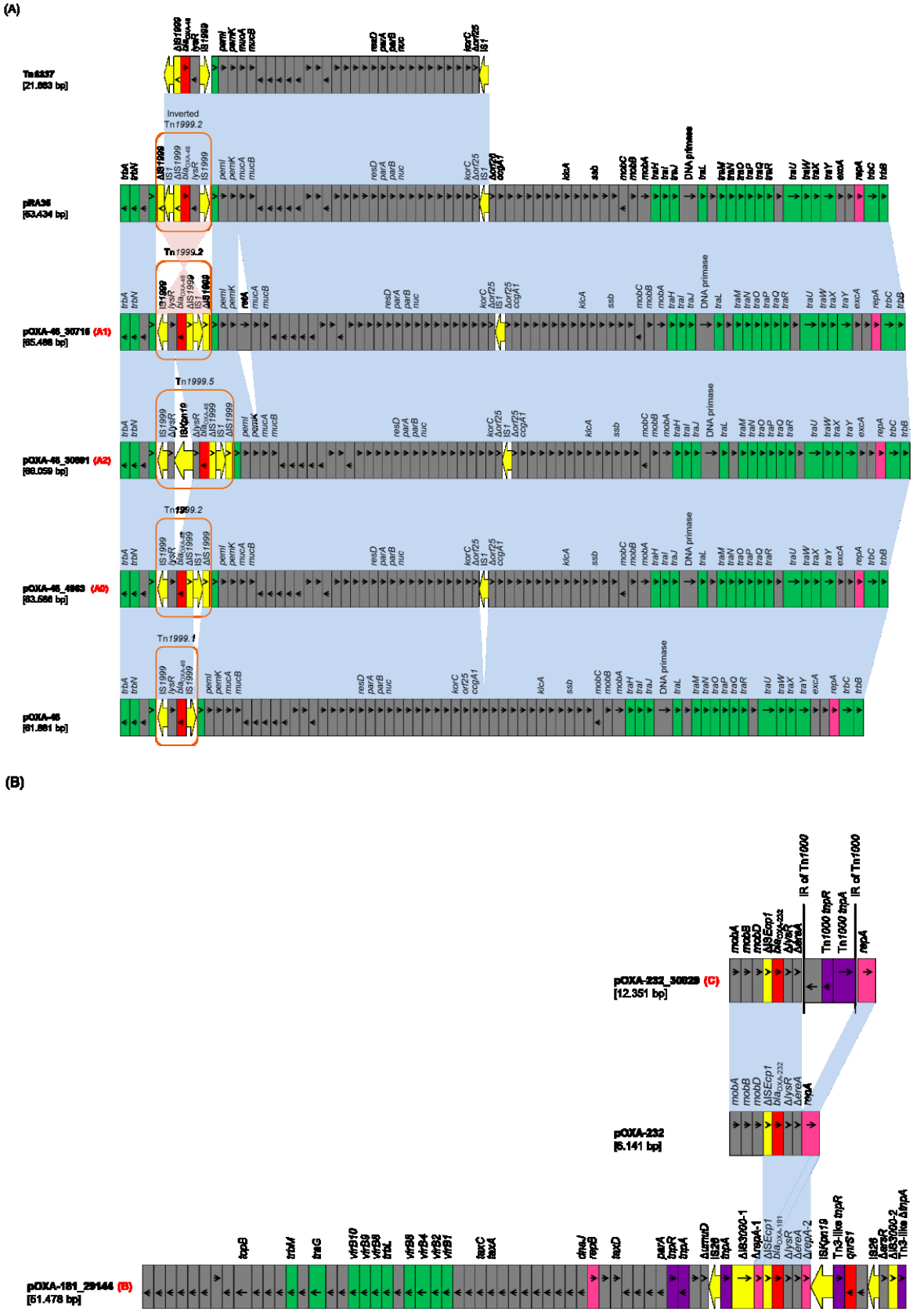


**Figure 8-1** Linear maps of the OXA-48-like-encoding plasmids. For each plasmid, the type of plasmid sequence is indicated in red next to the plasmid name. **(A)** Comparison of IncL $bla_{OXA-48}$-carrying plasmids pOXA-48,[50] pOXA-48_4963, pOXA-48_30715, pOXA-48_30891 and pRA35, and of the composite transposon Tn6237.[53] The boundaries of Tn1999-like transposons are also shown. **(B)** Comparison of the $bla_{OXA-232}$-carrying plasmids

pOXA-232[49] and pOXA-232_30929, and of the *bla*$_{OXA-181}$-carrying plasmid pOXA-181_29144. Open reading frames (ORFs) are shown as rectangles (arrows within rectangles indicate the direction of transcription). Intact insertion sequences are represented by arrows, while truncated insertion sequence elements appear as rectangles. Replicons of the plasmids are indicated as pink rectangles. Resistance genes, insertion sequence elements, and transposases are shown in red, yellow and purple, respectively. Green rectangles indicate genes responsible for conjugative transfer of the plasmids. The remaining genes, including plasmid scaffold regions, are indicated as grey rectangles. Homologous segments (representing ≥99% sequence identity) are indicated by light blue shading, while red shading shows inverted homologous segments.

pOXA-181_29144 (type B) (**Figure 8-1**), encoding OXA-181, was an IncX3-type plasmid that was identical to pOXA181_EC14828 (100% coverage, 100% identity), which to date has been described only in China from an ST410 *E. coli* strain (WCHEC14828), isolated in 2014.[52] Similar to pOXA181_EC14828, the *qnrS1* gene, conferring low-level resistance to fluoroquinolones, was identified in the sequence of pOXA-181_29144. Finally, plasmid-pOXA-232_30929 (type C) appeared to be a derivative of pOXA-232 (**Figure 8-1**), a ColE2-type plasmid originally described from an ST2968 *E. coli* isolate and two ST14 *K. pneumoniæ* isolates recovered from patients who transferred from India to France in 2011.[49] Only one difference between the two plasmids was observed. A 5,981 bp segment consisting of the Tn*1000* transposon was present in pOXA-232_30929 and was found 477 bp upstream of the *repA* gene.

Finally, *de novo* assembling obtained a unique contig containing *bla*$_{OXA-48}$ for ST45 *K. pneumoniæ* and ST38 *E. coli* isolates. Sequence analysis showed that these isolates harbored a 21.9 kb plasmid fragment containing *bla*$_{OXA-48}$ flanked by IS*1R* elements integrated into their chromosomes. This plasmidic fragment consisted of the IS*1R*-based composite transposon (**Figure 8-1**), Tn*6237*.[53] However, using the Illumina MiSeq platform, we were not able to identify the precise insertion site of Tn*6237*.

### 8.3.5 Concluding remarks

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

In four of the patients, two or three different OXA-48 producers were identified during their hospitalization, supposing the *in vivo* horizontal transfer of the *bla*$_{OXA-48}$-carrying plasmid. Sequencing data showed the presence of the same *bla*$_{OXA-48}$-carrying plasmid in three of these cases (**Table 8-1**), further confirming this hypothesis. In addition, the same *bla*$_{OXA-48}$-carrying plasmid

(type A0) was identified in all isolates recovered from patients that were involved in the outbreak, which took place in hospital B.

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

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

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We have no conflicts to declare.

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# 9 Characterization of NDM-encoding plasmids from *Enterobacteriaceæ* recovered from Czech hospitals

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The aim of the present study was to characterize sporadic cases and an outbreak of NDM-like-producing *Enterobacteriaceæ* recovered from hospital settings, in Czechia. During 2016, 18 *Entrobacteriaceæ* isolates including 9 *Enterobacter cloacæ* complex, 4 *Escherichia coli*, 1 *Enterobacter asburiæ*, 1 *Enterobacter intermedius*, 1 *Klebsiella pneumoniæ*, 1 *Klebsiella oxytoca*, and 1 *Raoultella ornithinolytica* that produced NDM-like carbapenemases were isolated from 15 patients. Three of the patients were colonized or infected by two different NDM-like producers. Moreover, an NDM-4-producing *Enterobacter*, isolated in 2012, was studied for comparative purposes. All *Enterobacter* isolates, recovered from the same hospital, were assigned to ST182. Additionally, two *E. coli* belonged to ST167, while the remaining isolates were not clonally related. Thirteen isolates carried *bla*$_{NDM-4}$, while six isolates carried *bla*$_{NDM-1}$ (n=3) or *bla*$_{NDM-5}$ (n=3). Almost all isolates carried *bla*$_{NDM}$-like-carrying plasmids being positive for the IncX3 allele, except ST58 *E. coli* and ST14 *K. pneumoniæ* isolates producing NDM-1. Analysis of plasmid sequences revealed that all IncX3 *bla*$_{NDM}$-like-carrying plasmids exhibited a high similarity to each other and to previously described plasmids, like pNDM-QD28, reported from worldwide. However, NDM-4-encoding plasmids differed from other IncX3 plasmids by the insertion of a Tn*3*-like transposon. On the other hand, the ST58 *E. coli* and ST14 *K. pneumoniæ* isolates carried two novel NDM-1-encoding plasmids, pKpn-35963cz and pEsco-36073cz. Plasmid pKpn-35963cz that was an IncFIB(K) molecule contained an acquired sequence, encoding NDM-1 metallo-β-lactamase (MβL), which exhibited high similarity to the mosaic region of pS-3002cz from an ST11 *K. pneumoniæ* from Czechia. Finally, pEsco-36073cz was a multireplicon

A/$C_2$+R NDM-1-encoding plasmid. Similar to other type 1 A/$C_2$ plasmids, the *bla*$_{NDM-1}$ gene was located within the ARI-A resistance island. These findings underlined that IncX3 plasmids have played a major role in the dissemination of *bla*$_{NDM}$-like genes in Czech hospitals. In combination with further evolvement of NDM-like-encoding MDR plasmids through reshuffling, NDM-like producers pose an important public threat.

**Keywords:** NDM, metallo-β-lactamases, *Enterobacter xiangfangensis*, ST182, IncX3

## 9.1 Introduction

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

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

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

## 9.2 Materials and methods

### 9.2.1 Bacterial isolates and confirmation of carbapenemase production

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

### 9.2.2 Susceptibility testing

The MICs of piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, meropenem, ertapenem, gentamicin, amikacin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, colistin, and tigecycline were determined by the broth dilution

method.[14] Data were interpreted according to the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org).

### 9.2.3 Typing

All isolates were typed by multilocus sequence typing (MLST).[15-18] The databases at https://pubmlst.org/ecloacae/, http://mlst.warwick.ac.uk/mlst/dbs/Ecoli, http://bigsdb.web.pasteur.fr/klebsiella and https://pubmlst.org/koxytoca/ were used to assign STs.

### 9.2.4 Detection of β-lactamases

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

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

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

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

### 9.2.6 Plasmid analysis

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

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

### 9.2.7 Detection of characteristic regions

Based on the results from Illumina sequencing (see below), six PCRs targeting characteristic regions of NDM-4-encoding IncX3 plasmids and ST182 *Enterobacter* genomes sequenced during this study were designed. The selected regions included: *(i)* a Tn*3*-like transposon found in NDM-4-encoding IncX3 plasmids, and *(ii)* four insertions identified in the genome of Encl-922 (see section **9.3.5**). All NDM-producing clinical or recombinant strains were screened for the presence of the regions described above by the use of specific primers (see **Table S1** in the supplemental material).

### 9.2.8 Plasmid and chromosome sequencing

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

Plasmid DNAs from transconjugants or transformants were extracted using a Qiagen Large-Construct kit (Qiagen, Hilden, Germany). Additionally, the genomic DNAs of *Enterobacter* isolates were extracted using a DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy). Multiplexed DNA libraries were prepared, using the Nextera XT Library Preparation kit, and 300-bp paired-end sequencing was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) using the MiSeq v3 600-cycle Reagent kit. Initial paired-end reads were quality trimmed using the Trimmomatic tool v0.33[30] with the sliding window size of 4 bp, required average base quality ≥17 and minimum read length of 48 bases Genomic DNA reads of *E. cloacæ* were consequently assembled using the de Bruijn graph-based *de novo* assembler SPAdes v3.9.1,[31] using k-mer sizes 21, 33, 55, 77, 99 and 127. For assembly of the plasmids, reads were mapped to the reference *E. coli* K-12 substrain MG 1655 genome (GenBank accession no. U00096) using the BWA-MEM algorithm (Li, 2013), in order to filter out the chromosomal DNA. Then, all the unmapped reads were assembled in the same way as described above. The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (www-is.biotoul.fr/), and the open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using the Mauve v2.3.1 program.[32]

Antibiotic resistance genes were identified using the ResFinder 2.1 tool (https://cge.cbs.dtu.dk/services/ResFinder/) with an identity threshold of >90%.[33]

### 9.2.9 Comparative analysis of *E. cloacæ* clinical isolates

Comparative genomic analysis of *Enterobacter* clinical strains was based on statistics calculated by QUAST v4.5[34] and VarScan v2.3.9[35] tools. All quality trimmed Illumina reads of Encl-922 were mapped to contigs of Encl-44578, employing BWA-MEM algorithm v0.7.12[36] and SAMtools v1.3,[37] for the format conversions and analysis of the results. Then, single nucleotide polymorphisms (SNPs) and indels were detected employing VarScan with parameters set as follows: minimum read depth at

a position =6, minimum base quality at a position =20 and minimum variant allele frequency threshold of 0.45. Moreover, SNPs and indels located in a region within 127 bp from any edge of a contig, as well as SNPs and indels harbored by contigs smaller than 2 kb were excluded from further analysis. Remaining SNPs and indels were also manually checked and refined by visualization of mapped data via Tablet v1.14.04.10.[38] Differences in assembly of *E. cloacæ* genomes were inspected using QUAST's Icarus viewer.[39] In order to examine whether SNPs and indels were located in intergenic or coding regions, as well as to find out what are the differences in genetic information between studied isolates, contigs of clinical strains were annotated using Prokka v1.10.[40] Genes harbouring SNPs were compared against NCBI's conserved domain database[41] via CD-Search[42] to identify conserved domain hits. Finally, sequencing data of clinical strains were examined for the presence of prophage sequences using PHAST web server.[43]

### 9.2.10 Nucleotide sequence accession numbers

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

## 9.3 Results

### 9.3.1 Carbapenemase-producing *Enterobacteriaceæ*

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

### 9.3.2 NDM-like-producing isolates

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

NDM-like producers were collected from five Czech hospitals located in three different Czech cities. In hospital B1, an outbreak that included ten patients diagnosed with NDM-like-producing *Enterobacteriaceæ* lasted the studied period. Additionally, two patients colonized or infected with NDM-like producers were reported in hospital B2. The three remaining cases were identified in three different hospitals. None of the patients, treated in hospital B1, had recently traveled abroad or had

been previously hospitalized. The patient treated in hospitals was directly repatriated from a hospital in China, while clinical data weren't available for the remaining patients.

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

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

**Table 9-1** Characteristics of NDM-like-producing *Enterobacteriaceæ*.

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

NA not applicable.

[a] White circles, black squares, and black triangles each indicate the NDM-like-producing isolates recovered from the same patient.

[b] Data for plasmids found in transconjugants are shown in bold; data for plasmids observed in transformants are underlined

The population structure of NDM-like-producing isolates studied by MLST is shown in **Table 9-1**. All *Enterobacter* isolates, which were recovered from hospital B1, belonged to ST182. Of note was that the NDM-4-producing *Enterobacter* that was isolated, in 2012, from the patient previously hospitalized in Sri Lanka[4] was also assigned to ST182. ST182 *Enterobacter* isolates were previously identified in Mexico and were associated with the production of NDM-1 enzyme.[44, 45] Two of *E. coli*, both of which were from hospital B2, belonged to ST167. *E. coli* ST167 was recently found among NDM-5-producing isolates from different healthcare institutions in China.[46, 47] The two remaining *E. coli* isolates were not clonally related and belonged to different STs (ST58 and ST69). The *K. pneumoniæ* isolate was assigned to the high-risk clone ST14,[48] while the *K. oxytoca* isolate was classified into ST2 that belongs to a growing international clonal complex (CC2).[49]

Sequencing of the PCR products revealed three *bla*$_{NDM}$-type genes encoding the NDM-1, NDM-4 and NDM-5 enzymes (**Table 9-1**).[1, 2, 50] NDM-5 is an NDM-1-related MβL variant that differs from NDM-1 by two amino-acid substitutions, Val88Leu and Met154Leu, the former one being its only change with NDM-4. Thirteen of the isolates, all of which were from hospital B1, were found to produce the NDM-4 MβL (**Table 9-1**). The three isolates from hospital B2 produced the NDM-5 enzyme, while the three remaining isolates that were recovered from sporadic cases in three different hospitals expressed NDM-1 carbapenemase. Additionally, most of *bla*$_{NDM}$-like-positive isolates were confirmed to coproduce the extended-spectrum β-lactamase CTX-M-15 (n=18) either alone or along with TEM-1 (n=13) and/or OXA-1 (n=13), whereas the *K. pneumoniæ* and *R. ornithinolytica* isolates also expressed the SHV-12 enzyme. The ST58 NDM-1-producing *E. coli* isolate coproduced CMY-16, CTX-M-15, OXA-10 and TEM-1 β-lactamases.

### 9.3.3 *bla*$_{NDM}$-like-carrying plasmids

The *bla*$_{NDM}$-like genes from all clinical strains were transferred by conjugation (n=14) or transformation (n=5) (**Table 9-1**). All *bla*$_{NDM}$-like-positive recombinants exhibited resistance to piperacillin, piperacillin-tazobactam, cephalosporins and ertapenem, while they remained susceptible to meropenem (**Table S2**). The three NDM-1-producing recombinants also exhibited resistance to aztreonam. Additionally, most of *bla*$_{NDM}$-like-positive recombinants (n=18) were susceptible to non-β-lactam antibiotics.

Plasmid analysis of NDM-4-producing donor and transconjugant strains revealed the transfer of plasmids, all of which were ~55 kb in size (**Table 9-1**). The three NDM-5-producing transformants harbored plasmids of ~45 kb, whereas the three remaining recombinants carried *bla*$_{NDM-1}$-positive plasmids of different sizes (~55 kb, ~150 kb and ~300 kb). Replicon typing showed seventeen of the plasmids, including those sizing ~45 kb, and ~55 kb, were positive for the IncX3 allele. The *bla*$_{NDM-1}$-positive plasmid of ~300 kb was positive for replicons R and A/C, whereas the one remaining *bla*$_{NDM-1}$-carrying plasmid was nontypeable by the PBRT method.[27, 28]

### 9.3.4 Structure of *bla*$_{NDM}$-like-carrying plasmids

The complete sequence of *bla*$_{NDM}$-like-carrying plasmids representative of different plasmid sizes, replicons, and resistance genes (n=10) was determined (**Table 9-1**). Sequence analysis revealed that all IncX3 *bla*$_{NDM}$-like-carrying plasmids exhibited a high similarity to each other and to previously described NDM-like-encoding plasmids, belonging to IncX3 group, reported from worldwide.[51-53] The *bla*$_{NDM-5}$-positive plasmids, pEsco-5256cz and pKlox-45574cz, were almost identical to NDM-5-encoding plasmid pNDM-QD28 (100% coverage, 99% identity) (GenBank accession no. KU167608) that was characterized from a ST167 *E. coli* in China.[52] Differences among these plasmids consisted in few SNPs (n=5), almost all located in mobile elements. Similar to pNDM-QD28, no other resistance genes were detected in these plasmids. Compared to other IncX3 NDM-encoding plasmids, all *bla*$_{NDM-4}$-encoding plasmids differed by the insertion of a Tn*3*-like transposon (nt 7108-14624 in pEncl-44578cz) downstream *topB* gene (**Figure 9-1**). The Tn*3*-like sequence was composed by the 38-bp inverted repeats (IR) of the transposon, *tnpA*, *tnpR* and two ORFs encoding hypothetical proteins. Target site duplications of 5 bp (GTACC) at the boundaries of the Tn*3*-like element indicated insertion by transposition. Of note was that the sequence of pEncl-922cz, isolated in 2012,[4] was identical to the respective sequences of NDM-4-encoding plasmids recovered in the same hospital, during 2016. PCR screening confirmed the presence of the Tn*3*-like transposon in all NDM-4-encoding IncX3 plasmids, isolated in hospital B1, while Tn*3*-like wasn't detected in the remaining *bla*$_{NDM}$-like-positive plasmids that belonged to IncX3 group. Furthermore, the *bla*$_{NDM-1}$-positive plasmid, pRor-30818cz, harbored an additional 7875-bp sequence (nt 40617-48491 in pRor-30818cz) encoding the extended-spectrum β-lactamase SHV-12 (**Figure 9-1**). A similar SHV-12-encoding region was found in the IncX3 *bla*$_{NDM-1}$-positive plasmid pKP04NDM (100% coverage, 99% identity) (GenBank accession no. KU314941) described from a *K. pneumoniæ* isolate in China.

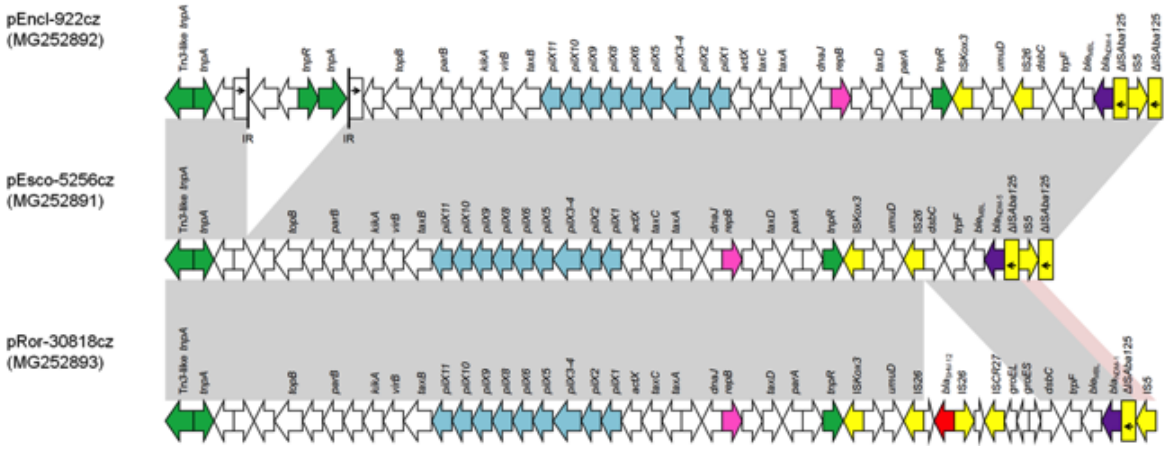
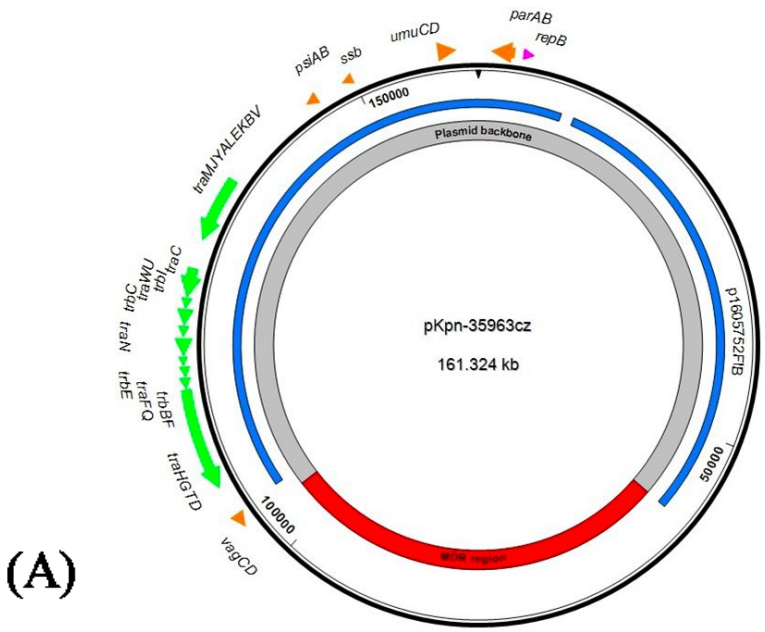



**Figure 9-1** Comparison of linear maps of the NDM-like-encoding IncX3 plasmids pEncl-922cz, pEsco-5256cz, and pRor-30818cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Replicons of the plasmids are shown in pink. *bla*$_{NDM-like}$ genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. Light blue arrows indicate genes responsible for the conjugative transfer of the plasmids. The remaining genes, including plasmid scaffold regions, are shown in white. Homologous segments (representing ≥99% sequence identity) are indicated by light gray shading, while pink shading shows inverted homologous segments.

The NDM-1-encoding plasmid pKpn-35963cz that was nontypeable by the PBRT method[27] was 161324 bp in size. Plasmid pKpn-35963cz was composed of two distinct parts: a contiguous plasmid backbone of 115998 bp (nt 1-58655 and 103982-161324) and an acquired sequence of 45326 bp (nt 58656-103981). The plasmid backbone, which shared similarities with the respective regions of plasmid p1605752FIB (GenBank accession no. CP022125) recovered from a pan-resistant isolate of *K. pneumoniæ* from the United States, harbored regions responsible for replication [*repB* gene; IncFIB(K) replicon], conjugative transfer (*tra* and *trb* genes) and plasmid maintenance (*vagCD*, *psiAB*, *umuCD* and *parAB* operons, and *ssb* gene) (**Figure 9-2**). The acquired sequence of pKpn-35963cz contained a 17836-bp segment (nt 77360-95195) encoding NDM-1, which was similar to the mosaic region of pS-3002cz (99% identity). pS-3002cs was characterized from an ST11 *K. pneumoniæ* isolate identified in Czechia.[5] The acquired sequence of pKpn-35963cz contained two additional segments that have also been described in pS-3002cz. The first segment (nt 65518-72935) included genes encoding an EcoRII methylase and EcoRII endonuclease, and the class 1 integron In191 carrying the *dfrA14* resistance gene. The second segment (nt 101342-103981) contained fragments of transposons Tn*1000* (ΔTn*1000*) and Tn*1331* (ΔTn*1331*). ΔTn*1331* comprised *tnpR* and *aacA4* resistance gene. Furthermore, the acquired sequence of pKpn-35963cz carried a macrolide resistance operon [*mph*(A)], and regions encoding OXA-1 and CTX-M-15 β-lactamases (**Figure 9-2**). In the acquired sequence of pKpn-35963cz, intact and truncated copies of several mobile elements that may have been implicated in the formation of this region were found.




(A)

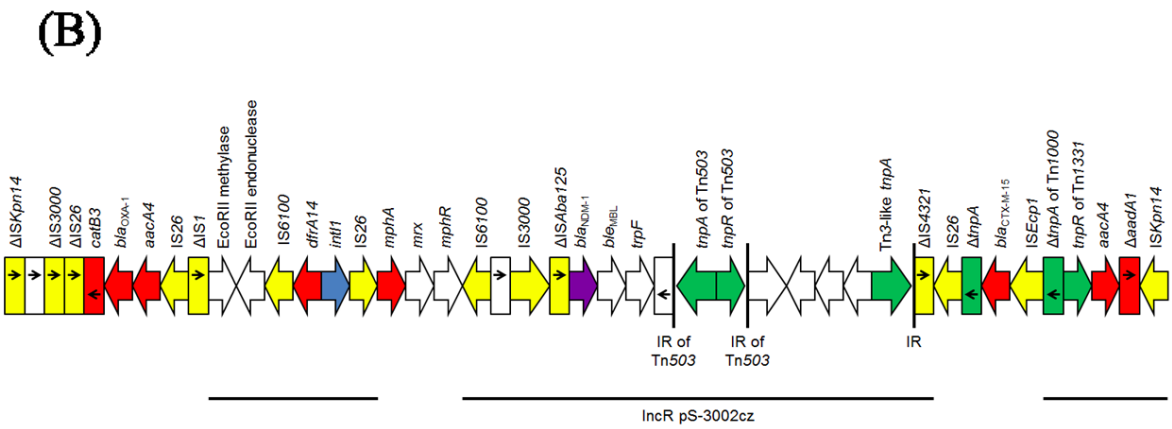



**Figure 9-2 (A)** Overview of the plasmid pKpn-35963cz. The innermost circles show the main regions of the plasmids. Similarities with other plasmids are shown in the next circle; each color represents a unique plasmid. In the outer circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in green and orange, respectively. **(B)** Linear map of the multidrug resistance region (MDR) of the plasmid pKpn-35963cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). *bla*$_{\text{NDM-like}}$ genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. intI1 genes are shaded blue. The remaining genes are shown in white. Thin lines below the map correspond to highly similar sequences from other plasmids.

The plasmid pEsco-36073cz, which encoded the NDM-1 carbapenemase, is 300958 bp in size. The plasmid showed a complex structure, being composed of sequences of diverse origin (**Figure 9-3**). A 170314-bp sequence (nt 232204-300958 and 1-101559) resembled the type 1 A/C$_2$ plasmid pRH-1238 (94% coverage, 99% identity) (**Figure 9-3**), characterized from a *Salmonella enterica* serovar Corvallis strain isolated from a migratory wild bird in Germany.[54] Analysis of A/C$_2$-associated sequence by the core gene PMLST (cgPMLST) scheme[55] indicated that it belonged to cgST3.4. The A/C$_2$ backbone was composed of regions responsible for replication (*repA* gene), conjugative transfer (Tra1 and Tra2 regions), and plasmid maintenance (*higBA* and *parAB* operons and *xerD*- and *kfrA*-like genes). Apart from the backbone, pEsco-36073cz carried the *bla*$_{\text{CMY-2}}$-like-containing region, and the ARI-B and ARI-A resistance islands, as previously described in other type 1 A/C$_2$ MDR plasmids.[56, 57] The *bla*$_{\text{NDM-1}}$ gene was located within ARI-A, in a genetic environment similar to those previously identified in pRH-1238.[54] However, unlike in pRH-1238, the ARI-A of pEsco-36073cz lacked the macrolide resistance determinant *mphA-mel-repAciN*. Furthermore, a class 1 integron with *aacA4* and *aphA1* gene cassettes was located between *resI* and *resII* sites of the Tn*1696* module. The ARI-A of pEsco-36073cz also carried a new integron, In1459, whose variable region comprised the *dfrA14*, *arr-2*, *cmlA1*, *bla*$_{\text{OXA-10}}$, *aadA1* cassettes. Additionally, pEsco-36073cz included fragments resembling the backbone of the recently described IncR plasmid pKP1780,[58] and sequences previously found in the plasmid pPSP-a3e[59] and in the chromosomes of several Gram-negative rods. Genes encoding for resistance to arsenate, cooper and mercury were identified in the three remaining acquired regions of pEsco-36073cz.

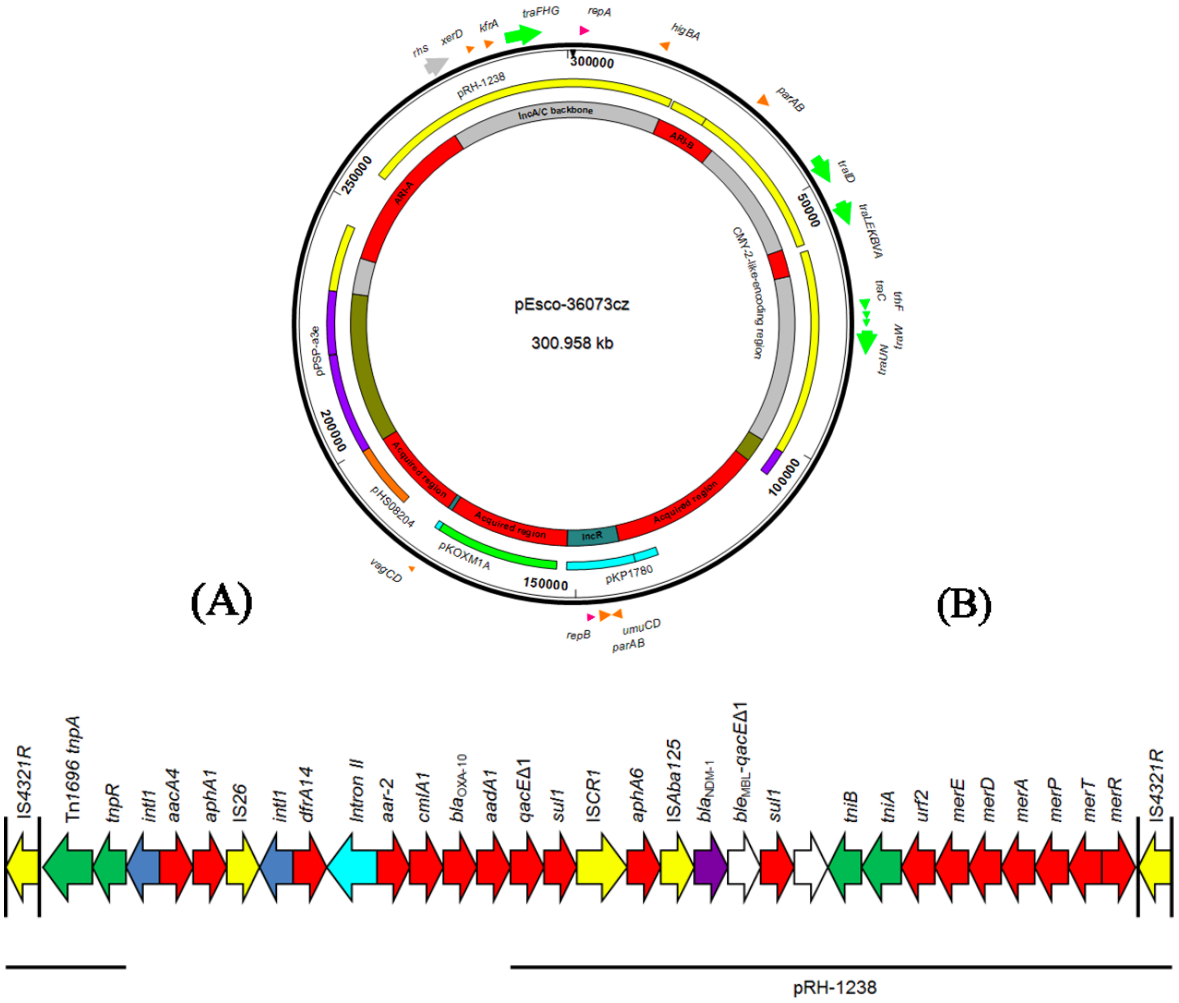



**Figure 9-3 (A)** Overview of the plasmid pEsco-36073cz. The innermost circles show the main regions of the plasmids. Similarities with other plasmids are shown in the next circle; each color represents a unique plasmid. In the outer circle, indicative genes and the direction of transcription are shown by arrows. Replicons of the plasmid are indicated as pink arrows. Genes responsible for plasmid transfer and maintenance are shown in green and orange, respectively. **(B)** Linear map of the ARI-A resistance island of the plasmid pEsco-36073cz. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). *bla*$_{\text{NDM-like}}$ genes are shaded purple, while other resistance genes are shown in red. IS elements and transposases are shown in yellow and green, respectively. intI1 genes are shaded blue; teal blue arrow indicates the group II intron. The remaining genes are shown in white. Thin lines below the map correspond to highly similar sequences from other plasmids.

### 9.3.5 Comparative analysis of *Enterobacter* isolates

'*In silico*' *hsp60* typing of the genome sequences[60] showed that both isolates belonged to the recently recognized *E. xiangfangensis* species.[61]

Since all *Enterobacter* isolates belonged to the same ST and carried the same IncX3 *bla*$_{\text{NDM-4}}$-carrying plasmid, the WGS data of clinical strains Encl-922 and Encl-44578 were compared, using QUAST and VarScan tools, in order to examine the phylogenetic relationship of the isolates recovered in 2012 and 2016.

Comparative analysis of *Enterobacter* clinical isolates revealed that the genome of Encl-922 exhibited extensive similarity (99.87% identity) to the genome of Encl-44578. Sixteen SNPs were identified in

the genome of Encl-922, compared to that of Encl-44578, five of which were located within prophage regions (**Table 9-2**). Interestingly, Encl-922 harbored three large insertions of 8933 bp (nt 439392-448324 in node 2), of 17903 bp (nt 17786-35688 in node 32) and of 13165 bp (nt 1-13165 in node 27; prophage sequence PHAGE_Salmon_SPN3UB_NC_019545). Additionally, Encl-922 harbored an insertion of 33-bp sequence (AACCCTCTCCCCAAAGGGGAGAGGGGACGATTA) located in an intergenic region. Moreover, Encl-922 showed a single nucleotide (G) deletion leading to CDS annotation change of general stress protein 39 to putative oxidoreductase YghA. Analysis of whole genome sequencing (WGS) data by PHAST web server found five intact prophage sequences (PHAGE_Haemop_HP2_NC_003315, PHAGE_Salmon_SPN3UB_NC_019545, PHAGE_Entero_mEp390_NC_019721, PHAGE_Pseudo_PPpW_3_NC_023006, and PHAGE_Salmon_SP_004_NC_021774) and one questionable prophage region (PHAGE_Entero_SfI_NC_027339), in both *Enterobacter* isolates. However, Encl-922 included one additional incomplete prophage region (PHAGE_Salmon_SPN3UB_NC_019545), which was absent from the Encl-44578 genome.

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

## 9.4 Discussion

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

The setting that was most affected was hospital B1, in which an outbreak of NDM-4-producing ST182 *E. xiangfangensis* isolates took place. Of note was that the *Enterobacter*, isolated in 2012 from a patient who had been previously hospitalized in Sri Lanka,[4] also belonged to ST182 and harbored an IncX3 $bla_{\text{NDM-4}}$-positive plasmid being identical to respective plasmids characterized from *Enterobacter* isolates recovered from patients treated in hospital B1 (**Table 9-1**), during 2016. However, comparative genome analysis revealed the presence of four insertions in the genome of *Enterobacter* Encl-922 isolate. These insertions were not found in the genomic DNA of *Enterobacter* isolates from 2016, suggesting a second insertion event of NDM-4-producing *Enterobacter* isolates in Czech hospitals.

**Table 9-2** Summary table of sixteen SNPs found between the genomes of *Enterobacter* isolates Encl-44578 (reference) and Encl-922 (query).

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

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

In three of the patients, two different NDM-like producers were identified during their hospitalization, supposing the *in vivo* horizontal transfer of *bla*$_{NDM}$-like-carrying plasmids. Sequencing and PCR screening data revealed the presence of the same *bla*$_{NDM-4}$- or *bla*$_{NDM-5}$-carrying plasmid in these isolates (**Table 9-1**). These results confirmed the hypothesis of the *in vivo* horizontal transfer of *bla*$_{NDM}$-like-carrying plasmids.

Results from Illumina sequencing showed that IncX3 plasmids have played a major role in the dissemination of *bla*$_{NDM}$-like genes in Czech hospitals, which is in agreement with the findings from previous studies from worldwide.[51-53] In the current study, three *bla*$_{NDM}$-type genes, encoding the NDM-1, NDM-4, and NDM-5 enzymes, were associated with IncX3 plasmids exhibiting high similarity to each other. Considering also the fact that NDM-1, NDM-4 and NDM-5 differ by one or two amino-acid substitutions may indicate the possibility that *bla*$_{NDM}$-like genes encoding NDM-1-related variants have evolved in the same plasmid type. Additionally, Illumina data showed the presence of a unique sequence, a Tn*3*-like transposon, in sequenced *bla*$_{NDM-4}$-carrying plasmids. PCR confirmed the presence of the Tn*3*-like sequence in all transconjugants, carrying *bla*$_{NDM-4}$-positive plasmids. Thus, the PCR targeting the Tn*3*-like sequence was able to distinguish *bla*$_{NDM-4}$-positive plasmids from other IncX3 plasmids carrying *bla*$_{NDM-1}$ or *bla*$_{NDM-5}$. On the other hand, two of the sporadic isolates carried novel NDM-1-encoding plasmids. Plasmid pKpn-35963cz that was an IncFIB(K) molecule contained an acquired sequence, encoding NDM-1 MβL, which exhibited high similarity to the mosaic region of pS-3002cz from an ST11 *K. pneumoniæ* from Czechia.[5] Whereas plasmid pEsco-36073cz was a multireplicon A/C$_2$+R NDM-1-encoding plasmid, being a fusion

derivative of sequences of diverse origin. Similar to other type 1 $A/C_2$ plasmids,[54, 56] the *bla*$_{NDM-1}$ gene was located within the ARI-A resistance island.

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

**Author Contributions**

CCP and JH played an important role in interpreting the results and in writing the manuscript. VJ, TB and ZH helped to acquired data. VP, MM, AS, KC, and IB carried out experimental work. CCP supervised the experiments and revised the final manuscript, which was approved by all authors.

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## 10 Molecular characterization of carbapenemase-producing *Pseudomonas æruginosa* of Czech origin and evidence for clonal spread of extensively resistant sequence type 357 expressing IMP-7 metallo-β-lactamase


Molecular Characterization of Carbapenemase-Producing *Pseudomonas aeruginosa* of Czech Origin and Evidence for Clonal Spread of Extensively Resistant Sequence Type 357 Expressing IMP-7 Metallo-β-Lactamase

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The objective of this study was to perform molecular surveillance for assessing the spread of carbapenemase-producing *Pseudomonas æruginosa* in Czech hospitals. One hundred thirty-six carbapenemase-producing isolates were recovered from 22 hospitals, located throughout the country. Sequence type 357 (ST357) dominated (n=120) among carbapenemase producers. One hundred seventeen isolates produced IMP-type (IMP-7 [n=116] and IMP-1 [n=1]) metallo-β-lactamases (MβLs), 15 produced the VIM-2 MβL, and the remaining isolates expressed the GES-5 enzyme. The

*bla*$_{IMP}$-like genes were located in three main integron types, with In-p110-like being the most prevalent (n=115). The two other IMP-encoding integrons (In1392 and In1393) have not been described previously. *bla*$_{VIM-2}$-carrying integrons included In59-like, In56, and a novel element (In1391). *bla*$_{GES-5}$ was carried by In717. Sequencing data showed that In-p110-like was associated with a Tn*4380*-like transposon inserted in genomic island LESGI-3, in the *P. æruginosa* chromosome. The other integrons were also integrated into the *P. æruginosa* chromosome. These findings indicated the clonal spread of ST357 *P. æruginosa*, carrying the IMP-7-encoding integron In-p110, in Czech hospitals. Additionally, the sporadic emergence of *P. æruginosa* producing different carbapenemase types, associated with divergent or novel integrons, punctuated the ongoing evolution of these bacteria.

**Keywords:** VIM, GES, ST235, ST111, Illumina sequencing, class 1 integrons, genomic islands (GIs), integrative conjugative element (ICE)

## 10.1 Introduction

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

MβLs are zinc-dependent enzymes commonly characterized by the ability to hydrolyze all β-lactams (with the exception of monobactams), including carbapenems. Their activity is not affected by the currently available β-lactamase inhibitors (i.e., clavulanic acid, tazobactam, sulbactam, or avibactam). Contrary to $bla_{NDM}$ genes, $bla_{VIM}$ and $bla_{IMP}$ occur as gene cassettes in class 1 integrons or, more rarely, integrons of class 2 or 3.[9-11] These integrons often also contain gene cassettes conferring resistance to other antibiotics, like aminoglycosides and trimethoprim. Integrons cannot mobilize themselves but often reside within transposon structures, which, in turn, may be found in plasmids or in chromosomes. Frequently, class 1 integrons found in chromosomes are associated with genomic islands (GIs) of pathogenic bacteria,[12, 13] like the *Salmonella* genomic island 1 (SGI1).[14] These islands may also contain other resistance- or virulence-associated genes.[15, 16]

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

In the Czech Republic, carbapenem-resistant *P. æruginosa* strains are currently a critical problem in the management of health care-associated infections. The first MβL-producing *P. æruginosa* isolates were identified in 2008. The isolates that produced the IMP-7 enzyme were assigned to ST357,[19, 20] which was previously reported in IMP-1-producing *P. æruginosa* from Japan.[21] In another study, carbapenemase-producing *P. æruginosa* isolated from a hospital in Brno,[22] during the period 2009-2011, belonged to STs 111 and 357 and carried VIM-2-encoding integrons In-p385 (*aacA29a-*$bla_{VIM-2}$)[22] and In56 ($bla_{VIM-2}$)[23] or the IMP-7-encoding integron In-p110 (*aacA4-orf105/orfD-*$bla_{IMP-7}$*-aacA4-*$bla_{OXA-2}$*-orfE*-like).[22] However, the previous data are the outcome of reports describing the sporadic emergence of MβL-producing *P. æruginosa* in Czech hospitals at the beginning of the spread of these bacteria.

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

## 10.2 Materials and methods

### 10.2.1 Bacterial isolates and confirmation of carbapenemase production

In 2015, 43 microbiological laboratories, covering all of the Czech Republic, collected nonrepetitive *P. æruginosa* isolates that were nonsusceptible to meropenem (MIC >2 µg/ml). All meropenem-nonsusceptible isolates were sent to the National Reference Laboratory (NRL) for Antibiotics for further analysis. Species identification was confirmed by matrix-assisted laser desorption ionization time-of flight mass spectrometry (MALDI-TOF MS) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). All isolates were tested for carbapenemase production by MALDI-TOF MS imipenem hydrolysis assay.[24] Isolates that were positive by MALDI-TOF MS imipenem hydrolysis assay were subjected to phenotypic detection of carbapenemases using double-disc synergy test (DDST) with EDTA and the phenylboronic acid disc test.[25, 26] Carbapenemase genes (*bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$-like) were detected by PCR amplification.[27-31] Additionally, 94 meropenem-susceptible *P. æruginosa* isolates (representative of all different hospitals and susceptibility profiles that are part of the European Antimicrobial Resistance Surveillance Network) recovered from positive blood cultures of patients treated in Czech hospitals during 2015 were included in this study for comparative purposes.

### 10.2.2 Susceptibility testing

MICs of piperacillin, piperacillin-tazobactam, ceftazidime, cefepime, meropenem, tobramycin, gentamicin, amikacin, colistin, and ciprofloxacin were determined by the broth dilution method.[32] Data were interpreted according to the criteria (version 7.1) of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org).

### 10.2.3 Typing

All *P. æruginosa* isolates were typed by multilocus sequence typing (MLST).[33] The database at https://pubmlst.org/pæruginosa was used for assigning STs. Based on allelic profiles, a graphical tool-based minimal spanning tree build in Bionumerics 7.6 (Applied-Maths, Austin, TX) was constructed.

### 10.2.4 Detection of virulence-associated genes

All isolates were PCR screened for the presence of genes encoding ExoS, ExoT, ExoU, and ExoY toxins of the *P. æruginosa* type III secretion system.[34] Primers and conditions for PCR amplification were used as described previously.[35]

### 10.2.5 Integron analysis

Variable regions of class 1 integrons with *bla*$_{IMP}$-, *bla*$_{VIM}$- and *bla*$_{GES}$-like genes were amplified in two parts, from the 5' conserved segment (5'CS) to carbapenemase-encoding cassette, and from carbapenemase-encoding cassette to the 3' conserved segment (3'CS).[22] Whole-gene arrays were sequenced using an ABI 3500 sequencer (Applied Biosystems, Foster City, CA). The Integrall integron database (http://integrall.bio.ua.pt)[36] was used to analyze and assign integron sequences.

### 10.2.6 Transfer of carbapenemase-encoding genes

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

Plasmid extractions from *P. æruginosa* isolates were carried out using a Qiagen Maxi kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Transformations were attempted by electroporation into *E. coli* DH5α cells. Transformants were selected on Luria-Bertani agar plates with ampicillin (50 μg/ml).

### 10.2.7 Whole-genome sequencing

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

The genomic DNAs of *P. æruginosa* were extracted using the DNA-Sorb-B kit (Sacace Biotechnologies S.r.l., Como, Italy), and were sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). Initial paired-end reads were quality trimmed using Trimmomatic tool v0.32[37] and assembled via de Bruijn graph-based de novo assembler SPAdes v3.6.0.[38] The sequence gaps were filled by a PCR-based strategy and Sanger sequencing. For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISFinder database (www-is.biotoul.fr/), and open reading frame (ORF) finder tool (www.bioinformatics.org/sms/) were utilized. Comparative genome alignments were performed using Mauve 2.3.1.[39]

Antibiotic resistance genes were identified using the ResFinder 2.1 tool (https://cge.cbs.dtu.dk/services/ResFinder/) with an identity threshold of >90%.[40]

### 10.2.8 Bayesian analysis

Additionally, Illumina data were used for phylogenetic analysis. Briefly, core genomes were extracted employing NUCmer v3.1[41] for detection of shared genome content among isolates, and a home-made script for extraction of core sequences. The resulting core genome sequences were 5.468 Mbp (range, 5,467,960 to 5,468,214 bp). They then were aligned by MAFFT v7.215[42] using default gap penalties, memsave parameter, and progressive FFT-NS-2 strategy. For the purposes of our analysis, we applied Bayesian statistics via MrBayes v3.2.6[43] using the following parameters: mixed model of nucleotide substitution, gamma-distributed rates among sites, four Monte Carlo Markov chains for 1,000,000 cycles, chains sampled every 1,000$^{th}$ generation, and first 25% of the samples were discarded as burn-in. The obtained summary statistics of Bayesian analysis were as following: average standard deviation of split frequencies, 0.0035; maximum standard deviation of split frequencies, 0.0301; average potential scale reduction factor, 1.000; and maximum potential scale reduction factor, 1.010. The final tree topology was generated using 50% majority-rule consensus and was visualized via iTOL v3.5.2[44] and edited by Inkscape v0.91 (www.inkscape.org).

### 10.2.9 Nucleotide sequence accession numbers

One nucleotide sequence representing each integron type was submitted to the GenBank, under accession numbers KY860566-KY860573.

## 10.3 Results and Discussion

### 10.3.1 Carbapenemase-producing *P. æruginosa*

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

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) imipenem hydrolysis assay[24] indicated carbapenemase production in 136 out of the 194 meropenem-nonsusceptible isolates. All MER-S isolates were negative in the MALDI-TOF MS imipenem hydrolysis assay. In 132 of the isolates, the EDTA-meropenem test[25] appeared to be positive, indicating MβL production. The remaining four isolates tested positive by the boronic acid-meropenem combined disc test,[26] suggesting carbapenemase production of the Ambler class A type. PCR screening showed that 117 of the isolates were positive for the presence of $bla_{\text{IMP}}$ and 15 isolates were positive for $bla_{\text{VIM}}$ genes. The four MβL-negative isolates were positive for the presence of $bla_{\text{GES}}$ genes. Nineteen of the carbapenemase producers, carrying $bla_{\text{IMP}}$ genes, were recovered from positive blood cultures. Carbapenemase producers were collected from 22 hospitals, located throughout the country (**Figure 10-1**).

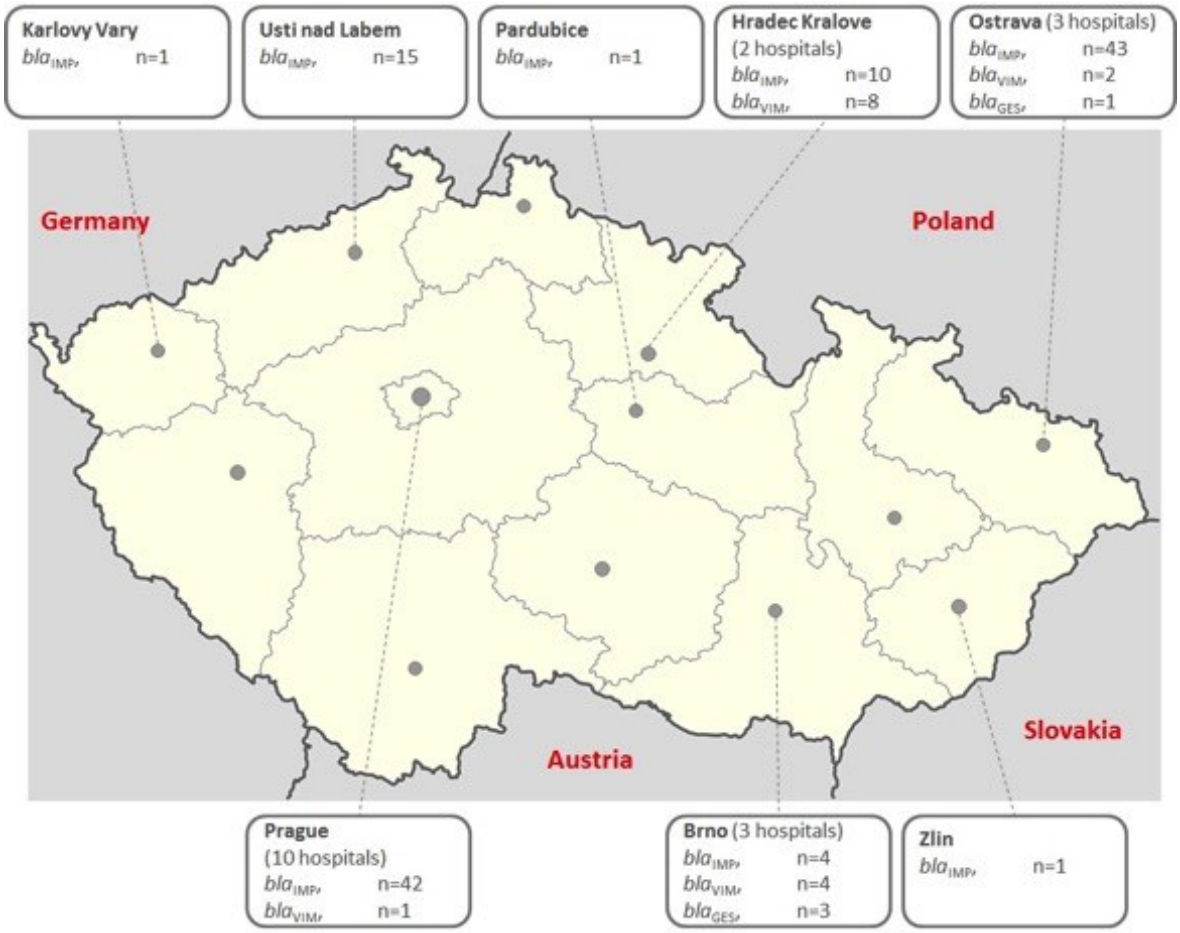


**Figure 10-1** Geographical map showing the locations of the hospitals as well as the number of the carbapenemase-producing *P. æruginosa* isolates (CPP) collected during the study.

Susceptibilities, in terms of MIC ranges, are presented in **Table 10-1**. $MIC_{50}$ and $MIC_{90}$ values for the group of 136 carbapenemase-producing *P. æruginosa* (CPP) isolates were >64 µg/ml for ceftazidime and cefepime and >32 µg/ml for meropenem. The majority of CPP isolates was resistant to ceftazidime (n=136, 100%), cefepime (n=134, 98.5%), meropenem (n=134, 98.5%), piperacillin (n=134, 98.5%) and piperacillin-tazobactam (n=127, 93.8%). One hundred thirty-four (98.5%) CPP isolates also exhibited resistance to ciprofloxacin, 131 (96.3%) were resistant to tobramycin, 117 (86.0%) were resistant to gentamicin, and 63 (46.3%) were resistant to amikacin, while only 5 (3.7%) isolates were resistant to colistin. The 58 non-carbapenemase-producing *P. æruginosa* (N-CPP) isolates exhibited lower MICs of ceftazidime, cefepime and meropenem than those observed for CPP isolates. Additionally, MICs of non-β-lactam antibiotics, except colistin, were higher for the groups of CPP and N-CPP isolates than for MER-S isolates. The majority of MER-S isolates were also susceptible to cefepime (n=89, 94.7%), ceftazidime (n=88, 93.6%), piperacillin-tazobactam (n=82, 87.2%), and piperacillin (n=81, 86.2%).

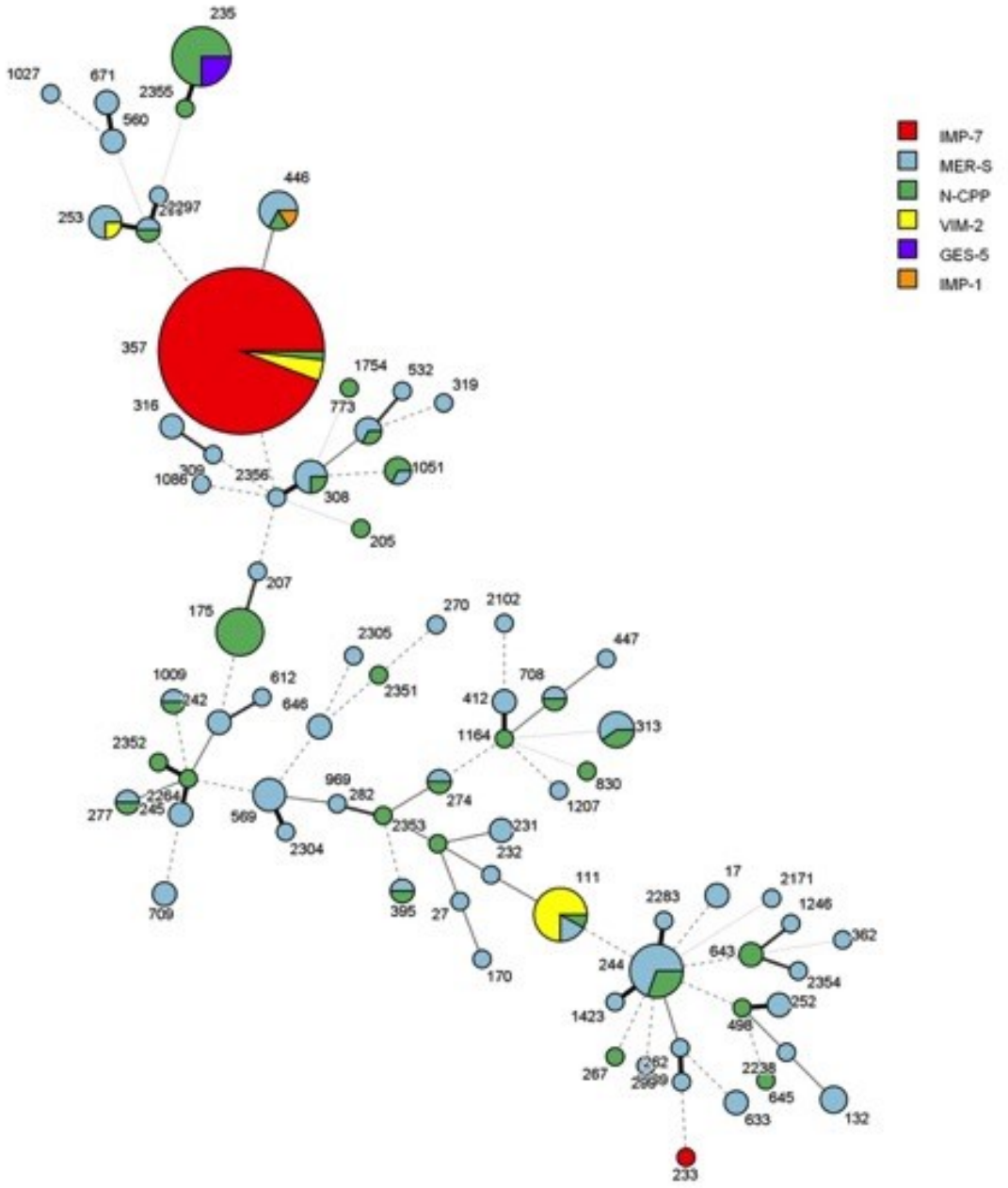


**Figure 10-2** Minimal spanning tree of 287 *P. æruginosa* isolates, recovered from Czech hospitals during 2015, showing sequence types (STs) versus carbapenemase content. Each circle corresponds to an ST. The area of each circle is proportional to the number of isolates. The style of the connecting lines between STs correspond to the number of allelic differences: up to 3 differences, solid lines; above 3 allelic differences, dashed lines.

The population structure of *P. æruginosa* isolates studied by MLST is shown in **Figure 10-2**. The CPP isolates comprised 7 STs. ST357 was the most prevalent, accounting for 120 CPP isolates (**Table 10-1**). The majority of ST357 isolates were associated with production of IMP-type MβLs (n=115), while only 5 out of the 15 isolates that produced VIM-type MβLs belonged to ST357. Thirteen of CPP isolates were distributed in STs 111 (n=9) and 235 (n=4), which have been considered high-risk clones.[17] The

three remaining CPP isolates belonged to distinct STs. However, significant genetic diversity was found in the groups N-CPP and MER-S isolates (see **Table S1** in the supplemental material). The group of 58 N-CPP isolates comprised 29 clones, with STs 175 (n=10) and 235 (n=12) accounting for 22 of the isolates. On the other hand, 58 different STs were identified among the group of 93 MER-S isolates. Most of the observed STs have been reported previously from *P. æruginosa* isolates of Czech origin.[45] However, five N-CPP and six MER-S isolates were assigned to novel STs (ST2297, ST2304, ST2305, ST2350, ST2351, ST2351, ST2352, ST2353, ST2354, ST2355, and ST2356). Of note was that high-risk clones ST235 and ST357 were not found among the MER-S isolates.

Furthermore, all *P. æruginosa* isolates were examined for the presence of the ExoS, ExoT, ExoU, and ExoY toxin-encoding genes. The *exoT* (n=287; 100%) and *exoY* (n=281; 97.9%) genes were present in the majority of the isolates (**Table S1**), while *exoU* was found in only 184 (64.1%) of the isolates. The *exoS* (n=110; 38.3%) gene was identified in less than half of the isolates. These results are in agreement with previous studies reporting that even if *exoY* and *exoT* are present in nearly all clinical isolates, a significant number lack either *exoS* or *exoU*.[46, 47] Additionally, our results confirmed that the high-risk clones belonging to STs 111 and 175 were associated with the copresence of *exoS*, *exoT*, and *exoY* genes, while those belonging to STs 235 and 357 were associated with the copresence of *exoT*, *exoU*, and *exoY*. Previous studies have demonstrated that the presence of ExoS, ExoT, or ExoU secretion correlates with a higher risk of mortality.[48] In particular, ExoU correlates with acute cytotoxicity and lung damage.[47]

### 10.3.2 Carbapenemase-encoding integrons

Characterization of the regions, flanking the carbapenemase-encoding genes, by PCR mapping and sequencing showed that *bla*$_{IMP}$-like genes were located in three main types of class 1 integrons (**Table 10-2**). The most prevalent were In-p110-like integrons, identified in 115 ST357 CPP isolates. The canonical In-p110[22] occurred in 94 ST357 isolates. However, the 21 remaining ST357 IMP-7 producers carried an In-p110 derivative, without the *orf105/D* cassette. Among ST233 and ST446 IMP-producing isolates were identified new integron types, designated In1393 and In1392, respectively. On the other hand, the most common (n=9) *bla*$_{VIM}$-carrying integron was an In59 derivative, differing from In59[49] by having a second copy of *aacA29a* instead of *aacA29b*. In59-like integrons were found in ST111 CPP isolates. Among ST357 VIM producers, the class 1 integron In56[23] was identified. The ST253 VIM-producing isolate carried a new integron, In1391. Finally, the class 1 integron In717[13] with *bla*$_{GES-5}$ was identified in the ST235 CPP isolates.

**Table 10-1** Susceptibility data of *P. æruginosa* isolates[a]

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

[a] Rows representing the total number of isolates per category are in bold.
[b] MIC50 and MIC90 values (MICs for 50% and 90% of the organisms, respectively) are also presented (in parentheses) for several groups of isolates. Pip, piperacillin; Tzp, piperacillin-tazobactam (inhibitor fixed at 4 μg/ml) Caz, ceftazidime; Fep, cefepime; Mer, meropenem; Gen, gentamicin; Tob, tobramycin; Amk, amikacin; Cip, ciprofloxacin; Col, colistin.

**Table 10-2** Integrons with carbapenemase-encoding genes identified in *P. æruginosa* isolates from Czech hospitals.

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

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

### 10.3.3 Chromosomal location of carbapenemase-encoding integrons

Repeated attempts to transfer carbapenemase-encoding genes from *P. æruginosa* isolates, representative of different STs and integron types (n=22), into *Escherichia coli* DH5α strain were unsuccessful. This finding suggested the chromosomal location of the carbapenemase-encoding integrons.

Thus, the complete sequence of these isolates (**Table 10-3**) was determined. Illumina sequencing revealed that, in all cases, carbapenemase-encoding integrons were inserted in the chromosomes of *P. æruginosa* isolates.

### 10.3.4 In-p110-like, In1392, and In717 integrons

In-p110-like, In1392, and In717 integrons were in LESGI-3, located in the chromosome (**Figure 10-3**). LESGI-3 was previously reported in *P. æruginosa* LESB58, an epidemic strain from the United Kingdom.[50] All three integrons were embedded in the mercury resistance transposon Tn*4380*. The IRi of the integrons were located downstream of the *resI* site of the Tn*4380* module in precisely the same position as In717 in Tn*6163*.[13] Similar to Tn*6163* in *P. æruginosa* C79, an IS*1071* element was inserted into the *tnpA* gene of the transposon.

The 3'conserved segment (3'CS) of In-p110 was truncated 526 bp after the start codon of *sul1*. Downstream of Δ*sul1*, a 2875-bp fragment of a Tn*3*-like transposon, consisting of the IRtnp of the transposon and the 3'-end of *tnpA*, was found. The *tnpA* gene was probably deleted due to insertion of an IS*6100* element.

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

Unlike in *P. æruginosa* C79, the 3'CS of In717 was truncated after the start codon of *sul1* in ST235 *P. æruginosa* Pae-30094cz and Pae-30653cz isolates. Downstream of Δ*sul1*, there was a *cmlA9*-*tetR*(G)-*tetA*(G)-IS*CR3*-*groEL*/*intI1*-*sul1*-*orf5* sequence. This sequence appeared as part of the *bla*$_{\text{VIM-2}}$-containing Tn*501*-like transposon from *P. æruginosa* isolates from northeast Ohio.[15] *orf5* was disrupted by an IS*6100* element.

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

**Table 10-3** Characteristics of 25 *P. æruginosa* isolates sequenced by Illumina platform.

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

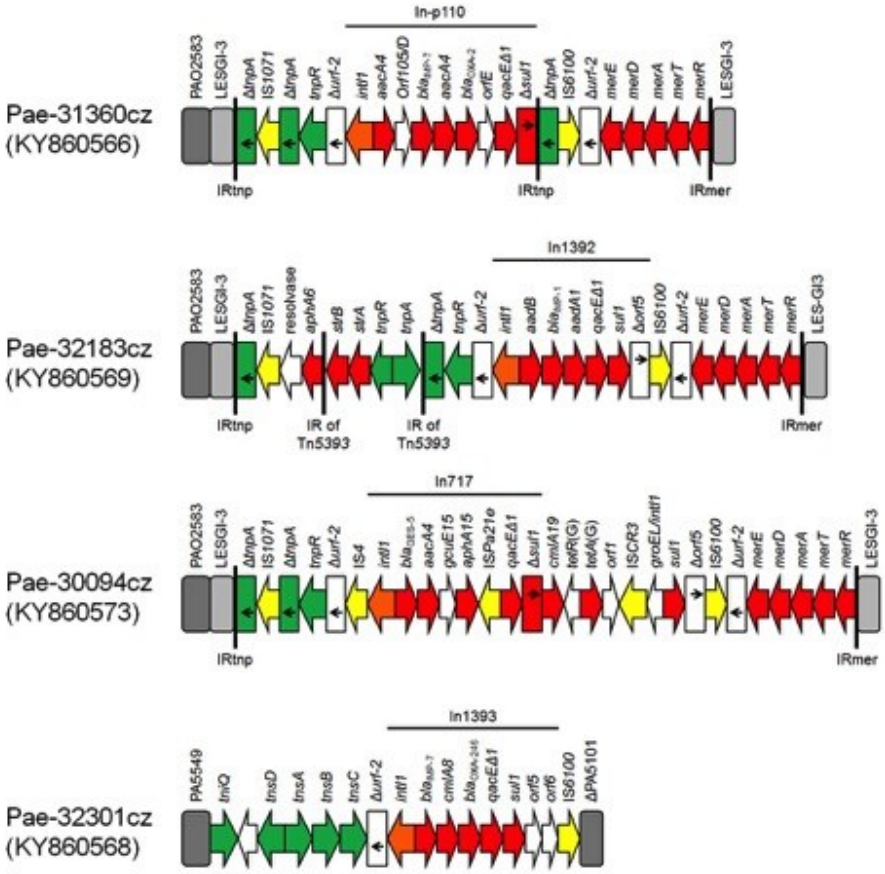


**Figure 10-3** Linear maps of the genetic context of carbapenemase-encoding class 1 integrons In-p110, In1392, and In717 inserted in LESGI-3 (**A**), and In1393 integrated into *P. æruginosa* chromosome (**B**). Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements, and transposases are shown in red, yellow, and green, respectively. intI1 genes are shaded orange. Sequences associated with GIs are shaded light gray; dark gray rectangles indicate the *P. æruginosa* chromosome. The remaining genes are shown in white.

### 10.3.5 In1393

Sequencing data showed that In1393 was also located in *P. æruginosa* chromosome (**Figure 10-3**). Both the 5'CS and 3'CS of the integron were intact. The IRi of In1393 was adjacent to a 9,685-bp sequence (nucleotide [nt] 9349 to 19033 in GenBank accession no. KY860568) exhibiting no identity with already characterized transposition modules. However, the putative products of this sequence showed high amino acid sequence similarity (from 95% to 99%) with TniQ-, TnsD-, TnsA, TnsB-, and TnsC-like transposition proteins from *Pseudomonas* sp. BAY1663 (GenBank accession no. AZSV01000008). This sequence was found at the boundary of *P. æruginosa* chromosome. An IS*6100* element, adjoining the 3'CS of In1393, was identified next to the other boundary of *P. æruginosa* chromosome.

### 10.3.6 In56

In both ST357 VIM-2 producers sequenced during this study, the integron In56 was associated with a Tn3-like transposon that was inserted in a novel genomic island (**Figure 10-4**), designated PAGI-56.

PAGI-56 included open reading frames encoding proteins of various functions (e.g., metabolic activities, DNA recombination, and regulation of gene expression) and hypothetical proteins of unknown function. GIs closely related to PAGI-56 have been previously reported in *P. æruginosa* isolates IOMTU 133 (77% coverage; 100% identity) (GenBank accession no. AP017302), PA7 (77% coverage; 99% identity),[51] and W36662 (55% coverage; 99% identity) (GenBank accession no. CP008870). PAGI-56 was inserted in the *P. æruginosa* chromosome, into a gene encoding for a DNA repair photolyase (PA0069 in GenBank accession no. AE004091), in the same position as that reported in the aforementioned isolates. PAGI-56 differed from the GI identified in *P. æruginosa* IOMTU 133 by the presence of an additional 8,002-bp sequence (nt 28607 to 36608 in GenBank accession no. KY860570) including a *mer* operon and coding sequences for proteins with unknown function. Additionally, a 19,505-bp segment (nt 115171 to 134675 in GenBank accession no. AP017302) was probably deleted due to insertion of the In56-carrying Tn*3*-like transposon. The IRi of In56 was located within the *urf-2* gene of the transposon, while an IS*6100* element was found downstream of *orf5* of integron 3'CS. IS*6100* was found at the boundary of PAGI-56.

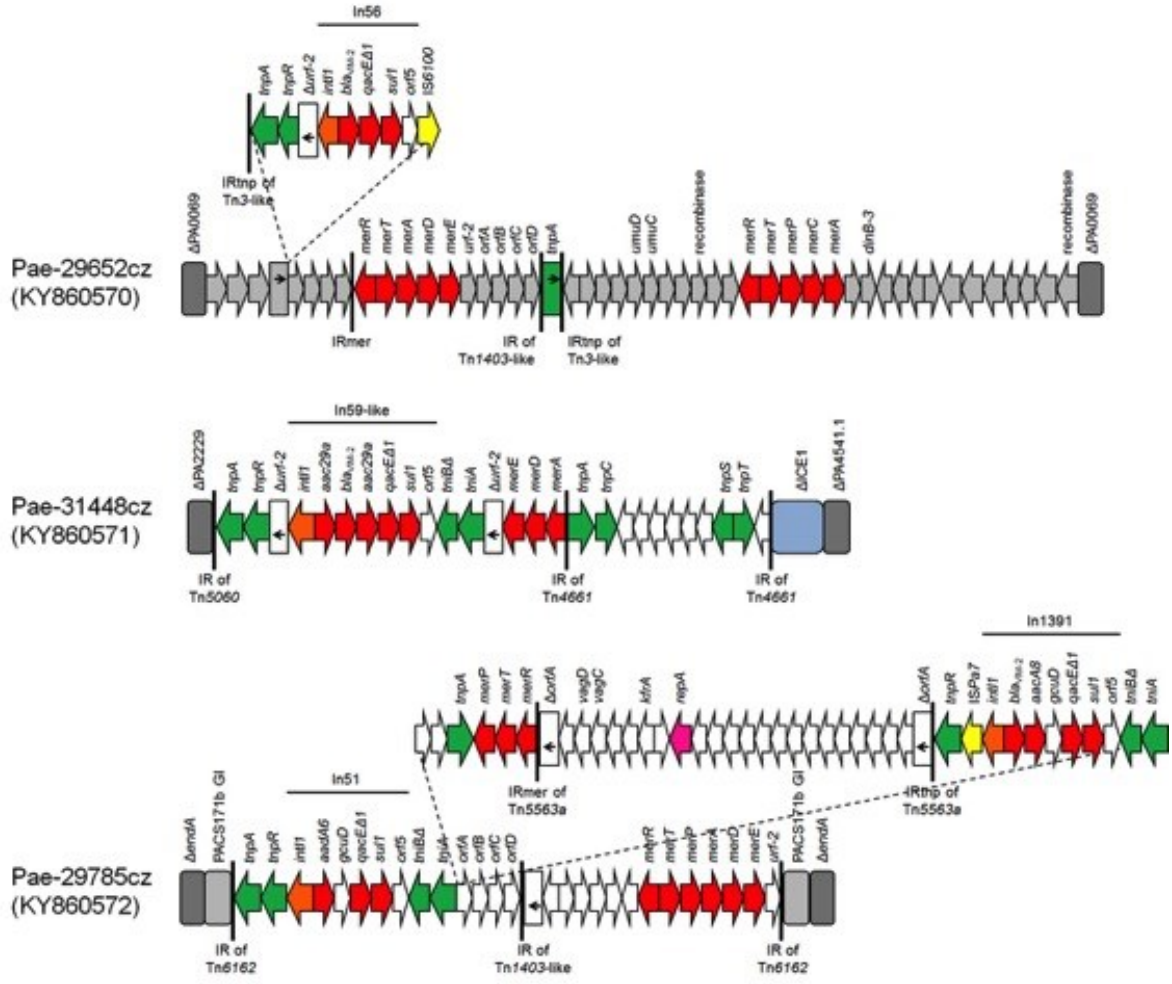


**Figure 10-4** Linear maps of the genetic context of carbapenemase-encoding class 1 integrons In56, located in the novel PAGI-56 **(A)**, In59-like, associated with ICE1 **(B)**, and In1391, inserted in the PACS171b GI **(C)**. Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription). Resistance genes, IS elements, and transposases are shown in red, yellow, and green, respectively. intI1 genes are shaded orange, while the repA gene of pAMBL2 is shown in pink. Sequences associated with GIs are shaded light gray; dark gray rectangles indicate *P. æruginosa* chromosome; ICE1 is shown in blue. The remaining genes are shown in white.

### 10.3.7 In59-like 1

In both ST111 isolates, sequenced by Illumina platform, the In59-like integron was inserted in a Tn*5060*-like transposon located in *P. æruginosa* chromosome (**Figure 10-4**). The mercury resistance Tn*5060*, which is most closely related to the ancestor of Tn*21* prior to integron acquisition, has been previously reported from *Pseudomonas* sp. A19-1 isolated from Siberian permafrost.[52] The IRi of In59-like was located within the *urf-2* gene of the transposon, while a defective Tn*402*-like *tni* module (ΔTn*402*-like) compsed of *tniB*Δ4 and *tniA* was found downstream of *orf5* and next to the *mer* operon of Tn*5060*. However, the Tn*5060 mer* module lacked an 1,847-bp fragment, including the *merC*, *merP*, *merT*, and *merR* genes, and the IRmer of the transposon (Δ*mer*). The Tn*3*-like transposon Tn*4661*, which carried two divergently transcribed *tnpS* and *tnpT* genes for cointegrate resolution,[53] was identified adjacent to Δ*mer* and to a partially deleted ICE1-like integrative conjugative element

(ΔICE1). Interestingly, a previous study showed that ICE1 was present in 67% of ST111 *P. æruginosa* isolates.[54] ΔICE1 was integrated into the $tRNA^{Lys}$ gene (PA4541.1 in GenBank accession no. AE004091), a similar site to that previously reported for most of analyzed *P. æruginosa* isolates. It is likely that insertion of the Tn*4661* transposon deleted the remaining parts of Δ*mer* and ΔICE1.

### 10.3.8 In1391

In1391 was in PACS171b GI (**Figure 10-4**) that includes open reading frames encoding proteins responsible for tellurite resistance, metabolic activities, DNA recombination, and gene regulation.[55] PACS171b GI was inserted into the chromosomal *endA* gene. In1391 was embedded in a mercury resistance Tn*6162*-like element, which is a Tn*1403* related transposon.[56] Similar to Tn*6162* in *P. æruginosa* C79,[13] 5-bp direct repeats of the target (GTCAT) were identified at the boundaries of the transposon, and the class 1 integron In51, containing *aadA6* and *gcuD* gene cassettes, was located in the Tn*6162* module. The 3'CS of In51 was followed by a partially deleted Tn*402*-like *tni* module carrying *tniB*Δ4 and *tniA*. Next to ΔTn*402*-like, a 20,045-bp (nt 44098 to 64142) sequence that comprised two fragments of Tn*5563a* transposon[57] flanking a central sequence, which exhibited extensive similarity to the VIM-1-encoding plasmid pAMBL2 from *P. æruginosa* PAO1 (GenBank accession no. KP873171), was found. The IRi of In1391 was located between *resII* and *resI* sites of the Tn*5563a* module. However, an IS*Pa7* element was present between the 3'-end of *intI1* and IRi. Similar to In51 in Tn*6162*,[13] a ΔTn*402*-like *tni* module followed by the *orfDCBA* and *mer* operons was identified adjacent to the 3'CS of In1391. However, the region intervening between *orfDCBA* and *mer* operons was in an inverted orientation. The described transposon may have resulted by integration of a pAMBL2-like plasmid, carrying a Tn*5563*-like with In1391, into Tn*6162*.

### 10.3.9 Further analysis of WGS data

Analysis of whole-genome sequencing (WGS) data by ResFinder 2.1 tool revealed that the majority of the sequenced isolates included additional genes for resistance to aminoglycosides, tetracyclines, trimethoprim, and chloramphenicol (**Table 10-3**). Fifteen out of the 16 ST357 isolates carried the *strA* and *strB* resistance genes. Interestingly, one IMP-7-producing ST357 isolate included the recently described *cmx* gene (GenBank accession no. U85507), conferring resistance to chloramphenicol, while one ST357 VIM-2 producer harbored the $bla_{LCR-1}$ oxacillinase gene.[58] Examination of quinolone resistance-determining regions of *gyrA*, *gyrB*, *parC,* and *parE* and of the *mexR*, *nfxB,* and *mexT* genes, which regulate the MexAB-OprM, MexCD-OprJ, MexEF-OprN multidrug efflux systems,[4] showed the presence of several amino acid substitutions (**Table S2**). The majority of these substitutions have been reported previously from both ciprofloxacin-susceptible and ciprofloxacin-resistant isolates. However, all ciprofloxacin-resistant isolates presented the T83I amino acid substitution in GyrA, which previously has been associated with increased quinolone resistance.[59] Finally, in 21 of the isolates, the sequence of *oprD* showed point mutations predicted to result in early termination of translation (**Table S2**), which is consistent with increased carbapenem resistance,[60] even in non-carbapenemase-producing isolates.

### 10.3.10 Phylogenetic analysis

Bayesian analysis of the 24 core genomes resulted in well-defined clusters (**Figure 10-5**), which corresponded to different STs. The largest cluster was composed of isolates which belonged to ST357. Isolates of STs 111 and 235 were grouped in monophyletic subgroups. The tree topology was greatly supported by 100% posterior probabilities for all clades representing different STs. Additionally, the results of Bayesian analysis indicated that IMP-7-producing ST357 *P. æruginosa* isolates, recovered from different geographical locations and hospitals, were closely related. This finding further supported the clonal spread of extensively resistant ST357 *P. æruginosa*, expressing IMP-7 MβL, in Czech hospitals.

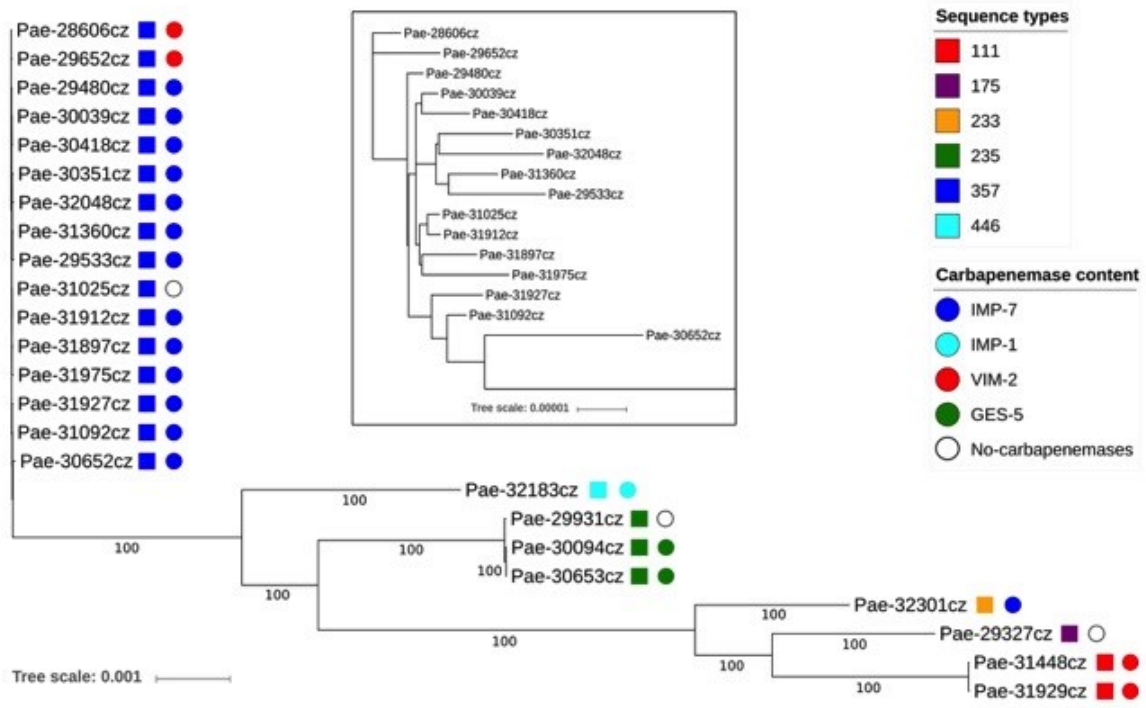


**Figure 10-5** Phylogenetic tree of *P. æruginosa* isolates, which were sequenced using Illumina platform, based on Bayesian statistics constructed from core genome alignment. The boxed portion shows the clade of ST357 isolates. Branch lengths indicate the number of base changes per site. STs and carbapenemase content are indicated as squares and circles, respectively, of different colors. Numbers at the branches represent their posterior probabilities.

### 10.3.11 Concluding remarks

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

inserted in LESGI-3, which was located in the same position of the *P. æruginosa* chromosome. Additionally, phylogenetic analysis showed that all ST357 isolates were clustered in a monophyletic group (**Figure 10-5**), indicating that they were close relatives. These findings further supported the hypothesis regarding the clonal spread of ST357 IMP-7-producing isolates in Czech hospitals. Analysis of WGS data revealed the presence of additional resistance genes, of T83I amino acid substitution in GyrA, and of premature stop codons in the *oprD* gene that can be implicated in the development of extensively multidrug-resistant bacteria,[4, 18, 42, 43] limiting therapeutic options. Additionally, all ST357 IMP-7 producers carried the virulence genes *exoT*, *exoU,* and *exoY*, which previously have been associated with increased pathogenicity and mortality of the bacterium.[29, 30] These data, which are in agreement with the results of previous studies,[17, 18] highlighted the important role of high-risk clones, such as STs 111, 175, 235, and 357, in the successful dissemination of clinically important resistance determinants.

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

**Supplemental material**

Supplemental material for this article may be found at http://aac.asm.org/content/61/12/e01811-17.

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# 11 Discussion

## 11.1 Validation of a novel automatic deposition of bacteria and yeasts on MALDI target for MALDI-TOF MS-based identification using MALDI Colonyst robot

The main focus of clinical microbiology is the isolation and identification of pathogenic microorganisms and subsequent determination of their susceptibility to antimicrobial agents to set appropriate treatment of a disease. Currently, the matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) method is the most common technique used for taxonomic identification in large clinical laboratories in many states. It is a high-speed, reproducible method with an extensive database allowing recognition of a broad scale of microorganisms, ranging from common bacteria to some filamentous fungi and Mycobacteria. Most bacteria only require direct deposition of colonies on a steel MALDI target, but for example majority of yeasts, mold, mycobacteria, or some anaerobes need prior protein extraction to improve spectra acquisition, either by direct on-plate semi-extraction by formic acid or in tube extraction with ethanol and formic acid/acetonitrile. However, direct deposition of cells is usually performed by wooden toothpick which does not allow standardization of the amount of microbes spotted on the target spot. This process is highly dependent on the technician's skills and significantly affects the resulting identification score of a microorganism. That is why we have tried to develop a universal automated method to apply a standard amount of cells on target spot directly into a droplet of formic acid to increase the score of an identified microbe.

The aim of this study was to compare results of average identification score for routinely used manual deposition (semi-extraction for yeasts) by a wooden toothpick with results of automatic deposition using MALDI Colonyst robot. Spotting by MALDI Colonyst significantly increased identification score of bacteria compared with the routine diagnostic process. In general, the lowest difference was found in *Enterobacteriaceæ* isolates which usually possess high identification score compared with other microbes. The identification score of yeasts, Gram-positives, and anaerobes was also higher than the score gained by manual deposition but not with such a big difference. Also, the high reproducibility of the method has been found, determined by calculation of standard deviation (SD) and relative standard deviation (SRD) after spotting each strain ten times on MALDI target from the same agar plate and processing by MALDI-TOF MS identification. Automatic deposition is a fast, well-monitored technique processing 96 MALDI target approximately in 45 minutes with standardized biomass amount in the spot, enabling retrospective quality control and without the need for pipette tips for formic acid deposition decreasing significantly the consumable cost. The disadvantage is the high purchase price, which cannot be afforded by small laboratories with a small circulation of identified samples per day.

Automated deposition of bacterial/yeasts cells on MALDI target follows the current effort to automate individual processes in microbiology, in the manner of hematological or clinical chemistry automated lines. An example may be, the newly offered automatic lines COPAN WASPLab® (COPAN Diagnostics Inc., Murrieta CA, USA) or BD Kiestra™ (Becton Dickinson, Franklin Lakes NJ, USA)

enabling a fully automated process in the bacteriological laboratory.[1, 2] Automatic lines currently include automation of sample inoculation process, smart incubation with possible documentation of microbial growth and workbenches allowing plate reading via high-resolution imaging.[3] Specifically, for automated cell sampling for MALDI-TOF, only one automat COPAN Colibri® (COPAN Diagnostics Inc., Murrieta CA, USA) has been developed so far, which was introduced during 25th ECCMID conference in 2015.[1] The automated process is expected to positively affect the quality of processed specimens and their standardization, including documentation of microbial cultures on plates.[4]

## 11.2 Characterization of KPC-encoding plasmids from *Enterobacteriaceæ* isolated in a Czech hospital

KPC-producing *Enterobacteriaceæ* have emerged as challenging pathogens causing healthcare-associated infections, due to their extremely drug-resistant phenotypes and ability to cause infections associated with high mortality. KPC producers are disseminated worldwide and currently constitute an important public health problem.[5] Nevertheless, their occurrence in the Czech Republic was rare until then. First sporadic case of KPC-2-producing *Klebsiella pneumoniæ* was recovered from a patient, who had been previously hospitalized in Greece, in the North Moravian region in 2009.[6]

The present study reports a "hidden outbreak" of KPC-producing *Enterobacteriaceæ*, which occurred in the 3rd Internal gerontometabolic clinic of University Hospital in Hradec Kralove (Czech Republic). From 2014 to 2016, ten non-repetitive KPC-producing isolates, obtained from seven patients, were detected. Interestingly, none of the patient had recently travelled abroad. All isolates were positive for presence of $bla_{KPC-2}$ gene. Most of the isolates were *Citrobacter freundii* belonging to three sequence types (STs), with ST18 being the predominant. In one of the patients, four KPC-2 producing isolates (*C. freundii*, *Klebsiella pneumoniæ*, *E. coli* and *Morganella morganii*) were identified during the hospitalization, implying *in vivo* horizontal transfer of $bla_{KPC-2}$-carrying plasmid. The same plasmid was also identified in two isolates from other patients, further supporting the hypothesis. The ten isolates carried $bla_{KPC}$-positive plasmids of different sizes (~30, ~45, and ~80 kb), of which eight were positive for the R allele. Whole-genome sequencing (WGS) revealed three types of plasmids (A to C). Type A plasmids, which were the most prevalent, comprised an IncR backbone and a KPC-2-encoding multidrug resistant (MDR) region. Type B appeared to be derivatives of type A plasmids carrying an IncN3-derived segment, while type C were IncP6 plasmids sharing the same KPC-2-encoding MDR region with type A and B plasmids. All three types of plasmids were not able to transfer via conjugation, due to partial deletion or absence of the transfer system genes. All plasmids carried $bla_{KPC-2}$ gene embedded in a Tn*4401a* transposon. Our findings underline the increasing clinical importance of the IncR plasmid family as well as spreading potential of large MDR segments through reshuffling of enterobacterial plasmids.

In our study a variety of typing methods including WGS have been used to obtain detailed information about the size of plasmids and the genetic background of the outbreak. A similar scheme of typing methods was used, for example, by Tofteland *et al.*[7] for the long-term outbreak of KPC-2 producers in Norway, or by Abboud *et al.*[8] in Brazil. But in recent years, a separate WGS has been used for these purposes, most often Illumina or PacBio platform.[9-11] WGS is particularly suited for short-term outbreaks, detailed mapping of chromosome/plasmid genes, for differentiation of clonal and non-clonal strains, and clustering. On the other hand, WGS is very financially demanding, relatively inappropriate for long-term investigations and in particular produces a huge amount of data to analyze.

## 11.3 Molecular characterization of OXA-48-like-producing *Enterobacteriaceæ* in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids

The class D β-lactamase OXA-48, which possess weak but significant carbapenemase activity, was first detected from *K. pneumoniæ* in Turkey in 2003.[12] At about the same time, the *bla*$_{\text{OXA-48}}$ gene was also identified in other Middle Eastern and North African countries, but only sporadically in Europe.[13, 14] At present, OXA-48 producers, are expanding across Europe, with the highest incidence, especially in southeastern Europe.[15] Since OXA-48 producers exhibit only a low level of resistance to carbapenems and are not distinguishable by inhibitor-based phenotypic tests, their identification may be quite difficult. Nevertheless, it has only been sporadically detected in the Czech Republic for a long time.

The aim of this study was to characterize the first cases, and outbreaks of OXA-48-like-producing *Enterobacteriaceæ* recovered from hospital settings in the Czech Republic. In the period from 2013 to 2015, twenty-two *Klebsiella pneumoniæ*, three *Escherichia coli* and one *Enterobacter cloacæ* producing OXA-48-like carbapenemases were isolated from 20 patients from hospitals throughout the Czech Republic. Five of the patients had recently traveled abroad, while, three OXA-48-like cases could be described as community-acquired since the patients had no history of previous hospitalization or traveling abroad. Four of the patients were colonized/infected by two or three different OXA-48-like producers, supporting the *in vivo* horizontal transfer of the *bla*$_{\text{OXA-48}}$-carrying plasmid. *K. pneumoniæ* isolates were classified into nine STs, with ST101 being the predominant (n=8). Thus, *K. pneumoniæ* ST101 is globally described in connection with outbreaks caused by OXA-48 producers.[16-19] *E. coli* were of different STs, while *E. cloacæ* isolate belonged to ST109. Results from Illumina sequencing showed that pOXA-48-like plasmids played a major role in the dissemination of *bla*$_{\text{OXA-48}}$ gene in Czech hospitals. Among our isolates, a highly conserved OXA-48-encoding plasmid, being identical to the previously described pE71T in Ireland,[20] was observed in a polyclonal population of *K. pneumoniæ* (five different STs). Plasmid pE71T was also found in two *E. coli* of different STs and one *E. cloacæ*. Additionally, two novel pE71T derivatives were characterized from *K. pneumoniæ* isolates of STs 11 and 891, respectively. Finally, sequencing data

showed that ST45 *K. pneumoniæ* and ST38 *E. coli* were chromosomally harbored. These findings support the horizontal transfer hypothesis of the pOXA-48-like plasmid, which allowed the *bla*$_{\text{OXA-48}}$ gene to disseminate in hospitals in the Czech Republic.

In our study a variety of typing methods including WGS have been used to obtain detailed information about the size of plasmids and the genetic background of the outbreak. By way of comparison, Cuzon *et al.*[21] used a similar scheme of methods for the *Klebsiella pneumoniæ* typing except the whole-genome sequencing. The genetic environment of the *bla*$_{\text{OXA-48}}$ gene was mapped only by PCR, revealing that this gene was also located in the Tn*1999.2* transposon. A similar study of the *K. pneumoniæ* outbreak was carried out by Wrenn *et al.*[22] in Ireland. In contrast, Perez-Vazquez *et al.*[23] used WGS and phylogenetic analysis of Spanish isolates to investigate the population structure of *bla*$_{\text{OXA-48-like}}$-expressing *K. pneumoniæ* ST11 and ST405 and to determine the distribution of resistance genes and plasmids encoding *bla*$_{\text{OXA-48-like}}$ carbapenemases. Based on these and other publications, it is clear that the OXA-48-producers are a significant threat to public health.

## 11.4 Characterization of NDM-encoding plasmids from *Enterobacteriaceæ* recovered from Czech hospitals

The NDM metallo-β-lactamase was first described in *K. pneumoniæ* and *E. coli* isolated in Sweden in 2008 from an Indian patient transferred from New Delhi hospital.[24] Since then, NDM-producing bacteria, including clinical isolates of *Enterobacteriaceæ* and *Acinetobacter baumannii*, have been reported worldwide. In the Czech Republic, the occurrence of NDM-producing bacteria was rare, with only three sporadic cases being detected in period from 2011 to 2013.[25-27]

The present study investigated sporadic case and an outbreak of NDM-like-producing *Enterobacteriaceæ* recovered from Czech hospitals, during 2016. In this period, eighteen *Enterobacteriaceæ* NDM-producing isolates including ten *Enterobacter cloacæ* complex, four *E. coli*, one *Kluyvera intermedia*, one *K. pneumoniæ*, one *Klebsiella oxytoca*, and one *Raoultella ornithinolytica*, were isolated from 15 patients. In three of the patients, two different NDM-like producers were identified during their hospitalization, which support the *in vivo* horizontal transfer of *bla*$_{\text{NDM-like}}$-carrying plasmid hypothesis. Gradually, all isolates were subjected to MLST, S1 nuclease plasmid profiling and whole-genome sequencing. Consequently, a comparative analysis of clinical isolates of *E. cloacæ* complex based on detection of SNPs, was performed. All isolates of *E. cloacæ* complex, except the *E. asburiæ*, recovered from the same hospital, were assigned to ST182. Additionally, two *E. coli* belonged to ST167, while the remaining isolates were not clonally related. Thirteen isolates were carried *bla*$_{\text{NDM-4}}$, while six isolates carried *bla*$_{\text{NDM-1}}$ or *bla*$_{\text{NDM-5}}$. Almost all isolates carried *bla*$_{\text{NDM-like}}$-carrying plasmids being positive for the IncX3 allele, except ST58 *E. coli* and ST14 *K. pneumoniæ* isolates producing NDM-1. Analysis of plasmid sequences revealed that all IncX3 *bla*$_{\text{NDM-like}}$-carrying plasmids exhibited a high similarity to each other and to previously described plasmids reported worldwide.[28-30] By way of comparison, Bocanegra-Ibarias *et al.*[31] used

only a plasmid analysis using S1 size profiling of plasmids for similar outbreak, and then subjected samples to MLST for clonal diversity detection. A similar scheme of typing methods was used by Torres-Gonzalez *et al.*[32] for outbreak investigation in Mexico, except that they did not perform S1 plasmid profiling but analyze the entire chromosome digested by *Xba*I endonuclease. Within both of these outbreaks, a number of isolates of *E. cloacæ* of ST182, as in our case, were detected. On the other hand, Stoesser *et al.*[33] in Nepal has subjected an entire collection of NDM-producers to next-generation sequencing using the HiSeq platform (Illumina, San Diego CA, USA).

Based on these and other publications, it is clear that *Enterobacter cloacæ* ST182 plays an important role in the global spread of the $bla_{\text{NDM-like}}$ gene. And in combination with the further evolvement of NDM-like-encoding MDR plasmids through reshuffling, NDM-like producers pose an important public threat.

## 11.5 Molecular characterization of carbapenemase-producing *Pseudomonas æruginosa* of Czech origin and evidence for clonal spread of extensively resistant sequence type 357 expressing IMP-7 metallo-β-lactamase

*Pseudomonas æruginosa* is one of the most clinically important opportunistic pathogens, characterized by intrinsic resistance to a wide variety of antimicrobials.[34] Sporadically, serine protease-producing *Pseudomonas* such as KPC, OXA, or GES have been reported, but they are more often associated with the production of metallo-β-lactamases (MβLs), mainly VIM and IMP types.[35] The MβLs are zinc-dependent enzymes commonly characterized by the ability to hydrolyze the majority of β-lactams, including carbapenems. In the Czech Republic, MβLs have been described so far sporadically.

This is the first national surveillance study on carbapenemase-producing *P. æruginosa* isolates from the Czech Republic using a deep molecular genetic typic procedure. During 2015, one hundred thirty-six carbapenemase-producing *Pseudomonas* have been collected from 22 hospitals located throughout the country. One hundred thirty-two isolates produced MβL-type carbapenemase and the remaining four isolates produced GES carbapenemase of the class A type. The vast majority of MβL-producing *Pseudomonas* belonged to sequence type (ST) 357 and carried the IMP-7-encoding integron In-p110, which indicates the clonal spread of these isolates in Czech hospitals. Among other STs, ST111 and 235 have been captured, which have been considered high-risk clones.[36] All isolates were subjected to PCR mapping of the regions flanking the carbapenemase-encoding genes which revealed seven types of class 1 integrons. Subsequently, whole-genome sequencing (WGS), and phylogenetic analysis were performed to determine population diversity based on difference in SNPs. The phylogenetic analysis showed that all ST357 isolates were clustered in a monophyletic group, indicating that they were close relatives (**Figure 10-5**). These findings further supported the hypothesis regarding the clonal spread of ST357 IMP-7-producing isolates in Czech hospitals. WGS also revealed additional genes for resistance to aminoglycosides, tetracyclines, trimethoprim, and

chloramphenicol. This result corresponds to the conclusions of a previous study of isolates from Czech and Polish hospitals. Most of these multidrug resistant isolates also belonged to ST357 producing IMP-7 metallo-β-lactamase, but in this case, the isolates were compared only by pulsed-field gel electrophoresis.[37] A phylogenetic analysis using WGS was conducted by the Miyoshi-Akiyama's team. Their set included strains isolated from hospital patients between 2001 and 2013 throughout Japan, as well as in other countries, among others from Poland. In this case, the dominant was high-risk clone ST235, followed again by ST357.[38]

These results indicate that it is likely to be regional clonal spread of ST357 producing IMP-7 metallo-β-lactamase in central Europe, because it is rarely found elsewhere. Therefore, appropriate preventive measures should be put in place to prevent further spread.

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## 12 Conclusion

This dissertation thesis summarizes the results of five selected publications. The first work led to the development and validation of a novel automatic technique for bacterial and yeast deposition on MALDI target using "wet-deposition" into a droplet of 70% formic acid. This automatic deposition was compared to conventional manual spotting using a wooden toothpick, semi-extraction routinely used for yeasts, and manual "wet-deposition". Spotting by MALDI Colonyst robot significantly increased identification score of bacteria compared with the routine diagnostic process. The largest differences in identification scores were seen in yeasts and anaerobes, on the contrary, the smallest differences were in *Enterobacteriaceæ*. As our data has shown, it is a robust method that allows rapid identification of bacteria and yeasts, resulting in an increase of MALDI-TOF MS identification scores and reproducibility and allowing further use within automated lines in laboratories of clinical microbiology.

Following four publications are focusing on the epidemiology of several of the most common carbapenemases in the Czech Republic (KPC, OXA-48, NDM, and IMP), either within hospital outbreaks or their occurring across the country. In particular, we focused on detecting the possible clonal spread and tracking the path and the source of their transmission. In KPC-2-type carbapenemases, a predominantly non-conjugative plasmid of the IncR type was identified which is not described in this area but is often associated with the spread of carbapenemases in Southeast Asia.[1, 2] All plasmids carried *bla*$_{\text{KPC-2}}$ gene embedded in a Tn*4401a* transposon in the MDR region. Our findings underline the increasing clinical importance of the IncR plasmid family as well as spreading potential of large MDR segments through reshuffling of enterobacterial plasmids.

In the case of OXA-48 carbapenemase producers, twenty-six *Enterobacteriaceæ* isolates were obtained from twenty patients from hospitals throughout the Czech Republic. In the vast majority, *Klebsiella pneumoniæ* isolates with predominant sequence types (ST) 101 and 461 were detected, but also high-risk clones ST11 have been identified. In total, a plasmid of the same type (IncL/M, pE71T) carrying *bla*$_{\text{OXA-48}}$-like gene was found in most isolates. This plasmid genetically coincided with the plasmid first identified in 2011 in Ireland.[3] These findings support the horizontal transfer hypothesis of the pOXA-48-like plasmid, which allowed the *bla*$_{\text{OXA-48}}$ gene to disseminate in hospitals in the Czech Republic.

Within the epidemiological study of NDM-4-producers, eighteen *Enterobacteriaceæ* isolates from 15 patients from Czech hospitals were obtained, with predominance of *Enterobacter cloacæ* ST182. Almost all isolates carried *bla*$_{\text{NDM-like}}$-carrying plasmids being positive for the IncX3 allele, and their analysis revealed that all IncX3 *bla*$_{\text{NDM-like}}$-carrying plasmids exhibited a high similarity to each other and to previously described plasmids, reported worldwide, often in association with ST182.[4-6] Based on these and other publications, it is clear that *Enterobacter cloacæ* ST182 plays an important role in the global spread of the *bla*$_{\text{NDM-like}}$ gene.

In the last included study, we focused on carbapenemase-producing *P. æruginosa* with using deep molecular-genetic typing techniques. During 2015, one hundred thirty-six carbapenemase-producing *Pseudomonas* were collected from 22 hospitals located throughout the country. The vast majority of metallo-β-lactamase-producing *Pseudomonas* belonged to ST357 and carried the IMP-7-encoding integron In-p110, which indicates the clonal spread of these isolates in Czech hospitals. Therefore, appropriate preventive measures should be put in place to prevent further spread.

Previous conclusions show the importance of epidemiological studies of individual resistance genes following their pathways around the world, hand in hand with the development of new powerful typing techniques and international online databases.

## References


1. Xiang, D.-R.; Li, J.-J.; Sheng, Z.-K.; Yu, H.-Y.; Deng, M.; Bi, S.; Hu, F.-S.; Chen, W.; Xue, X.-W.; Zhou, Z.-B.; Doi, Y.; Sheng, J.-F.; Li, L.-J., Complete Sequence of a Novel IncR-F33:A-:B- Plasmid, pKP1034, Harboring fosA3, bla(KPC-2), bla(CTX-M-65), bla(SHV-12), and rmtB from an Epidemic Klebsiella pneumoniæ Sequence Type 11 Strain in China. *Antimicrobial Agents and Chemotherapy* **2016,** *60* (3), 1343-1348.
2. Ageevets, V.; Sopova, J.; Lazareva, I.; Malakhova, M.; Ilina, E.; Kostryukova, E.; Babenko, V.; Carattoli, A.; Lobzin, Y.; Uskov, A.; Sidorenko, S., Genetic Environment of the bla(KPC-2) Gene in a Klebsiella pneumoniæ Isolate That May Have Been Imported to Russia from Southeast Asia. *Antimicrobial Agents and Chemotherapy* **2017,** *61* (2).
3. Power, K.; Wang, J.; Karczmarczyk, M.; Crowley, B.; Cotter, M.; Haughton, P.; Lynch, M.; Schaffer, K.; Fanning, S., Molecular Analysis of OXA-48-Carrying Conjugative IncL/M-Like Plasmids in Clinical Isolates of Klebsiella pneumoniæ in Ireland. *Microbial Drug Resistance* **2014,** *20* (4), 270-274.
4. Zhu, Y.-q.; Zhao, J.-y.; Xu, C.; Zhao, H.; Jia, N.; Li, Y.-n., Identification of an NDM-5-producing Escherichia coli Sequence Type 167 in a Neonatal Patient in China. *Scientific Reports* **2016,** *6*.
5. Krishnaraju, M.; Kamatchi, C.; Jha, A. K.; Devasena, N.; Vennila, R.; Sumathi, G.; Vaidyanathan, R., Complete sequencing of an IncX3 plasmid carrying bla(NDM-5) allele reveals an early stage in the dissemination of the bla(NDM) gene. *Indian Journal of Medical Microbiology* **2015,** *33* (1), 30-38.
6. Pal, T.; Ghazawi, A.; Darwish, D.; Villa, L.; Carattoli, A.; Hashmey, R.; Aldeesi, Z.; Jamal, W.; Rotimi, V.; Al-Jardani, A.; Al-Abri, S. S.; Sonnevend, A., Characterization of NDM-7 Carbapenemase-Producing Escherichia coli Isolates in the Arabian Peninsula. *Microbial Drug Resistance* **2017,** *23* (7), 871-878.

# 13 Annexis

## 13.1 Curriculum Vitæ

| | | |
|---|---|---|
| **Name** | Katerina Chudejova, Mgr. (MSc.) | |
| **Date & Place of Birth** | 9th December, 1989, Vsetin, Czech Republic | |
| **Address** | Elisky Krasnohorske 741/22, Plzen 323 00, CZ | |
| **Sex** | Woman | |
| **Present Position** | Since 2018 | Specialist in laboratory methods, Department of Microbiology, University Hospital in Pilsen |
| | Since 2014 | Ph.D. student – Department of Clinical Microbiology, Biomedical Center, Faculty of Medicine in Pilsen, Charles University |
| **Work Address and Contacts** | Alej Svobody 76, 323 00 Plzen, Czech Republic | |
| **Education** | 2012–2014 | Faculty of Pharmacy in Hradec Kralove, Charles University; specialization: *Medical bioanalytics – Laboratory technician* |
| | 2009–2012 | Faculty of Pharmacy in Hradec Kralove, Charles University; specialization: *Medical bioanalytics – Expert in laboratory methods* |
| | 2005–2009 | Secondary Medical School and College of Higher Medical Education in Brno; specialization: *Laboratory technician assistant* |
| **Language Skills** | Czech | – mother tongue |
| | English | – B1 – B2 |
| | Spanish | – A2 |
| | German | – passive |
| **Technical Skills** | Microbiology: Experienced in Microbiology techniques. Isolation, identification and characterization of bacteria (mainly *Enterobacteriaceæ*, *Pseudomonas* spp.); experienced in Clinical microbiology, especially in bacteriology and molecular-genetic diagnostics, molecular epidemiology. | |
| | Molecular Biology: Experienced in molecular biology techniques, e.g., real time quantitative PCR, RT-PCR and related molecular biology works based on DNA amplification, DNA sequencing, Southern blotting, hybridization, transformation, conjugation, PFGE, MLST. | |
| | Protein Analysis: Isoelectric focusing, MALDI-TOF mass spectrometry techniques. | |
| **H-Index** | 3 | (22.06.2018, Scopus) |
| **Sum of Times Cited without Self-Citations** | 25 | (22.06.2018, Scopus) |
| **Number of Publications in Journals with IF** | 9 + 2 articles in press | |
| **Publications** | See list of publications | |

**Professional experiences**

**Posters:**

*European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2018*

- Poster – Characterization of KPC-encoding plasmids from *Enterobacteriaceæ* in a Czech hospital (Chudejova K, Kukla R, Papagiannitsis C. C, Medvecky M, Habalova K, Hobzova L, Bolehovska R, Pliskova L, Hrabak J, Zemlickova H)
- Poster – Characterization of NDM-like-producing *Enterobacteriaceæ* isolated in Czech hospitals (Rotova V, Bitar I, Medvecky M, Skalova A, Chudejova K, Jakubu V, Bergerova T, Zemlickova H, Papagiannitsis C. C, Hrabak J)

*European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2017*

- ePoster – Evaluation and validation of newly developed spectrophotometric HRC assay for detection of carbapenemases in *Enterobacteriaceæ* (Rotova V, Papagiannitsis C. C, Skalova A, Chudejova K, Hrabak J)
- Poster – Molecular characterization of MβL-producing *Pseudomonas æruginosa* isolates in Czech hospitals (Papagiannitsis C. C, Chudejova K, Medvecky M, Skalova A, Rotova V, Jakubu V, Zemlickova H, Hrabak J)
- Poster – Automatic deposition of bacteria and yeast on MALDI target using MALDI Colonyst robot (Chudejova K., Hrabak J., Rotova V, Papagiannitsis C. C, Bohac M, Skalova A, Bergerova T)
- Poster – Molecular-epidemiological characteristics of antituberculotic-resistant *Mycobacterium tuberculosis* isolates identified in West Bohemian region of the Czech Republic (Amlerova J, Kralova D, Chudejova K, Hrabak J)

*European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2016*

- ePoster – Molecular epidemiological analysis of OXA-48 producing *Enterobacteriaceæ* in the Czech Republic with an evidence of horizontal gene transfer (Skalova A., Chudejova K., Rotova V, Bergerova T, Jakubu V, Zemlickova H, Papagiannitsis C. C, Hrabak J)
- Poster – Complete nucleotide sequences of three IncA/C2-type plasmids carrying In416-like integrons with blaVIM genes from *Enterobacteriaceæ* isolates of Greek origin (Papagiannitsis C. C, Dolejska M, Izdebski R, Giakkoupi P, Skalova A, Chudejova K, Dobiasova H, Vatopoulos A, Derde L. P. G, Bonten M. J, Gniadkowski M, Hrabak J)

## 13.2 List of publications

| Publications | Impact Factor (Web of Science) | Citations (Web of Science, 22.06.2018) | Citations (Scopus, 22.06.2018) |
|---|---|---|---|
| Paskova, V, Medvecky, M, Skalova, A, **Chudejova, K**, Bitar, I, Jakubu, V, Bergerova, T, Zemlickova, H, Papagiannitsis, C.C, and Hrabak, J. "Characterization of NDM encoding plasmids from *Enterobacteriaceæ* recovered from Czech hospitals" *Frontiers in Microbiology* (**2018**): **In press** | 4.076 | – | – |
| Jamborova, I, Johnston, B, Papousek, I, Kachlikova, K, Micenkova, L, Clabots, C, Skalova, A, **Chudejova, K**, Dolejska, M, Literak, I, and Johnson, J. "Extensive genetic commonality among wildlife, wastewater, community, and nosocomial isolates of *Escherichia coli* sequence type 131 (*H*30R1 and *H*30Rx subclones) That Carry $bla_{CTX\text{-}M\text{-}27}$ or $bla_{CTX\text{-}M\text{-}15}$" *Antimicrobial agents and chemotherapy* (**2018**): **In press** | 4.302 | – | – |
| Papagiannitsis, C.C, Paskova, V, **Chudejova, K**, Medvecky, M, Bitar, I, Jakubu, V, Zemlickova, H, Jirsa, R, and Hrabak, J. "Characterization of pEncl-30969cz, a novel ColE1-like plasmid encoding VIM-1 carbapenemase, from an *Enterobacter cloacæ* sequence type 92 isolate." *Diagnostic microbiology and infectious disease 91.2* (**2018**): 191-193. doi: **10.1016/j.diagmicrobio.2018.01.024**. | 2.401 | 0 | 0 |
| Kukla, R*, **Chudejova, K***, Papagiannitsis, C.C, Medvecky, M, Habalova, K., Hobzova, L, Bolehovska, R, Pliskova, L, Hrabak, J, and Zemlickova, H. "Characterization of KPC-encoding plasmids from *Enterobacteriaceæ* isolated in a Czech hospital." *Antimicrobial agents and chemotherapy 62.3* (**2018**): e02152-17. doi: **10.1128/AAC.02152-17**. | 4.302 | 0 | 0 |
| **Chudejova, K**, Paskova, V, Skalova, A, Medvecky, M, Adamkova, V, Papagiannitsis, C.C, and Hrabak, J. "Emergence of sequence type 252 *Enterobacter cloacæ* producing GES-5 carbapenemase in a Czech hospital." *Diagnostic microbiology and infectious disease 90.2* (**2018**): 148-150. doi: **10.1016/j.diagmicrobio.2017.10.011**. | 2.401 | 0 | 0 |
| **Chudejova, K**, Bohac, M, Skalova, A, Paskova, V, Papagiannitsis, C.C, Hanzlickova, J, Bergerova, T, and Hrabak, J. "Validation of a novel automatic deposition of bacteria and yeasts on MALDI target for MALDI-TOF MS-based identification using MALDI Colonyst robot." *PloS one 12.12* (**2017**): e0190038. doi: **10.1371/journal.pone.0190038**. | 2.806 | 0 | 0 |
| Paskova, V, Papagiannitsis, C.C, **Chudejova, K**, Medvecky, M, Skalova, A, Adamkova, V, and Hrabak, J. "First description of the emergence of *Enterobacter asburiæ* producing IMI-2 carbapenemase in the Czech Republic." *Journal of global antimicrobial resistance 11* (**2017**): 98. doi: **10.1016/j.jgar.2017.10.001**. | 1.276 | 0 | 0 |
| Papagiannitsis, C.C, Medvecky, M, **Chudejova, K**, Skalova, A, Paskova, V, Spanelova, P, Jakubu, V, Zemlickova, H, and Hrabak, J. "Molecular Characterization of Carbapenemase-Producing *Pseudomonas æruginosa* of Czech Origin and Evidence for Clonal Spread of Extensively Resistant Sequence Type 357 Expressing IMP-7 Metallo-β-Lactamase." *Antimicrobial agents and chemotherapy 61.12* (**2017**): e01811-17. doi: **10.1128/AAC.01811-17**. | 4.302 | 0 | 0 |
| Paskova, V, Papagiannitsis, C.C, Skalova, A, **Chudejova, K**, and Hrabak, J. "Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity." *Journal of microbiological methods 137* (**2017**): 30-33. doi: **10.1016/j.mimet.2017.04.003**. | 1.790 | 6 | 7 |

| | | | |
|---|---|---|---|
| Skalova, A, **Chudejova, K**, Paskova, V, Medvecky, M, Studentova, V, Chudackova, E, Lavicka, P, Bergerova, T, Jakubu, V, Zemlickova, H, Papagiannitsis, C.C, and Hrabak, J. "Molecular characterization of OXA-48-like-producing *Enterobacteriaceæ* in the Czech Republic and evidence for horizontal transfer of pOXA-48-like plasmids." *Antimicrobial agents and chemotherapy 61.2* (**2017**): e01889-16. doi: **10.1128/AAC.01889-16**. | 4.302 | 7 | 12 |
| Papagiannitsis, C.C, Dolejska, M, Izdebski, R, Giakkoupi, P, Skalova, A, **Chudejova, K**, Dobiasova, H, Vatopoulos, A.C, Derde, L.P.G, Bonten, M.J.M, Gniadkowski, M, and Hrabak, J. "Characterisation of IncA/C2 plasmids carrying an In416-like integron with the blaVIM-19 gene from *Klebsiella pneumoniæ* ST383 of Greek origin." *International journal of antimicrobial agents 47.2* (**2016**): 158-162. doi: **10.1093/jac/dkv304**. | 4.307 | 6 | 6 |

* R.K. and K.CH. contributed equally to this work