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ABSTRACT

Carbapenemase-producing (CP) Enterobacterales (CPE) are a growing menace to healthcare systems worldwide. CPE infections are difficult to treat because few antimicrobials remain effective. One of the new drug combinations, ceftazidime-avibactam (CAZ-AVI), was launched in Europe in 2016, however, a few years later concerns rose about CAZ-AVI resistance. Currently, whole genome sequencing (WGS) is an essential tool for public health surveillance and for studying the molecular epidemiology of CPE.

Molecular surveillance data of WGS-characterized CPE in Finland during 2012-2018 were analyzed. In addition, isolates belonging to clusters caused by CP *Citrobacter freundii* were studied up to April 2020. The largest CPE clusters and discovered resistance to CAZ-AVI were investigated in detail in collaboration with regional infection control teams.

The annual number of CPE isolates increased from 9 in 2012 to 70 in 2018 and different sequence types from 7 to 33. Of the isolates, 59% were found by screening and of the cases 61% had preceding travelling or hospitalization abroad. The most common CPE species were *Klebsiella pneumoniae* (45%), *Escherichia coli* (40%) and *C. freundii* (6%), and the most prevalent carbapenemase genes were $bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$ and $bla_{\text{KPC-like}}$. We detected 10 CPE clusters; three of them were linked to abroad. Seven clusters were caused by *K. pneumoniae* (2-23 cases per cluster) and three by *C. freundii* (2-16 cases per cluster). All *K. pneumoniae* sequence types belonged to international high-risk clones. The largest clusters spread via patient transfers to several healthcare facilities with environmental contamination most likely playing a role in the persistence of the clusters. We describe a patient case developing CAZ-AVI resistance during CAZ-AVI treatment and showed that the resistance was probably due to a mutation in *K. pneumoniae* carbapenemase gene.

During 2012-2018 a remarkable increase in CPE isolates was observed and most were detected by screening. The hospital environment can be a CPE reservoir prolonging the outbreaks, hence the national guidelines of CPE control were updated for terminal cleaning and screening. Real-time nationwide surveillance using WGS and team collaboration were crucial when identifying clusters, tracing back transmission chains and controlling the spread.

KEYWORDS: CPE, carbapenemase-producing Enterobacterales, antimicrobial resistance, molecular epidemiology, whole genome sequencing

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TIIVISTELMÄ

Karbapenemaaseja tuottavat (CP) Enterobacterales -bakteerit (CPE) ovat kasvava uhka terveydenhuoltojärjestelmälle maailmanlaajuisesti. CPE:n aiheuttamien infektioiden hoito on hankalaa, sillä tehokkaita mikrobilääkkeitä on vähän. Yksi uusista lääkeyhdistelmistä on 2016 Euroopassa markkinoille tullut keftatsidiimi-avibaktaami (CAZ-AVI). Huoli resistenssin kehittymisestä CAZ-AVI kohtaan alkoi jo muutama vuosi käyttöönoton jälkeen. Nykyisin kokogenomisekvensointi (WGS) on keskeinen työväline CPE:n molekyyliepidemiologisessa seurannassa.

Väitöskirjassa tutkittiin WGS:lla tuotettua CPE:n molekulaarista seurantatietoa Suomesta vuosilta 2012–2018. Lisäksi CP *Citrobacter freundii* bakteerin aiheuttamien rypäiden kantoja tutkittiin huhtikuuhun 2020 asti. Suurimmat CPE-rypäät ja havaittu CAZ-AVI resistenssi tutkittiin tarkemmin yhteistyössä alueellisten infektioiden torjuntatiimien kanssa.

CPE-tapausten määrä kasvoi vuoden 2012 9:stä vuoden 2018 70:een ja erilaiset sekvenssityypit 7:stä 33:een. Tapauksista 61 %:lla oli taustalla edeltänyt matkustus tai sairaalahoito ulkomailla ja 59 %:ia löytyi seulonnalla. Yleisimmät bakteerilajit olivat *Klebsiella pneumoniae* (45 %), *Escherichia coli* (40 %) ja *C. freundii* (6 %). Vallitsevat karbapenemaasigeenit olivat $bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$ and $bla_{\text{KPC-like}}$. Tutkimuksen aikana havaittiin 10 CPE-ryvästä, joista kolmella oli linkki ulkomaille. *K. pneumoniae* aiheutti rypäistä seitsemän (2–23 tapausta/ryväs) ja *C. freundii* kolme (2–16 tapausta/ryväs). Kaikki rypäitä aiheuttaneet *K. pneumoniae* sekvenssityypit kuuluivat kansainvälisiin korkeanriskin klooneihin. Suurin ryväs levisi potilassiirtojen välityksellä useisiin terveydenhuollon yksiköihin ja ympäristön kontaminaatiolla todennäköisesti oli merkitystä ongelman pitkittymiseen. Kuvasimme potilastapauksen, jossa CAZ-AVI-resistenssi kehittyi hoidon aikana ja osoitimme, että resistenssin aiheutti mahdollisesti mutaatio *K. pneumoniae* karbapenemaasigeenissä.

Vuosina 2012–2018 CPE-kannoissa nähtiin merkittävä lisääntyminen ja valtaosa löytyi seulonnalla. Sairaalaympäristö voi toimia CPE-kantojen lähteenä pitkittäen epidemioita, minkä vuoksi kansallista torjuntaohjetta päivitettiin loppusiivousta ja seulontaa koskien. Reaaliaikainen, kansallinen WGS-seuranta ja eri tahojen yhteistyö oli ratkaisevaa rypäiden havaitsemisessa sekä jäljitys- ja torjuntatyössä.

AVAINSANAT: CPE, karbapenemaasia tuottava Enterobacterales, mikrobilääke-resistenssi, molekyyliepidemiologia, kokogenomisekvensointi

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Abbreviations

AmpC	Ampicillinase C
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
cgMLST	Core Genome Multi Locus Sequence Typing
cgSNP	Core genome single nucleotide polymorphism
CAZ-AVI	Ceftazidime-avibactam
CC	Clonal Complex
CMY	Cephamycin-hydrolyzing β-lactamase
CP	Carbapenemase-producing
CPE	Carbapenemase-producing Enterobacterales
CRE	Carbapenem-resistant Enterobacterales
CTX-M	Cefotaximase-Munich
DNA	Deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
ESBL	Extended-spectrum β-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GES	Guiana extended-spectrum carbapenemase
HAI	Hospital-acquired infection
HCF	Health care facility
IMI	Imipenem-hydrolyzing β-lactamase
IMP	IMP-type metallo-β-lactamase
Inc	Incompatibility group
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metallo-β-lactamases
MDR	Multidrug-resistant
MIC	Minimal inhibitory concentration
MLST	Multi Locus Sequence Typing
MRSA	Methicillin-resistant *Staphylococcus aureus*

NDM	New Delhi metallo-β-lactamase
NGS	Next-generation sequencing
NIDR	National Infectious Disease Registry
NMC	Not Metalloenzyme carbapenemase
OECD	Organisation for Economic Co-operation and Development
OXA	Oxacillinase
PBP	Penicillin-binding protein
PCR	Polymerace chain reaction
PDR	Pandrug-resistant
PFGE	Pulsed-field gel electrophoresis
SBL	Serine β-lactamases
SHV	Sulfhydryl variable
SME	*Serratia Marcescens* enzyme
SNP	Single nucleotide polymorphism
ST	Sequence Type in MLST
TEM	Temoneira
THL	Finnish Institute for Health and Welfare
VIM	Verona integron-encoded metallo-β-lactamase
WGS	Whole Genome Sequencing
wgMLST	Whole Genome Multilocus Sequence Typing
WHO	World Health Organization
XDR	Extensively drug-resistant

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Räisänen K, Lyytikäinen O, Kauranen J, Tarkka E, Forsblom-Helander B, Grönroos J.O, Vuento R, Arifulla D, Sarvikivi E, Toura S, Jalava J. Molecular epidemiology of carbapenemase-producing Enterobacterales in Finland, 2012–2018. *European Journal of Clinical Microbiology & Infectious Diseases*, 2020; 39(9): 1651–1656.

II van Beek J, Räisänen K, Broas M, Kauranen J, Kähkölä A, Laine J, Mustonen E, Nurkkala T, Puhto T, Sinkkonen J, Torvinen S, Vornanen T, Vuento R, Jalava J, Lyytikäinen O. Tracing local and regional clusters of carbapenemase-producing *Klebsiella pneumoniae* ST512 with whole genome sequencing, Finland, 2013 to 2018. *Eurosurveillance*, 2019; 38:1800522.

III Räisänen K, Sarvikivi E, Arifulla D, Pietikäinen R, Forsblom-Helander B, Tarkka E, Anttila V-J, Grönroos J.O, Rintala E, Kauranen J, Ahlsved M, Broas M, Mikkola J, Sieberns J, Jalava J, Lyytikäinen O. Three clusters of Carbapenemase-producing *Citrobacter freundii* in Finland, 2016-20, *Journal of Antimicrobial Chemotherapy,* 2021; 10:2697–2701.

IV Räisänen K, Koivula I, Ilmavirta H, Puranen S, Kallonen T, Lyytikäinen O, Jalava J. Emergence of ceftazidime-avibactam-resistant *Klebsiella pneumoniae* during treatment, Finland, December 2018. *Eurosurveillance*, 2019; 19:1900256.

1 Introduction

Antimicrobial resistance (AMR) is an enormous health risk and the World Health Organization (WHO) has estimated that globally AMR now causes 700, 000 extra deaths per year (Bryan-Wilson, 2016). Carbapenemase-producing (CP) Enterobacterales (CPE) often have other resistance genes in addition to carbapenemase genes and are frequently extensively drug-resistant (XDR) or even pandrug-resistant (PDR) (Magiorakos et al., 2012). This multidrug-resistance (MDR) limits treatment options, delays proper treatment and causes high fatality (Falagas et al., 2014). The few drugs remaining that can deal with bacteria exhibiting carbapenem resistance are generally less effective or have excessive side effects and resistance to these drugs occurs increasingly. Transmission of CPE primarily occurs in hospitals or other health care facilities (HCF) and the CPE situation varies considerably between different countries, from sporadic cases to an endemic situation.

Carbapenems and polymyxins are considered some of the last-line antimicrobial groups against infections caused by gram-negative bacteria such as Enterobacterales (Nang et al., 2021). Last-line antimicrobials mean the last treatment options for patients infected with bacteria resistant to other available antimicrobials (Nang et al., 2021). Resistance to carbapenems among Enterobacterales has been highlighted as one of the most significant threats (World Health Organization (WHO), 2022). Development of resistance to carbapenems may be due to intrinsic or acquired resistance mechanisms or both. From an infection control point of view the carbapenemases, enzymes that break carbapenems, are the most important. Carbapenemases spread via plasmids and can transfer between bacteria and different bacterial species as well. In addition to these aforementioned antimicrobial groups novel β-lactam-β-lactamase inhibitor combinations are also important promising new antimicrobials against MDR Enterobacterales and the resistance against these should be monitored carefully. Ceftazidime-avibactam (CAZ-AVI) is one of these currently most promising new combinations with activity against many CPE (van Duin & Bonomo, 2021).

For bacterial typing the main method in several high-income countries is whole genome sequencing (WGS) and the use of WGS in surveillance allows precise

detection of resistance genes and very fine-tuned phylogenetics on bacteria (World Health Organization (WHO), 2020). Resistance spreads, among other things, via bacterial clones and these are studied via phylogenetics. A clone in microbiology is defined as bacterial isolates that are genetically indistinguishable or highly similar to each other with a common ancestor, as identified using genetic tests (F. C. Tenover et al., 1995). When analyzing the WGS data it is important to include the epidemiological background information and clinical data. Combining data enables linkages during the early detection of outbreaks, accurate tracing of transmission chains, precise definition of the geographical spread of an outbreak and identification of sources of infection (World Health Organization (WHO), 2020).

This thesis gives an overview of the molecular epidemiology of CPE in Finland and a detailed cluster analysis of detected CPE clusters. In addition, we describe the emergence of CAZ-AVI resistance in a *Klebsiella pneumoniae* carbapenemase (KPC)-2-producing *K. pneumoniae* strain. WGS gives us accurate information of the bacterial characterization. The information is compared to publicly available international databases and our comprehensive national genomic database. Molecular epidemiological surveillance follows the trends in CPE, species distribution, resistance genes and clusters spreading nationally in comparison to internationally spread clones.

MDR	Multidrug-resistance among Enterobacterales means the isolate is non-susceptible to at least one agent in three or more of 17 antimicrobial categories; intrinsic resistance is not addressed.
PDR	Pandrug-resistance is defined as non-susceptibility to all agents in all antimicrobial categories.
XDR	Extensively drug-resistance is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (for example bacterial isolates remain susceptible to only one or two categories).

2 Review of the Literature

2.1 What is antimicrobial resistance and how to measure it?

AMR is defined as the ability of a microbe (e.g. bacteria, virus, parasite, fungi) to resist a certain antimicrobial. Therefore, antimicrobials cannot be used to treat infections caused by the resistant microbes. Resistance can be quantified by the Minimal Inhibitory Concentration (MIC), which can be defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism (Andrews, 2001). In clinical settings MIC is usually used for the determination of resistance or susceptibility of a microbe to antimicrobials and is used for estimating if an antimicrobial can be used for patient therapy although it is measured in a laboratory which may not reflect the efficacy of the antimicrobial in vivo. In MIC determination, bacteria are exposed to different concentrations of antimicrobials, and their ability to grow is measured using different standardized techniques.

The two most used MIC techniques are E-tests and micro broth dilution method. In addition to MIC determination resistance can be measured using indirect methods like disk diffusion test where diameter of the growth inhibition zone around the antimicrobial disk is measured. Results from MIC determination or a disk diffusion test are typically interpreted using internationally standardized breakpoints (European Committee on Antimicrobial Susceptibility Testing, EUCAST and Clinical and Laboratory Standards Institute). Assessing these breakpoints for clinical use EUCAST has a scientific process in which the knowledge of dosage, mode of administration, type and severity of infection, pharmacodynamic of the agent, MIC distribution, epidemiological cut-off values, resistance mechanism and zone diameter distribution are all considered (EUCAST, 2021).

2.1.1 AMR and its burden in the public health

Discovery of antimicrobials was one of the largest interventions during the last century and our modern medicine with cancer therapy, organ transplant and surgery, relies on effective antimicrobials. Now the curative and lifesaving power of

antimicrobials are at risk due to AMR. Current evidence shows that resistance to all antimicrobials occurs sooner or later after taking one into use, which means that developing new antimicrobials is not a solution to the AMR problem (Wright & Poinar, 2012).

The WHO have estimated that on a global scale AMR is currently causing 700, 000 extra deaths annually (Bryan-Wilson, 2016). The European Centre for Disease Prevention and Control (ECDC) have estimated that in the European Union (EU)/European Economic Area (EAA) resistance is causing 33 000 deaths and in Finland around 100 extra deaths annually (Cassini et al., 2019). These figures are expected to grow if resistance continues to increase. If AMR continues to increase, the total of annual deaths globally is estimated to be 10 million by the year 2050 (Jim O'Neill, 2016). This is more deaths than cancer causes today.

AMR is a global threat to health, but also a serious threat to the economy. In addition to the loss of lives and healthy days AMR prolongs treatments, increases hospital days and decrease the quality of life causing economic costs. The economic and social costs of AMR are mainly (75%) due to hospital-acquired infections (HAI) caused by AMR pathogens (OECD, 2018). The impact of AMR on the global Gross Domestic Product is estimated to be a 4-percent decrease by the year 2050 (Jim O'Neill, 2016). Estimated costs due to resistance are around EUR 1,1 billion yearly in Europe (OECD, 2018). The amount of AMR costs corresponds to 10 percent of expenses in health care costs caused by communicable diseases. Based on the calculations of the Organization for Economic Co-operation and Development (OECD) the burden of AMR could be prevented if 1.5 euros per capita per year were invested in infection prevention (OECD, 2018). In Finland the necessary investment would be a total of 8.3 million euros per year.

The most cost-effective measure to prevent infections and spread of resistance is hand hygiene. In addition, improvement in HCF hygiene, improvement in diagnostics, prudent use of antimicrobials and education/information campaigns about AMR for doctors and citizens are all cost-effective measures to reduce the burden of AMR. The earlier prevention is financed, the less expensive it will be. In addition, it is calculated that a major economic benefit will be gained by prevention. The cost of prevention is difficult to estimate, and it is therefore often overlooked. (World Bank, 2016)

2.1.2 One Health approach

AMR is complex and it is created and spread across all sectors and that is why a One Health approach is necessary (McEwen & Collignon, 2018). A One Health approach recognizes that the health of people is closely connected to the health of animals and our shared environment. Resistant microbes circulate among human and animal

population via touch, food, water and the environment (Liu et al., 2018). It has been shown that patients, retail chicken meat and poultry shared the same extended-spectrum β-lactamases (ESBL) genes, plasmids and strains suggesting transmission of AMR from poultry to humans, probably via the food chain (Leverstein-van Hall et al., 2011). Livestock workers are recognized to be at a significantly higher risk of having livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) colonization and subsequent infection (C. Chen & Wu, 2020). People are in even more close contact with companion animals than farmed animals and the detected rise and rapid spread of CPE among companion animals is alarming (Sellera et al., 2021). A case report from Finland showed two hunting dogs and their owner sharing genetically related CPE (Grönthal et al., 2018).

The AMR problem is changing over time. Gram-positive pathogens were in the spotlight fifteen years ago and are still a problem, but now there are gram-negative bacteria and especially MDR Enterobacterales. Enterobacterales normally reside within human and animal gut microbiomes and the dissemination is wide (T. R. Walsh, 2018). Spreading of resistance seems to be accelerated simultaneously when people travel more, and it is definitely a global problem. International traveling and direct hospital transfers from a foreign hospital have been recognized as spreading MDR bacteria (Arcilla et al., 2017; Kajova et al., 2021; Kantele et al., 2016; Laupland et al., 2008). In addition environmental factors, such as livestock care, food distribution, sewage, and recreational water have been involved in global dissemination of MDR Enterobacterales (Hansen, 2021). The rise of gram-negative MDR bacteria and their rapid increase have changed how we perceive AMR.

2.2 The order Enterobacterales

2.2.1 Species, clinical infections and resistance

The Enterobacterales are gram-negative, non-spore forming, rod-shaped, facultative anaerobes. The order contains seven families and the family of Enterobacteriaceae is the largest one (Adeolu et al., 2016). Clinically the most important species are *Escherichia coli* and *K. pneumoniae* which are commensals and generally colonize the intestinal canal of humans and animals. However, they can be opportunistic pathogens in certain circumstances. Both species can survive for long periods in the environment and can form biofilms (Smismans et al., 2019).

Enterobacterales easily harbor resistance genes, and by acquiring multidrug-resistance, they are a common cause of health care-associated infections, causing high morbidity and mortality rates. When these abovementioned infections are acquired in hospitals, they are called hospital-acquired infections (HAI).

E. coli

Some *E. coli* strains have virulence factors and are able to cause an infection in a healthy person (Allocati et al., 2013). Most common infections caused by *E. coli* are urinary tract infections and infections of the intestines including serious diarrhea (Allocati et al., 2013). *E. coli* is the leading cause of bloodstream infections in the community and the second leading cause of infections in hospitals globally (OECD, 2018). According to the National Infectious Disease Registry (NIDR) *E. coli* is the most common cause of invasive infections of working-age and elderly people in Finland.

Infections caused by third-generation cephalosporin-resistant *E. coli* are responsible for most of the burden of AMR in the Europe (Cassini et al., 2019). Invasive infections caused by *E. coli* are usually treated with third-generation cephalosporins in Europe (OECD, 2018). Resistance to third-generation cephalosporins among invasive *E. coli* was 14.9% and ranged from country to country between 5.8–41.4% according to surveillance carried out by the European Antimicrobial Resistance Surveillance Network (EARS-Net) in Europe in 2020 (ECDC, 2022). Higher resistance was reported from the south and east of Europe and lower from northern Europe (ECDC, 2022). The Finnish Finres report stated that the proportion of ESBLs among invasive *E. coli* was 6.6% in 2020 (THL, 2021a). Resistance to carbapenems among invasive *E. coli* was 0.2% (country range 0.0–0.8%) in Europe and 0.0% in Finland in 2020 (ECDC, 2022; THL, 2021a). Invasive *E. coli* isolates resistant to carbapenems were found one isolate per year in 2016, 2018 and 2020 (THL, 2021a).

K. pneumoniae

K. pneumoniae causes a wide range of infections from urinary tract and upper respiratory tract infections to sepsis and meningitis (Podschun & Ullmann, 1998). It is also a significant cause of HAIs (Podschun & Ullmann, 1998). Antimicrobial treatment depends on the infection site and local resistance profiles but can include third-generation cephalosporins, carbapenems, aminoglycosides, and quinolones (OECD, 2018). Resistance to third-generation cephalosporins among invasive *K. pneumoniae* was 33.9% with a country range of 0.0–79.1%, according to EARS-Net surveillance in Europe in 2020 (ECDC, 2022). The proportion of ESBLs among invasive *K. pneumoniae* was 6.0% in Finland in 2020 (THL, 2021a). Resistance to carbapenems among invasive *K. pneumoniae* was 10.0% (country range 0.0–66.3%) in Europe and 0.1% in Finland in 2020 (ECDC, 2022; THL, 2021a). Invasive *K. pneumoniae* isolates resistant to carbapenems were found respectively, one in 2014, two in 2016, five in 2018, two in 2019 and one in 2020 (THL, 2021a). Again higher resistance was reported from the south and east and a lower resistance from northern

Europe (ECDC, 2022). High variation in *K. pneumoniae* carbapenem resistance in EU is problematic and highlights the need for close monitoring of the situation.

Other species

Other often encountered genera in the family Enterobacterales are *Enterobacter* and *Citrobacter*. *Enterobacter* species like *E. cloacae* seldom cause infections in healthy people (Sanders & Sanders, 1997) but can cause HAIs in intensive care patients, especially to those receiving mechanical ventilation (Davin-Regli & Pagès, 2015) *E. cloacae* resistant to third-generation cephalosporins is often due to inducible mechanism developing during treatment (Davin-Regli & Pagès, 2015). In Finland resistance to third-generation cephalosporins among invasive *E. cloacae* isolates was 28.5% in 2020, and one invasive *E. cloacae* isolate resistant to carbapenems was detected in 2014, 2018 and 2019, respectively (THL, 2021a). However, the genus *Citrobacter* species like *C. freundii* are mainly responsible for causing infections in immunocompromised patients or infants, for example respiratory, urinary, gastrointestinal and bloodstream infections (Samonis et al., 2009). So far, the resistance of *C. freundii* has not been under surveillance in Europe nor in Finland on national level.

2.2.2 Structure of the cell wall

The bacterial cell wall is an important target for antimicrobials (Dörr et al., 2019). It has a unique structure specific to bacteria, that is not found in any other cellular organisms including, most importantly, animal cells (Dörr et al., 2019). The bacterial cell wall is a layer that gives bacterium a characteristic shape and prevents it from osmotic and mechanical stresses (Dörr et al., 2019). Enterobacterales are gram-negative bacteria having a double lipid bilayer (inner and outer membrane) separated by periplasm and peptidoglycan. The inner plasma membrane envelopes a thin peptidoglycan cell wall (in gram-positive bacteria this is thicker), which is surrounded by a second lipid membrane consisting of lipopolysaccharides and lipoproteins, called the outer membrane (Beveridge, 1999). The space between the outer membrane and inner membrane is called the periplasmic space (Beveridge, 1999). The outer membrane is an additional protective layer in gram-negative bacteria and protects the bacteria from many substances (Beveridge, 1999). The channels located in the outer membrane are called porins, which enable the entry of molecules important for bacterial growth, but also other molecules such as antimicrobials (Winterhalter, 2021).

2.3 β-lactam antibiotics

Antimicrobials came into clinical use in the 1940s and have since been one of the most efficient medicines to use against infections caused by bacteria (Bush & Bradford, 2016). The most well-known β-lactam antibiotic is penicillin, which was discovered by Sir Alexander Fleming in 1928 (Ligon, 2004). Penicillin and other early β-lactam antibiotics are active against gram-positive bacteria (Ligon, 2004). Further development produced broad-spectrum β-lactam antibiotics like aminopenicillins, which were also active against gram-negative bacteria (Bush & Bradford, 2016). Currently β-lactam antibiotics are the most widely used group of antimicrobials because of their particular effectiveness, low cost, simple delivery, and minimal side effects (Bush & Bradford, 2016). The main β-lactam groups are penicillins, cephalosporins, carbapenems, and monobactams (Bush & Bradford, 2016).

2.3.1 Structure and function

β-lactam antibiotics are molecules that contain a β-lactam ring, a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom (C. Walsh, 2003). β-lactam antibiotics are bactericidal which means they kill bacteria (C. Walsh, 2003). β-lactam antibiotics act by inhibiting cell wall biosynthesis leading to the death of a bacterial cell (C. Walsh, 2003). They disturb cell wall formation as a result of covalent binding to essential penicillin-binding proteins (PBP), the enzymes involved in the last steps of peptidoglycan cross-linking (Bush & Bradford, 2016). The effectiveness of β-lactam antibiotics relies on the ability to reach and bind to the PBP (Bush & Bradford, 2016).

2.3.2 Cephalosporins

Cephalosporins are classified into generations based on the antibacterial activity. Different substitutions to the side chain of cephalosporins alter the activity and pharmacokinetic properties. The first generation cephalosporins, such as cephalotin, have best activity against gram-positive bacteria and only activity to some gram-negative bacteria. The second generation cephalosporins, such as cefoxitin, are less active against gram-positive bacteria and have broader activity against gram-negative bacteria. The third generation, also called the broad-spectrum cephalosporins, such as ceftriaxone and ceftazidime, have been optimized and extended to cover *Pseudomonas aeruginosa* and still retain activity against gram-positive bacteria, except ceftazidime. The fourth generation, also broad-spectrum cephalosporin is cefepime. Cephalosporins are extensively prescribed and best-selling class of the β-lactam antibiotics. (C. Walsh, 2003)

2.3.3 Carbapenems

Carbapenems are the broadest spectrum β-lactam antibiotics currently available and they are stable against hydrolysis by ESBLs and Ampicillinase C (AmpC) β-lactamases (Papp-Wallace et al., 2011). Structurally, carbapenems have a carbon atom in position 1 of the β-lactam ring, whereas penicillin have a sulphur atom, and hence the name carbapenems (C. Walsh, 2003). Commonly carbapenems bind strongly to PBP2 but may also bind to PBP1a, 1b and 3 in gram-negative bacteria (Bush & Bradford, 2016). The first carbapenem was thienamycin and this discovery was reported in 1976 (Papp-Wallace et al., 2011). Thienamycin was not found to be useful as a drug and the first clinically and widely used carbapenem was imipenem (approved for medical use in 1985), the second was meropenem (1996), the third ertapenem (2001) and the fourth doripenem (2007) (Bush & Bradford, 2016).

2.3.4 β-lactamase inhibitors

After the appearance of β-lactamase enzymes, which hydrolyze the β-lactam ring and cause resistance to β-lactam antibiotics, the search for β-lactamase inhibitors started in the 1970s. β-lactamase inhibitors are drugs that are co-administered with β-lactam antibiotics to prevent hydrolytic action of β-lactamase enzymes and hence restore the activity of β-lactam antibiotics. (Bush & Bradford, 2016)

β-lactamase inhibitors with a β-lactam core are divided into two groups: clavulanic acid and penicillanic acid sulfones. The three inhibitors approved for clinical use are clavulanic acid, tazobactam, and sulbactam. They have similar structure as β-lactam antibiotics, but the inhibitors are not active enough to be used as monotheraphy. Typically, clavulanic acid acts synergistically with different penicillins and cephalosporins, whereas penicillanic acid sulfones and, sulbactam act synergistically with ampicillin and tazobactam with piperacillin. (C. Walsh, 2003)

β-lactamase inhibitors without a β-lactam core are avibactam used with ceftazidime (active against ESBLs, KPCs, AmpCs, and Oxacillinases (OXA)), vaborbactam used with meropenem (active against ESBLs, KPCs, and AmpCs), and relebactam used with imipenem (active against ESBLs, KPCs, and AmpCs). (van Duin & Bonomo, 2021)

CAZ-AVI is one of the most recent promising β-lactam antibiotic inhibitor combinations with activity against CPE that produces enzymes from class A, class C and some from class D (van Duin & Bonomo, 2021). However, avibactam does not inactivate class B enzymes like NDM (van Duin & Bonomo, 2021). Avibactam is a diazabicyclooctane non-β-lactam β-lactamase inhibitor, a completely new molecule unrelated to the old β-lactam type inhibitors (Ehmann et al., 2012). Avibactam targets the active site of serine β-lactamases by binding covalently and reversibly to β-lactamases (van Duin & Bonomo, 2021). CAZ-AVI was first

approved for clinical use by the United States (US) Food and Drug Administration in 2015 and by the European Medicines Agency in 2016.

2.4 Development and mechanisms of antimicrobial resistance

Microbes accumulate genetic changes over time and some of the changes can cause resistance against antimicrobials (N Woodford & Ellington, 2007). The use of antimicrobials causes the selection of these resistant microbes and resistance genes in humans, animals and environment ecosystems (Fletcher, 2015). The more antimicrobials used, the greater the selection pressure. Antimicrobials used for treating bacterial infections have especially started to lose efficacy, but the same is true for other drugs used to treat infections caused by fungi, viruses or protozoan parasites (Levy & Marshall, 2004).

Resistance against antimicrobials can be either intrinsic or acquired. Intrinsic resistance is the innate ability of a bacterial species to resist activity of a particular antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class (N Woodford & Ellington, 2007). Intrinsic resistance is expressed in all members of a species and is independent of previous antimicrobial exposure (Cox & Wright, 2013). Intrinsic resistance can be due to: 1) altering the target protein to which the antimicrobial agent binds by modifying or eliminating the binding site, 2) upregulating the production of enzymes that inactivate the antimicrobial agent, 3) downregulating or altering an outer membrane protein channel that the drug requires for cell entry, 4) upregulating pumps that expel the drug from the cell (F. Tenover, 2006).

Acquired resistance occurs when a particular bacteria obtains the ability to resist the activity of an antimicrobial agent to which it was previously susceptible. Acquired resistance is present only in certain isolates of a species and it can be temporary or permanent. Acquired resistance develops through gene mutation or acquired new genetic material via horizontal gene transfer (transformation, transposition, or conjugation). Mutations continuously occur in bacteria but most of these are lethal and do not give a selective advantage. Mutations causing AMR usually occur in genes encoding drug targets, drug transporters, regulators that control drug transporters, and genes encoding enzymes that can modify antimicrobials. Usually, mutations conferring AMR cause some other disadvantages to bacteria. Plasmid-mediated transmission of resistance genes is the most common route for gaining genetic material from outside of the cell. (C Reygaert, 2018)

A plasmid is a small circular, extra-chromosomal piece of DNA that occurs naturally in bacteria and can contain genes causing AMR. Plasmids have an ability to replicate autonomously within a suitable host. They have specific regions,

replicons, that regulate their replication and can be used for the classification of plasmids into different incompatibility groups (Inc). Plasmids can spread horizontally between bacterial strains and even between bacterial species. Plasmids with AMR genes can be grouped into the narrow-host-range group, which usually belongs to the incompatibility group F (IncF), and the broad-host-range group, which belongs to the incompatibility groups IncA/C, IncL/M, and IncN. The most common incompatibility group disseminating among Enterobacterales is IncF and two known MDR clones, *E. coli* sequence type (ST) 131 and *K. pneumoniae* ST258 high-risk clone causing pandemics have plasmids belonging to this incompatibility group. The plasmid-mediated β-lactamase resistance is the most critical resistance issue among Enterobacterales, because the plasmids have made the rapid global spread of AMR genes possible. (Mathers et al., 2015b)

The main resistance mechanisms are: 1) limiting the uptake of a drug, 2) modification of the drug target, 3) inactivation of the drug, 4) active efflux of the drug. Gram-negative bacteria use all four mechanisms. Some of these mechanisms can be intrinsic or acquired and sometimes intrinsic can become acquired, for example efflux pumps causing MDR. Genes encoding efflux pumps are present in all bacteria chromosomes and this is considered an intrinsic resistant mechanism. However, some efflux pumps move, via plasmids, between bacteria and this is considered an acquired resistance mechanism. (C Reygaert, 2018)

2.4.1 β-lactam resistance mechanisms

Today β-lactamases are the most important cause of antibiotic resistance and almost 2,800 unique proteins exist (Bush, 2018). Resistance to β-lactam antibiotics is caused by production of β-lactamases, changes in outer membrane permeability, changes in drug target or efflux systems (examples of efflux systems in previous chapter) (D M Livermore, 1998).

β-lactamases are a large group of enzymes that act by inactivating a drug by hydrolyzing the β-lactam ring structure, causing the ring to open. When the ring is open, it is no longer able to bind to the target PBP. The β-lactamase enzymes are classified based on their molecular structure and/or functional characteristics. The Ambler classification based on amino acid sequences is commonly used and it divides these enzymes into four categories A (serine-β-lactamases), B (metallo-β-lactamases), C (cephaloporinases), and D (oxacillinases) (Figure 1). Some species of the Enterobacterales order have these β-lactamase genes in the chromosome and some can acquire these via plasmid. These β-lactamases were relatively rare in clinical isolates until the use of β-lactam antibiotics started in medicine. Each time, after a new class of β-lactam has been clinically introduced, new β-lactamases were detected that also hydrolyzed the antimicrobial. (David M. Livermore, 1995)

Gram-negative bacteria have an outer membrane serving as a barrier and substances often use the porin channels to enter the cell. Cells can limit the drug uptake by decreasing the number of porins present or by mutations that cause changes to the porin selectivity. Enterobacterales can achieve resistance to β-lactam antimicrobials by reducing the number of porins, and it is possible that they even stop the production of some porins. The porins are divided into specific and non-specific. (C Reygaert, 2018)

Alterations in PBPs can cause β-lactam resistance if affinity for the drug is reduced. Every bacterial species has its own selection of PBPs. The level of resistance depends on the size of alterations and is more significant in gram-positive bacteria. Typical alterations causing mutations are point mutations in PBP genes, remodeling of PBP and or acquisition of other set of PBPs. (C Reygaert, 2018)

2.4.2 Carbapenem resistance mechanisms

Carbapenemases are β-lactamases that are active against carbapenems and other β-lactam antibiotics and are found primarily in Enterobacterales. Moreover, carbapenemases can be classified by Ambler´s classification as shown in Figure 1. Carbapenemases are found in classes A, B and D. (van Duin & Doi, 2017)

Different carbapenemases differ greatly from each other and this affects how easily they are detected and how the infections they cause are treated. Different carbapenemase and bacterial species combinations are found to cause different levels of carbapenem resistance (usually meropenem is recommended for screening). For example, *K. pneumoniae* with the KPC-gene usually has clear meropenem resistance (high meropenem MIC values) but *E. coli* with an OXA-48-like-gene has a wider range of meropenem resistance and a considerable number are susceptible to meropenem (Fattouh et al., 2016). The carbapenemase gene OXA-244 is one of the OXA-48-like-gene variants with very low carbapenem resistance (Rima et al., 2021). This low-level carbapenem resistance is considered a problem when detecting these organisms by screening and needs to be considered in laboratory diagnostic technologies.

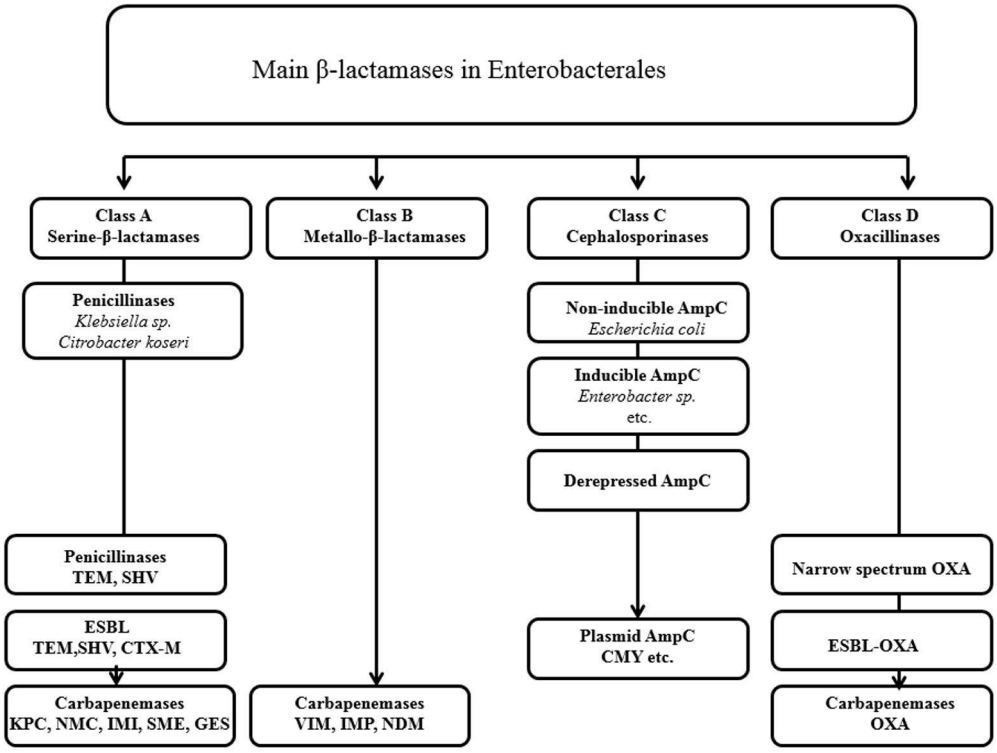

Figure 1. Mechanisms of antimicrobial resistance in Enterobacterales according to Ambler´s classification. Figure modified from Ruppé et al. (2015) and reprinted with the permission of the copyright holders, licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

Awareness of the epidemiological situation and which carbapenemase types are dominant regionally or nationally is essential to avoiding the spread of CPE and to selecting the right treatment. One example of this is when considering treating an infection caused by CPE with a β-lactamase inhibitor combination like CAZ-AVI. It has been tested and shown that CAZ-AVI is probably effective against KPC and OXA-48 producers, but not against MBL producers like NDM (David M Livermore et al., 2020). In addition some small mutations can affect the activity of certain drug, once again the example is CAZ-AVI, where an Asp179Tyr substitution decreases the activity of CAZ-AVI (Gaibani et al., 2018). This can lead to a situation where the clinician needs even more accurate detection of the resistance mechanism in CPE isolates to select the right drug.

Class A carbapenemases

Class A carbapenemases require a serine active site, and have the ability to hydrolyze carbapenems, penicillins, aztreonam, and cephalosporins. These are genetically high

diverse carbapenemases and may be chromosomally encoded (SME, NMC-A) or plasmid-encoded (KPC, GES) or both (IMI). Chromosomally encoded class A carbapenemases have been usually recognized in rare species and sporadically. SME abbreviated from *Serratia marcescens* enzyme is usually detected in *S. marcescens* and first isolates with SME were collected in England in 1982. IMI/NMC-A called imipenem-hydrolyzing β-lactamase/not metalloenzyme carbapenemase A has two subgroups, IMI and NMC-A, respectively, and these have been detected occasionally in *E. cloacae.* The IMI-2 variant is plasmid-encoded and spreads among Enterobacterales. The Guiana extended-spectrum carbapenemase (GES) -type enzymes are increasingly found in different gram-negative bacterial species, including *P. aeruginosa, Acinetobacter baumannii, E. coli* and *K. pneumoniae*, and these enzymes are spread worldwide. (Naas et al., 2016)

KPC is the abbreviation of *K. pneumoniae* carbapenemase, and is the most prevalent class A carbapenemase. The first report of KPC being detected in *K. pneumoniae* was published in 2001 and it was found in an isolate collected in the US in 1996 (Yigit et al., 2001). Globally, at least 18 variants of KPC-gene are known to exist (N Stoesser et al., 2017). KPC is mostly described as plasmid associated, but can be chromosomally integrated as well and the rate of chromosomal integration is unknown (Mathers et al., 2017). It causes resistance to all β-lactam antibiotics, and contrary to other class A carbapenemases, and β-lactamase inhibitors, like clavulanic acid, sulbactam, and tazobactam are also ineffective against KPC (Papp-Wallace et al., 2010). Some new β-lactamase inhibitors like avibactam and relebactam are active against KPC. However, resistance to these newer β-lactamase inhibitors, for example CAZ-AVI, has been described and can develop during treatment (Shields et al., 2017).

The spreading of class A carbapenemases among Enterobacterales is of great clinical concern because these pathogens cause HAIs and are often the reason for treatment failures (Naas et al., 2016).

Class B carbapenemases

Class B carbapenemases require a zinc ion at their active sites and have the ability to hydrolyze carbapenems, penicillins, and cephalosporins but do not hydrolyze aztreonam. These metallo-β-lactamases (MBL) are mainly chromosomally encoded but can also be found on plasmids of Enterobacterales, *Pseudomonas*, and *Acinetobacter* species (Rotondo & Wright, 2017). MBLs confer resistance to most β-lactam antibiotics and at the moment all β-lactamase inhibitors are inactive against MBLs. This unavailability of effective inhibitors against MBLs is a growing problem. However, monobactams such as aztreoname are active against MBLs. Clinically the most important MBLs are the plasmid-mediated IMP-type metallo-β-lactamase (IMP), Verona integron-encoded metallo-β-lactamase (VIM) and New

Delhi metallo-β-lactamase (NDM). In 1991, IMP was the first MBL to be described (Rotondo & Wright, 2017). The second MBL found was VIM in 1999 and the latest is NDM, found in *K. pneumoniae* in 2009 from a Swedish patient after having preceding hospitalization in India (Rotondo & Wright, 2017; Yong et al., 2009). Unlike IMP and VIM, NDM spread rapidly worldwide after its discovery.

Class D carbapenemases

Class D enzymes, oxacillinases (OXA), are the most diverse and less understood class of all the β-lactamases. Some OXA enzymes only hydrolyze only penicillins while others are active against cephalosporins and carbapenems. Inhibitors that are active against other classes of β-lactamases are mostly ineffective against class D enzymes (Leonard et al., 2013). Of particular concern are plasmid-mediated OXA-23 and OXA-24/40 mainly in *Acinetobacter* species and OXA-48 groups in Enterobacterales, because these are responsible for carbapenem resistance (Tooke et al., 2019). The difference between the OXA-48-like carbapenemases group is based on one to five specific amino acid substitutions of the enzyme that can impact the efficiency of carbapenem hydrolysis (Pitout et al., 2020). The most common OXA-48-like carbapenemases are OXA-48, OXA-162, OXA-181, OXA-204, OXA-232 and less common are OXA-245, OXA-484 and OXA-519 (Pitout et al., 2020). Clonal dissemination plays a minor role in the spread of OXA-48 but it is associated with certain high-risk clones; *K. pneumoniae* ST147, ST307, ST15, and ST14 and *E. coli* ST38 and ST410 (Pitout et al., 2020). The first carbapenem hydrolyzing OXA-enzyme, OXA-48, in Enterobacterales, *K. pneumoniae*, was reported in 2001 in Turkey (Poirel et al., 2004). Compared to other carbapenemases, OXAs are often weak carbapenemases and they are more difficult to identify. OXAs are still important from the perspective of infection control and this difficulty in identification increases the likelihood of patient to patient transmission in HCFs.

2.4.3 Other clinically important β-lactamases causing carbapenem resistance

ESBLs (class A β-lactamases)

ESBLs are enzymes which have the ability to hydrolyze extended-spectrum cephalosporin antibiotics and monobactams but not cephamycin and carbapenems (Ur Rahman et al., 2018). They are inhibited by clavulanic acid and with classical definition ESBL genes spread via plasmids (Mazzariol et al., 2017). They belong to Ambler´s class A group of β-lactamases. Although there are β-lactamases which belong to different classes and they can share an extended spectrum of β-lactam

hydrolysis (Giske et al., 2009). The clinically most important ESBLs belong to the following families: temoneira (TEM), sulfhydryl variable (SHV) and cefotaximase-Munich (CTX-M) (David M. Livermore, 2008). Numerous variants of TEM and SHV exist but all are not ESBLs, because some variants (e.g. TEM-1, TEM-12, SHV-1 and SHV-11) have only slightly increased activity against extended-spectrum cephalosporins (Lee et al., 2012; Liakopoulos et al., 2016).

TEMs are generally encoded by gram-negative bacteria and approximately 90% of the ampicillin resistance among them is due to TEM encoded genes. TEM ESBLs are often plasmid mediated and mutations from the classic TEM (TEM-1 and TEM-2) genes by single or multiple amino acid substitutions around the active site. TEM-1 is only able to hydrolyze penicillin and hence not considered an ESBL. TEM-1 was first reported in 1965 in Greece. The first TEM-type ESBL detected was TEM-12 in *Klebsiella oxytoca* in England, 1982. Over 200 different TEM-variants have been reported. (Ur Rahman et al., 2018)

SHV types of enzymes are usually found in *K. pneumoniae* and located in plasmids. However, numerous species carry the SHV-1 gene in the chromosome. The first SHV-type ESBL detected was SHV-2 in *Klebsiella ozaenae* in Germany, 1983. SHV has almost the same number of reported variants as TEM. (Ur Rahman et al., 2018)

CTX-M is the most recent discovery of ESBL-type enzymes, and the most increasingly reported. CTX-M enzymes are plasmid-mediated and the fastest growing family of ESBLs (Ur Rahman et al., 2018). There are somewhat fewer CTX-M variants reported than SHV variants (Ur Rahman et al., 2018). It is worrisome that CTX-M is often associated with other resistance elements as well. The most common variants of CTX-M detected in important microbes are CTX-M-15 and CTX-M-14 (Ur Rahman et al., 2018). ESBLs are a public health concern because of the high prevalence in *E. coli* especially in the community. *E. coli* with CTX-M isolated from urinary tract infections are common. CTX-M producing *K. pneumoniae* strains are increasingly also found among HAIs (Jalava et al., 2019; "Surveillance of Antimicrobial Resistance in Europe 2018.," 2019).

AmpC (class C) β-lactamases

AmpC β-lactamases are enzymes which cause resistance to penicillins, cephalosporins, and β-lactamases/β-lactam inhibitor combinations. AmpC in *E. coli* was the first bacterial enzyme reported to destroy penicillin (Jacoby, 2009). The AmpC resistance mechanism can be divided into three groups: 1) inducible resistance via chromosomally encoded AmpC genes, 2) stable derepression due to mutations in ampC regulatory genes 3) or plasmid-mediated resistance (Tamma et al., 2019). For example *Enterobacter* sp. and *C. freundii*, produce inducible chromosomal AmpC (É. Ruppé et al., 2015). This mechanism is only effective under

certain conditions (e.g. the presence of amoxicillin, clavulanic acid, cefoxitin and first-generation cephalosporins) and can exhibit high levels of resistance due to overexpression of the enzyme (É. Ruppé et al., 2015). Overexpression is problematic especially in *Klebsiella aerogenes* and *E. cloacae* where susceptible isolates may become resistant during therapy (Jacoby, 2009). 2) mutations in the induction system can cause permanent overexpression of AmpC, even in the absence of a β-lactam trigger (É. Ruppé et al., 2015; Tamma et al., 2019). 3) plasmid-mediated AmpC have been found worldwide, but are less common than ESBLs (Jacoby, 2009). AmpC-producing bacteria are usually susceptible to carbapenems, but carbapenem resistance can arise by the combined effect of AmpC with mutations in porins, reducing influx or enhancing efflux of carbapenems. The interactions of carbapenems with β-lactamases of class C remain less understood except for the recently identified plasmid-mediated CMY-10. CMY-10 has shown carbapenemase activity due to deletion leading to an expansion of the active site (Tooke et al., 2019).

2.5 Surveillance of antimicrobial resistance

Surveillance of AMR gives information about the AMR situation and enables the monitoring of threats to public health (World Health Organization (WHO), 2015). Information can guide clinical decisions about empirical treatment and is an essential tool that can inform policies for infection prevention and control responses (World Health Organization (WHO), 2015). The estimations of the spread of AMR provide information for local, national and global strategies and are based on data from surveillance (World Health Organization (WHO), 2015). Surveillance data can also be used to raise awareness among the scientific community and among the general public. The importance of AMR surveillance is highlighted in both the WHO European Strategic Action Plan on Antibiotic Resistance for the period 2011–2020 (Committee, 2011) and the European Commission's European One Health Action Plan against Antimicrobial Resistance (European Commission, 2017). AMR surveillance in Europe is based on both the decision No 1082/2013/EU of the European parliament of preparedness and response to serious cross-border health threats, including AMR and of the Commission Implementing Decision (EU) 2018/945 of 22 June 2018 on communicable diseases and related special health issues to be covered by epidemiological surveillance. In addition, in Finland the national legislation regulates AMR surveillance by the communicable diseases act (1227/2016).

The objective of surveillance is to collect comparable, representative and accurate AMR data. From the collected data the temporal and spatial trends of AMR can be analyzed and reported. Surveillance of AMR is based on the characterization of AMR pathogens and their distribution in the population. Different methods can be used to address different aspects. Phenotypic tests are used to characterize how bacteria

respond in the presence of an antimicrobial and WGS can be used to characterize the genome of the isolate. Currently, the international AMR surveillance is largely based on routine clinical antimicrobial susceptibility (AST) data from invasive isolates being reported clinical laboratories. (World Health Organization (WHO), 2020)

This traditional surveillance based on AST requires an organized and wealthy society to perform the data collection. AMR surveillance data from Europe is of good quality, but the WHO stated in its report in 2014 that globally the surveillance of AMR is not coordinated or harmonized and there are many gaps in the information. One possibility in the future might be sewage-based surveillance where the surveillance would be independent of the income level of a country (Aarestrup & Woolhouse, 2020). It has been shown that AMR in European wastewater reflects the pattern of clinical AMR prevalence (Pärnänen et al., 2019).

GLASS

The Global Antimicrobial Resistance Surveillance System (GLASS) is the WHOs worldwide AMR surveillance system launched 2015. In the first data call in 2017, 22 countries provided AMR data and 42 countries were enrolled (World Health Organization (WHO), 2017). GLASS focuses on eight pathogens (*Acinetobacter* spp., *E. coli, K. pneumoniae, Neisseria gonorrhoeae, Salmonella* spp., *Shigella* spp., *S. aureus*, and *Streptococcus pneumoniae*) with different specimen types (blood, urine, stool, urethral and cervical swabs) and is based on AST data. The objectives of the GLASS are to foster national surveillance systems, harmonize global standards, estimate the extent and burden of AMR globally, analyze and report data regularly, detect emerging resistance and its international spread. GLASS aims to support global surveillance and research and help decision-making by providing information.

EARS-Net

EARS-Net is ECDCs Europewide surveillance system and it comprises the data from countries within the EU and EEA (2019:30) (ECDC, 2020). EARS-Net is the first international AMR surveillance program, whose predecessor (EARSS) was launched in 1998. It collects routine AST data from invasive isolates (blood and cerebrospinal fluid) and it is limited to seven bacterial pathogens (*E. coli, K. pneumoniae, P. aeruginosa, Acinetobacter species, S. pneumoniae, S. aureus, Enterococcus faecalis, E. faecium*) commonly causing infections in humans. EARS-Net produces yearly reports in which data is analyzed from the last five years and it is updated in the Atlas information service < https://www.ecdc.europa.eu/en/surveillance-atlas-infectious-diseases>. EARS-Net provides Europewide AMR data for temporal and spatial analyses and timely data

for policy decisions. In addition, EARS-Net supports national systems in their efforts to improve diagnostic accuracy by offering annual external quality assessments. EARS-Net AMR data for year 2019 showed wide variations across Europe.

ECDC has proposed widening the AMR surveillance by integrating genomic typing into European disease and genomic surveillance, and multi-country outbreak investigations (European Centre for Disease Prevention and Control (ECDC), 2021). The aim of one of the ECDCs surveys The European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net) was to monitor the trends in MDR Enterobacteriaceae of public health importance. The objectives of the EURGen-Net were to determine the occurrence, geographic distribution and population dynamics of high-risk CRE or colistin resistant Enterobacteriaceae clones, and/or transmissible resistance/genetic elements within the European healthcare setting to inform risk assessment and control policies.

CAESAR

The Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR) Network <https://www.euro.who.int/en/health-topics/disease-prevention/antimicrobial-resistance/surveillance/central-asian-and-european-surveillance-of-antimicrobial-resistance-caesar> collects data from countries and areas within the WHO European Region that are not included in EARS-Net (eastern Europe and central Asia). CAESAR is a joint initiative of the WHO Regional Office for Europe, the European Society of Clinical Microbiology and Infectious Diseases and the Dutch National Institute for Public Health and the Environment. CAESAR is part of the Global initiative GLASS. The first CAESAR report was published in 2015 and currently, 19 countries participate in CAESAR.

Finres

Finres is the Finnish national AMR surveillance system maintained by THL. The Finnish clinical microbiology laboratories (called FiRe-laboratories) send routine annual AST results to the Finres database and THL produces the Finres report. AST data has been collected by Finres since 1997 and first report was published in 2012. The report describes the 10-year rolling AMR situation in Finland and in 2019, 22 laboratories participated (Räisänen, K., Ilmavirta, 2020). The report covers widely different bacterial isolates mainly from infections and comprehensively different antimicrobials. The Finres 2019 -report covers 20 different bacterial species. Coverage of the report is on average 98% from blood culture results collected during the past ten years (also non-invasive isolates are included in the report). The Finres report is able to monitor and give timely information on AMR trends and the effects

of retortions or vaccines (10-valent pneumococcal conjugate vaccine) in Finland. In addition to Finres, the following bacterial isolates obtained from all types of specimens are notified to the National Infectious Disease Registry: MRSA, VRE, third-generation cephalosporins non-susceptible *E. coli* and third-generation cephalosporins non-susceptible *K. pneumoniae*, and carbapenem non-susceptible *K. pneumoniae, E. coli* and *E. cloacae.*

2.6 Carbapenem-resistant Enterobacterales

2.6.1 Background

CRE are a public health concern and one of the critical pathogens the WHO has identified to guide the research, discovery, and development of new antibiotics (Shrivastava et al., 2018). Carbapenems are considered the last-line drug against Enterobacterales with ESBLs, which makes resistance to this drug class extremely worrisome. Carbapenem resistance is a clinical problem mainly caused by CPE.

CPE as a concept is complex, because it covers many species and many types of enzymes compared to, for example, methicillin-resistant *S. aureus* (MRSA), which is a single species and almost always with only one mechanism. CPE typically harbor other resistance genes as well; these are often XDR or even PDR, which cause delays, limit proper treatment, or produce a rise in fatality when causing infections (Falagas et al., 2014; Perez et al., 2016). Currently, transmission of CPE primarily occurs in hospitals and other HCFs and are able to cause outbreaks (David et al., 2019). CPE has the potential to initiate an epidemic and has caused numerous large clonal hospital outbreaks worldwide with *K. pneumoniae* being reported as the most common organism in the outbreaks (French et al., 2017). The source of a CPE outbreak (in low prevalence country) is often indexed patients previously hospitalized abroad, an environmental reservoir like a hospital sink or a contaminated hospital instrument (French et al., 2017). Asymptomatic CPE carriage among hospitalized patients increases the risk of spreading CPE and developing an infection caused by CPE (Brink, 2019). At the same time, NDM-1-producing bacteria have also been reported both in hospital and community-acquired infections and environmental sources have been shown to spread NDM-producing bacteria in lower-income countries (Poirel et al., 2014; T. R. Walsh et al., 2011). A study from India showed that 4% of the drinking water and 30% of seepage samples included NDM-1-producing bacteria (T. R. Walsh et al., 2011).

Cassini et al. estimated the burden of infections caused by different resistant bacteria on public health concerns in Europe during the years 2007-2015. During this period the burden increased for all the studied antibiotic-resistant bacteria, but the burden of carbapenem-resistant *K. pneumoniae* had increased the most in terms

of the number of infections and the number of deaths, with the second largest increase among the carbapenem-resistant *E. coli*. The burden highlighted the geographical heterogeneity of CRE, noted in EARS-Net surveys, as being high in Italy and Greece and lower in the Nordic countries. (Cassini et al., 2019)

CRE	Enterobacterales that test phenotypically resistant to at least one of the carbapenem antibiotics (for example ertapenem, meropenem, doripenem, or imipenem) are called CRE. This is a phenotypic definition based on the antibiotic susceptibility pattern of the organism. There are many different mechanisms that can result in carbapenem resistance.
CPE	Carbapenem-resistant Enterobacterales (CRE) that produce carbapenemases, enzymes that break down carbapenems and related antimicrobials making them ineffective, are called CPE. CPE are a subset of CRE, but there are some CPE (like some OXA carbapenemases) that confer low level resistance and might be susceptible to carbapenems. It is important to confirm whether CRE is also CPE, because acquired resistance requires more attention in infection prevention.

2.6.2 Epidemiology of carbapenemase-producing Enterobacterales

The first report of carbapenemase from an *Aeromonas hydrophil*a isolate was presented in Japan in 1986 (Shannon et al., 1986). In the early 1990s, plasmid-mediated carbapenemases were found in multiple species in clinical isolates; IMP-1 in *P. aeruginosa* in 1991, OXA-23 in *A. baumannii* in 1993, and KPC-1 in *K. pneumoniae* in 1996 (Brink, 2019). After these first findings CPE were infrequently reported in a single country or region until 2013, after which they have spread exponentially worldwide (Hansen, 2021). Today the most common species of Enterobacterales having transmissible carbapenemase genes is *K. pneumoniae* (Brink, 2019). Carbapenemase types are not evenly distributed in the world and some countries or continents are endemic to certain carbapenemases (Figure 2). IMP-type carbapenemases are endemic or highly distributed in Japan, Taiwan and Greece, VIM-type only Greece, OXA-type Turkey, Morocco, and Malta, KPC-type China, the US, Israel, Italy, and Greece, and NDM-type especially Asia (Logan & Weinstein, 2017). Nonetheless all known carbapenemases have spread worldwide.

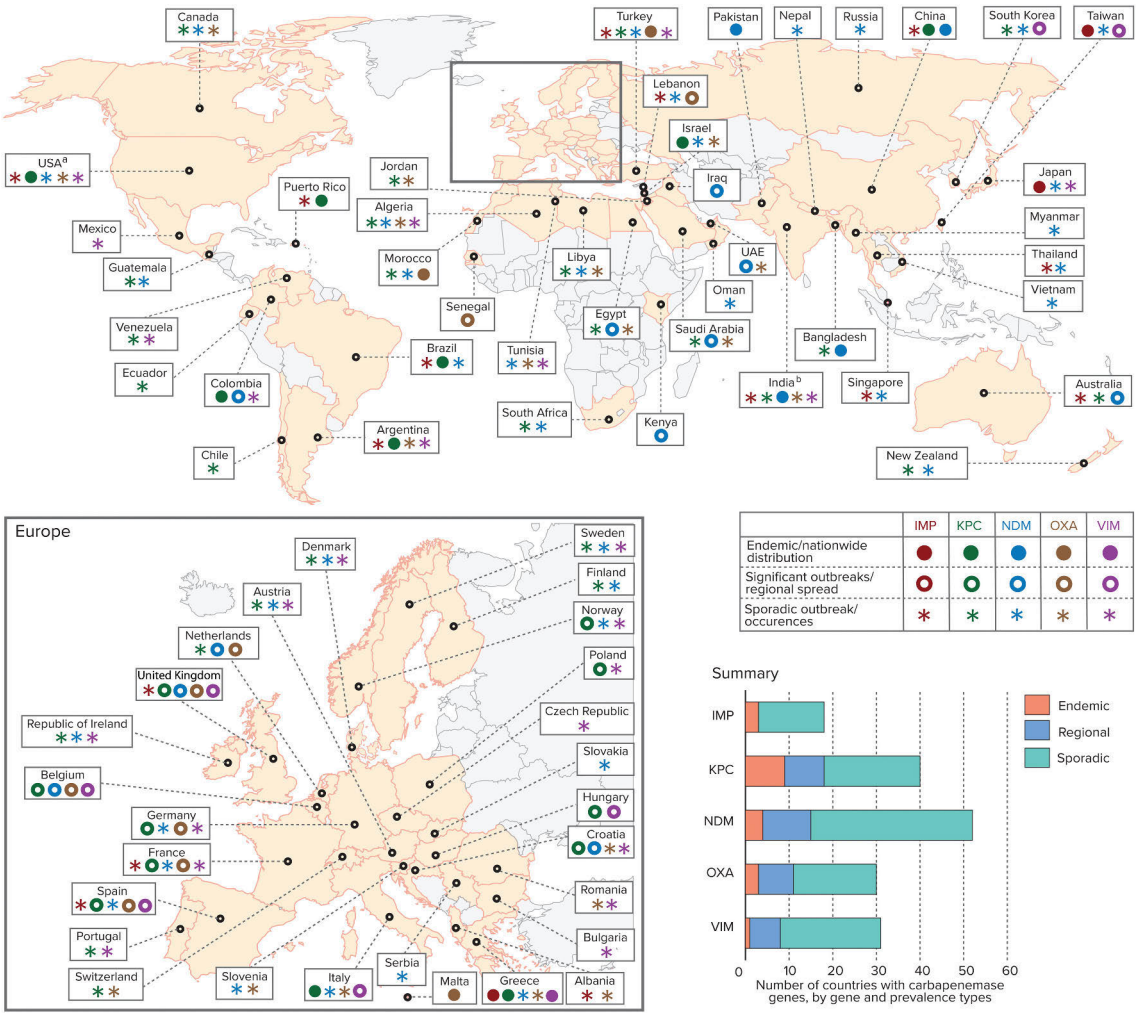

Figure 2. Global distribution of carbapenemases in Enterobacterales. Figure is reprented from Logan and Weinstein, 2017 with the permission of Oxford University Press.

Epidemiological data on CPE from different parts of the world varies, as some areas have sophisticated surveillance systems, while others lack such systems, and this creates challenges when comparing the data. In addition, the state of the CPE situation in a country can affect the surveillance system. In a high-income country with low levels of CPE cases detailed molecular analysis is feasible (Lane et al., 2020) but if a high-income country has a high prevalence of CPE or in low-income country (despite CPE prevalence) it may be impossible to analyze sufficient number of isolates to obtain a complete picture of the situation. The WHO has produced the GLASS report on the AMR situation and the current state of AMR surveillance globally in 2019 (Agnew et al., 2021). The report has gaps in the CRE information

in most WHO regions. The majority, 49 out of 69 datasets came from the US and European regions. Because *K. pneumoniae* is the most common species having a carbapenemase gene, it´s carbapenem resistance rates are used here to compare different regions (Figure 3). CRE is not same as CPE for instance; at large study from the US showed that only half of all submitted CRE isolates were found to have a carbapenemase gene (Guh et al., 2015) and this can also differ between countries. At the same time all CPE are not CRE.

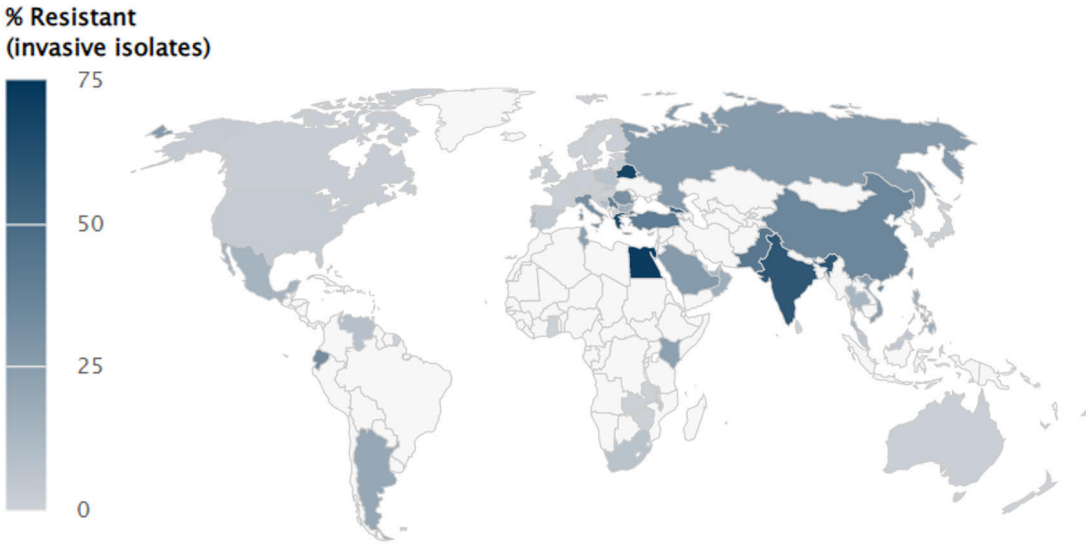

Figure 3. Resistance of *K. pneumoniae* to carbapenems 2014-2017. Data from several sources (including EARS-Net and GLASS 2017) compiled by Center for Disease Dynamics, Economics and Policy, https://resistancemap.cddep.org. Data show aggregated resistance rates for isolates from blood and cerebrospinal fluid. Due to differences in sample collection and testing methods, rates should be interpreted with caution. For some countries rates are based on less than 100 isolates. Light grey countries lack data. Figure is reprented with the permisson, (*Cent. Dis. Dyn. Econ. Policy. Resist. Antibiot. Resist. 2021*, 2021).

Geographic distribution of CRE/CPE

North America

Carbapenem resistance among *K. pneumoniae* in the US is between 3.1–4.9% (Cai et al., 2017). In the US, CRE incidence is 0.3-2.9 infections per 100 000 persons per year, and infection rates were highest in long-term acute-care hospitals, hospitals specializing in treating patients requiring extended hospitalization (Livorsi et al., 2018). Reports of colonization and infection with CPE are increasing across the US (Livorsi et al., 2018). The first KPC-producing *K. pneumoniae* was discovered in a North Carolina hospital in 1996, and today, KPC is endemic in many regional parts of the US (Yigit et al., 2001). Furthermore, other carbapenemases like NDM, VIM,

and OXA-48 are being detected in the US (Nordmann & Poirel, 2019). CRE strains in Canada showed that the most common carbapenemases were KPC-type and NDM-1 (Nordmann & Poirel, 2019).

South America

Latin America has a carbapenem resistant *K. pneumoniae* rate of 1.3–28.6% and virtually all resistance mechanisms have spread in this area (Nordmann & Poirel, 2019). One of the first reports of CPE in Latin America was the description of IMP-producing *K. pneumoniae* from Brazil in 2005 (Lincopan et al., 2005).

Africa

Comprehensive data from Africa is missing and estimations should be evaluated with caution. Estimating the overall carbapenem resistance in *E. coli* and *K. pneumoniae* in the African region was generally low (<1%) to moderate (1–5%), when all available data was aggregated (Mitgang et al., 2018). A review of East African studies summarized that the mean prevalence of carbapenem resistant *K. pneumoniae* was 15% (Ssekatawa et al., 2018). A surveillance report from South Africa collected invasive isolates between 2015–2018 (Perovic et al., 2020). Most of (70%) the CRE cases were hospital-acquired, the main species was *K. pneumoniae* (78%) and the prevalent carbapenemase genes were OXA-48 and it´s variants, and NDM (Perovic et al., 2020).

Oceania

In the whole Asia, carbapenem resistance among *K. pneumoniae* ranges from 0–52% (Nordmann & Poirel, 2019). The prevalence rate of CRE colonization in the Asia region ranged from 13–22.7% (H. Y. Chen et al., 2021). The most common carbapenemases in Asia were NDM and other metallo-β-lactamases, and OXA-48-type (Suwantarat & Carroll, 2016). An Australian report on antimicrobial use and resistance in humans showed low levels (0.3–0.6%) of carbapenem resistance among *K. pneumoniae* isolates from infections in 2019 (Australian Commission on Safety and Quality in Health Care, 2021) and the incidence of CPE was 0.45–0.60 infections per 100 000 in 2016–2018 (Lane et al., 2020). Usually, the carbapenemases like NDM, OXA-48-like, and KPC where related to traveling abroad compared to the IMP cases which were often from local outbreaks (Lane et al., 2020).

Europe

ECDC produces high-quality AMR data from Europe with yearly EARS-Net reporting. The AMR situation in Europe varies between countries and generally carbapenem resistance is rare in *E. coli,* while carbapenem resistance in *K. pneumoniae* was over 10% in several countries (https://www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc). The European mean of carbapenem resistance among *K. pneumoniae* isolated from invasive infections is 7.9% and the country range is 0.0–

58.3% (ECDC, 2020). This means that in some countries carbapenem antibiotics would not be effective in more than half of the patients treated for *K. pneumoniae* infections. For CRE a north-to-south and west-to-east gradient is evident (Figure 4). CPE is endemic or at least there are high levels in Italy, Greece, Cyprus and Romania (Iacchini et al., 2019).

The first KPC-producing *K. pneumoniae* findings in Europe were made in France 2004 (Naas et al., 2005), Greece 2007 (Cuzon et al., 2008), and the United Kingdom 2007–2008 (Neil Woodford et al., 2008). In Greece, CRE was at low levels and VIM-type carbapenemases were prevalent before 2001 (Hansen, 2021). After the introduction of the first KPC finding, the CRE increased dramatically and this was attributable to the spread of the KPC-2 gene in *K. pneumoniae* in a single dominant sequence type, ST258 (Hansen, 2021). In the Nordic countries, Switzerland, and Netherlands (van der Zwaluw et al., 2020) CPE cases are still mainly sporadic (Albiger et al., 2015; Ramette et al., 2021; Samuelsen et al., 2017).

In Europe, the mean incidence of CPE is 2.5 infection per 100 000 hospital patient days (Grundmann et al., 2017) and the CPE trend in Europe during 2015–2019 is rising ("Surveillance of Antimicrobial Resistance in Europe 2018.," 2019). In Europe the most common carbapenemases were KPC (42%) and OXA-48 (38%) (Grundmann et al., 2017).

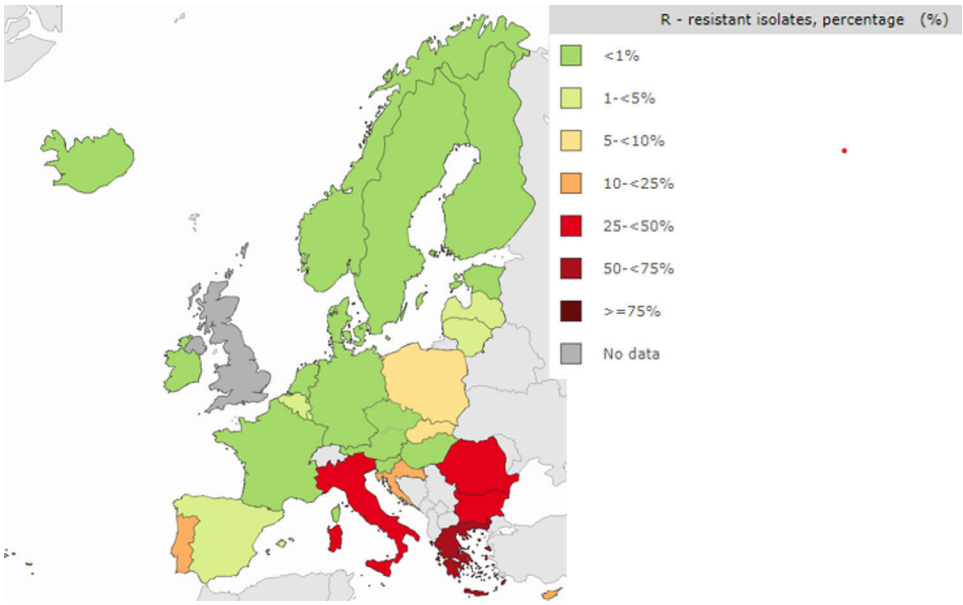

Figure 4. Percentage of invasive isolates resistant to carbapenems (imipenem/meropenem) among *Klebsiella pneumoniae*, by country, EU/EEA, 2020. the figure has been reprinted with the permission of the copyright holders, licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/) from: www.ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc.

In Finland, in addition to the Finres report, an annual communicable disease report also covers detected CRE and CPE cases (Räisänen, K., Ilmavirta, 2020; THL, 2020b). CPE cases in Finland are still rare but the trend is increasing (THL, 2020b). CPE cases in Finland are often sporadically detected (THL, 2020b), but the epidemiological stage for the spread of CPE has changed from a single hospital outbreak in 2015 to a regional spread in 2018 (Brolund et al., 2019). In 2020, 71 CRE notifications were made to the national infectious disease registry; 89 CRE isolates were sent to THL for further characterization and 65 of the isolates were CPE. There was a small decline compared to 2018 where 53 CPE isolates were detected (THL, 2021b). The most common carbapenemases in 2020 were KPC-2, OXA-48 and NDM-1 (THL, 2021b). During 2016-2020 the dominant CPE species was *E. coli* (approximately 43%), and notifiable species (*K. pneumoniae, E. coli, E. cloacae*) covered 87% of the detected CPE species. (THL, 2021b)

2.6.2.1 International high-risk clones

Clones are another mechanism, in addition to horizontal plasmid transfer, by which AMR in Enterobacterales spread globally and locally. The high-risk clones are special cases of these clones. It has been shown that the spread of well-established clones has a central role for intra- and inter-hospital transmission of CPE (David et al., 2019). Successful high-risk clones are common in all parts of the world. The spread of high-risk clones is a threat under the selective pressure of antimicrobials particularly in HCFs, and successful high-risk CPE clones are often associated with outbreaks (Hardiman et al., 2016). International high-risk clones have to have; 1) a global distribution, 2) resistance mechanisms, 3) the ability to colonize and persist in hosts for long time intervals, 4) enhanced pathogenicity and fitness, and 5) the ability to cause severe and/or recurrent infections (Mathers et al., 2015b). They spread AMR vertically with offspring and, moreover, they act as efficient donors and recipients of plasmid-mediated AMR because of the characteristics listed above (Mathers et al., 2015b).

After international successful clones start to spread locally, the clone varies and exchanges plasmids. This is how new clones emerge. These new clones can stay local or can become new successful or even high-risk clones and continue spreading. Locally, the clones are prevented by focusing on infection control measures. The most important successful clones in the 2000s have been *E. coli* ST131 and *K. pneumoniae* ST258. (Mathers et al., 2015b)

E. coli ST131 clone

The *E. coli* ST131 clone was first identified in 2008 and it is responsible for global distribution of CTX-M-15 during the early to mid-2000s (Pitout & Finn, 2020; Neil Woodford et al., 2011). Retrospective studies have implicated that it may have risen as early as 2003 (Nicolas-Chanoine et al., 2014). *E. coli* ST131 is often linked with community-acquired infections, like urinary tract infections. The clone has been reported accounting for as high as 70% of the total ESBL-producing isolates and is frequently fluoroquinolone resistant (Mathers et al., 2015a; Pitout & Finn, 2020). The prevalence of the clone among *E. coli* from clinical isolates varies by geographic region and host population from 13% to nearly 30% (Mathers et al., 2015a). As awareness of the clone increased it started to be found in clinical specimens, companion animals, poultry and occasionally in other food-producing animals, and wildlife, but also in environmental sources like soil, beach water and wastewater (Pitout & Finn, 2020; Platell et al., 2011).

Population genetics show that ST131 consists of different clades (A, B, and C) (Pitout & DeVinney, 2017) and the C2 clade, subdivision of clade C, has been the principle clone (Ellaby et al., 2019). This C2 acquired ESBL production due to the acquisition of an IncF plasmid harboring the gene CTX-M-15 (Pitout & Finn, 2020). However, no answer exists as to why certain alleles of the ESBL family have become more successfully established than others. This clone has seldom been associated with carbapenemases, but concerning reports have increased in recent years (Ellaby et al., 2019; Gong et al., 2020; Welker et al., 2020). If carbapenemases become more prevalent among this clone, it would create a significant public health concern.

K. pneumoniae clones ST258 and ST512

K. pneumoniae ST258 is a successful high-risk clone. The clone dominantly has a KPC-2 or KPC-3 gene and it also appeared in the early to mid-2000s (Pitout et al., 2015). The clone has two lineages, namely, clade I (associated with KPC-2) and clade II (associated with KPC-3), and presumably it is a hybrid clone from a large recombination event between ST11 and ST442 (Pitout et al., 2015). *K. pneumoniae* ST258 belongs to the clonal complex 258, which also includes ST11, ST340, ST437 and ST512 (Mathers et al., 2015b; Wyres & Holt, 2016). *K. pneumoniae* ST258 was first identified among isolates from the New York area collected during 2005 (Mathers et al., 2015b). Four years later this clone accounted for 70% of *K. pneumoniae* isolates with a KPC-gene in the US (Mathers et al., 2015b). Subsequently, the clone was detected in Israel and Greece, and genetic analyses support that its onward spreading to Europe occured mostly from the Greece lineage probably via travel (David et al., 2019).

ST512 is single-locus variant in the *gap*A locus of ST258. This clone often carries the KPC-3 gene and has caused outbreaks globally (Baraniak et al., 2017; Migliavacca et al., 2013; Piccirilli et al., 2020). ST258 and ST512 are often grouped together because overall these STs have less diversity than each other (David et al., 2019). It is predicted that ST512 emerged initially in Israel and was introduced from Israel to Italy (David et al., 2019). In Italy ST512 is reported to be widely disseminated and was considered the dominating clone among invasive *K. pneumoniae* infections during 2012–2013 (Conte et al., 2016). The clone ST258 and its derivative, ST512, is now endemic in the US, Israel, and some southern European countries, particularly Greece and Italy (David et al., 2019). Clones ST258 and ST512 have been shown to spread from endemic countries and induce outbreaks elsewhere (David et al., 2019). The characteristics that make this clone high-risk are that it is associated with MDR determinant, it spreads successfully in HCFs for a long period of time and causes outbreaks (Mathers et al., 2015b). A study from Italy showed that all studied CAZ-AVI resistant *K. pneumoniae* strains during a one-year period from six hospitals were all from successful clones and ST512 was the most common (Venditti et al., 2021). In Italian hospitals especially, blood stream infections caused by rapidly spreading resistant clones, like ST512, cause infections that are very difficult to treat and that as a consequence deaths are increasing (Fontana et al., 2020).

2.7 Methods used for molecular epidemiology

Molecular epidemiology, in the field of infectious diseases, has been defined as the use of molecular typing methods for infectious agents in order to study distribution, dynamics, and determinants of health and disease in human populations (Hall, 1996). Molecular epidemiology combines traditional epidemiological methods with analysis of genome polymorphism of pathogens over periods of time, across locations and individuals in human populations and relevant reservoirs. The objective is to identify host-pathogen interactions and infer hypotheses about host-to-host or source-to-host transmission.

Molecular epidemiology evolves as new tools are developed. Different molecular microbiology methods are used for different purposes, which can be, for example, bacterial typing for global surveillance or in local outbreak investigation (Riley & Blanton, 2018). For global epidemiological surveillance multi locus sequence typing (MLST) has been used from 1998 and it is still an operational method (M. C. J. Maiden, 2006). For local outbreak investigation the pulsed-field gel electrophoresis (PFGE) was for several decades the golden standard for bacterial typing (Kaufmann, 1998). PFGE was widely used before 2008, when next-

generation sequencing (NGS) technology and whole genome sequencing (WGS) became generally accessible (Koboldt et al., 2013).

Sequencing methods of nucleic acids have developed rapidly in the last twenty years. The first (generation) sequencing technique was Sanger sequencing, discovered in 1977, and it was the most used technique for more than thirty years (Sanger et al., 1977). Sanger sequencing has high accuracy and is still widely used when individual genes are sequenced, but it is not the choice for sequencing an entire bacterial genome. Next-generation sequencing (NGS) was available for research settings in 2008 and has subsequently been the main method in large scale applications because of high throughput and low costs (Koboldt et al., 2013).

2.7.1 Pulsed-field gel electrophoresis (PFGE)

PFGE was developed in 1984 by Schwartz and Cantor and it involves enzyme digestion of bacterial DNA, where the restricted DNA bands are separated using a pulsed-field electrophoresis chamber then stained, after which the banding pattern is analyzed visually (Goering, 2010). These electrophoretic pulses by constantly changing the directions of the electric field make it possible to separate large DNA fragments compared to conventional agarose-gel electrophoresis (Goering, 2010). An estimation is that the average bacterial PFGE pattern represents more than 90% of the total genome (Goering, 2010). The intra- and inter-laboratory reproducibility of PFGE was and is a challenge, but there are a number of standardized typing approaches developed to ease the difficulty. Tenover et al. created the widely used interpretation of PFGE banding patterns which show how to sort bacterial strains that are closely related, possibly related or unrelated (F. C. Tenover et al., 1995). PFGE was used as the gold standard for more than twenty years and it is still used in some countries or hospitals. Compared to WGS, PFGE is a time consuming, laborious and low resolution method and WGS has already mostly replaced it (Miro et al., 2020). The implementation of WGS is underway across the world.

2.7.2 Multilocus sequence typing (MLST)

MLST is a molecular microbiology method ideal for global scale epidemiology for revealing global clones, defining epidemic strains or grouping into clonal complexes or linages as an improved understanding of the biological population structure emerges (M. C. J. Maiden et al., 2013). MLST was introduced in 1998 and it follows the DNA sequence variation of usually five to seven housekeeping genes (loci) and characterizes strains by allelic profiles (M. C. Maiden et al., 1998). Allelic profiles are compared and collected in an international database (containing the reference allele sequences of certain bacterium). The database provides standardized

nomenclature giving a certain sequence type (ST) to the strain with certain allelic combination. MLST enables inferring evolutionary relationships from sequence data. Two isolates with the same numerical ST (for example ST11) have identical sequences in the seven housekeeping genes. For different bacterial species there can exist more than one MLST schemes and curated databases (such as those found in the PubMLST database collection < https://pubmlst.org/>). Prior to WGS, MLST was conducted with first amplification housekeeping genes with PCR, and then the nucleotide base sequence was determined by the Sanger sequencing technique (Sanger et al., 1977). Currently, MLST information can be extracted from WGS data. MLST is highly unambiguous and portable, but the discriminatory power compared to WGS is low (Miro et al., 2020).

2.7.3 Whole genome sequencing (WGS)

WGS is currently the gold standard for bacterial typing and can be used for molecular epidemiology at different levels. It is a comprehensive method for analyzing the genomic DNA of a bacterium at a single time by using sequencing techniques like Sanger sequencing or high throughput NGS sequencing. The method has the highest possible discriminatory power of the known methods and it can be applied to any bacterial species and it has largely replaced the old molecular methods used. WGS provides almost complete information on the genome of an isolate. In addition to discriminatory power, the detection of AMR determinants or other determinants like virulence factors can be performed. These determinants can be located, on the bacterial chromosome or even in plasmids, and the information of location provides valuable information on how AMR is spread. (Croucher & Didelot, 2015)

Next-generation sequencing (NGS) technologies

At present WGS is performed mainly using NGS technology (Besser et al., 2018). NGS is a massively parallel sequencing technology that has high throughput (Besser et al., 2018). Today, most NGS technologies are relatively low cost and rapid (Besser et al., 2018). The difference between Sanger sequencing (first generation sequencing) or so called conventional capillary sequencing is that, instead of sequencing a single DNA fragment at a time, NGS sequences millions of fragments simultaneously per run (McGinn & Gut, 2013). This process translates into sequencing hundreds to thousands of genes at one time (McGinn & Gut, 2013).

Several different NGS technologies or platforms exist for producing sequences and WGS data. NGS technologies can be divided in relation to the read length for short (fever than 300 base pairs) and long (more than 10,000 base pairs). Compared to Sanger sequencing, the error rate in NGS technologies is higher. Moreover, third

generation technologies are today available, and these produce longer reads than second generation sequencing, between 10,000 and 100,000 base pairs in one run, without the need to cut and amplify the DNA samples. The benefits of long-read sequencing are that the genome sequence is assembled from much larger pieces, therefore opportunities for error and uncertainty are reduced. These longer reads are more reliable where large sections of DNA are inserted, deleted or moved around. Long-read sequencing is considered better for sequencing repetitive DNA because it is less error-prone compared to shorter reads where repeats can be overlooked or duplicated during reassembling. At the same time, long-read sequencing clearly produces more errors than short-read sequencing and is more expensive. When constructing complete genomes or studying plasmids long-read sequences are valuable, particularly when used in combination with short reads. Short read NGS technology is the most popular one for producing WGS data from bacterial isolates. (McGinn & Gut, 2013)

Use of WGS in public health practices

Nowadays WGS is widely used in national reference laboratories in high- and middle-income countries. WGS produces a considerable amount of information. One of the key advantages of using WGS data is its superior resolution in phylogenetic analyses compared to older methods like PFGE (Miro et al., 2020). Data is portable, available for direct analysis and relatively easy to store. Analysis can be done with computer software and the analysis can be harmonized and automated. No visual interpretation of the results is needed as in PFGE.

The advantage of WGS is that the many different properties that used to require several different laboratory methods, can now be obtained from the WGS data. First, taxonomic analysis can be done or confirmed from the WGS data. Second, AMR determinants like resistance genes can be detected using reliable and comprehensive databases. In addition, phenotypic prediction can be made from genotypic resistance gene results. For surveillance, virulence factors and mobile genetic elements can also be determined. Third, outbreak detection can be done by subtyping and following the spread of internationally successful or high-risk clones with the MLST method or even more closely with core genome (cg)MLST method integrated with epidemiological background information.

WGS has changed the nature of CPE surveillance and outbreak investigation. Before WGS, PFGE was, at least in Finland, conducted for isolates during any suspicion of an outbreak because typing of all collected isolates was not feasible. The suspicion that the isolate was part of an outbreak was usually based on epidemiological data and attempts were to put all the suspected isolates on the same PFGE run to minimize the differences between the runs and help subjective analysis

(Kanerva et al., 2015). Today, when WGS is used it is possible to add a new isolate to the database and conduct a comparison to previous isolates. Furthermore, WGS data can guide the contact tracing, mark the timetable and focus the screening in outbreak investigations. Genetic data can reveal unexpected modes of transmission and motivate a thorough search for linkages between patients suspected of transmitting the strain (Snitkin et al., 2012). In addition, linking patient and environmental or infrastructure isolates together may enhanced efficient cleaning of shared equipment and vacated patient rooms (Snitkin et al., 2012). It is also confirmed that WGS can be used to identify relationships between strains during outbreaks even when prior data are minimal and limited for example to the index strain (E. Ruppé et al., 2017). Nevertheless, surveillance, tracing and controlling transmission of AMR with WGS data requires reference databases with good quality genomic and AST data. WGS data combined with epidemiological and clinical background information enables linkages during the early detection of outbreaks, accurate tracing of transmission chains, precise definition of the geographical spread of an outbreak and identification of sources of infection. It has been shown that a coordinated, statewide collaborative genomics and epidemiology approach to CPE has been invaluable to define the burden of CPE and direct public health and hospital infection control interventions (Sherry et al., 2019). To gain the best advantage from WGS data it should be utilized and shared nationally and internationally (European Centre for Disease Prevention and Control, 2016; World Health Organization (WHO), 2020).

Adopting WGS for global surveillance can provide information on the early emergence and spread of high-risk AMR clones. Additionally, WGS is essential for a One Health approach in AMR surveillance, because it can show the relatedness of human and animal isolates (Jagadeesan et al., 2019). The WHO has published a document which addresses the applications of WGS for AMR surveillance, including the benefits and limitations of current WGS technologies (World Health Organization (WHO), 2020).

ECDC has also started projects towards European wide genomic-based surveillance: The European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net) which concentrates on healthcare-associated MDR pathogens and Reference Laboratory Capacity Building (EURGen-RefLabCap) which focuses on harmonizing protocols for CRE surveillance and outbreak detection using WGS data. The use of WGS data internationally and replacing other (older) methods will in the future improve the accuracy and effectiveness of disease surveillance, outbreak investigation and the evaluation of prevention policies. ECDC has two main surveillance objectives for WGS-based comparative genome analysis internationally: 1) phylogenetic analysis and 2) prediction of clinically and epidemiologically relevant bacterial phenotypes. 1) In phylogenetic analyses WGS provides optimal resolution of the near-complete genomic sequence comparison for

measuring inter-genomic sequence similarity and inferring the most probable phylogenetic lineages of descent between isolates to infer the direction and route of pathogen transmission, from environmental, animal or human sources and reservoirs. 2) The in silico prediction of phenotype and, in particular, acquired AMR mechanisms, pathogenicity and virulence determinants as well as correlates of epidemiological/ecological fitness associated with epidemic spread, also described as high-risk clones. (European Centre for Disease Prevention and Control, 2016)

Before international or in some countries even national surveillance is adopted, there are still challenges concerning data analyzing systems, data storage, and data sharing between organizations. WGS technologies require expensive investment and the training of staff to use bioinformatics is also essential. In addition, clinicians and epidemiologists needs extra education. Locally, and more favorably globally, standardized nomenclature, standard operating procedures and quality assurance protocols are needed. (Gwinn et al., 2017)

2.7.4 Core genome multi locus sequence typing (cgMLST)

Phylogenetic approaches based on WGS data rely on calculating genetic distances based on either single nucleotide polymorphism (SNP) or allele differences known as core or whole genome MLST (cg/wgMLST) (Schürch et al., 2018). These three typing methods (cgSNP, cgMLST and wgMLST) have their own strengths and weaknesses (Miro et al., 2020). cgSNP includes most of the available genome sequence data and thus has the highest resolution power (Schürch et al., 2018). On the other hand, cgMLST and wgMLST lose genetic data because only alleles are compared and not every mutation (Schürch et al., 2018). However, for the same reason they are not as prone to conflicting signals of recombination as cgSNP. One recombination event can produce a gene with several mutations although from the view of evolution there is only one event (Friedrich et al., 2016). In addition, cgMLST or wgMLST do not need as much calculation power as cgSNP (Schürch et al., 2018). cgMLST utilize the least amount of genetic data of the three methods having the lowest resolution power but being the most stable because only core genes that can be found from all isolates of the species are included. In practice these three typing methods produce similar results (Miro et al., 2020).

cgMLST has the same principle for bacterial typing as MLST: the use of alleles as the unit of comparison, but in a wider scale and with greater discriminatory power (Zhou et al., 2017). While in MLST typically seven housekeeping genes were compared, in cgMLST thousands of genes are used in comparison, among Enterobacterales usually 2000-3000 (Zhou et al., 2017). The cgMLST typing schema includes the core genome targets, which are a fixed set of conserved genome-wide genes among all members of the species (Zhou et al., 2017). The cgMLST typing

schemes can be developed at genus level, genetic-complex level, or species level, depending on the number of available complete reference genomes (Friedrich et al., 2016). When even more accuracy is needed, wgMLST can be used (Schürch et al., 2018). wgMLST includes accessory targets in addition to cgMLST targets (genes) (Schürch et al., 2018). These accessory targets are not included in the cgMLST targets because they are less conserved but by including them in wgMLST it enables the capture of the full complement of protein-coding genes in the genome for analysis (Schürch et al., 2018). Technical issues favor cgMLST over wgMLST for global surveillance because the cgMLST based database is easier to curate. cgMLST is more suitable for outbreak investigation and longitudinal surveillance.

Genome assembly

The short read NGS technique is the method most commonly used to produce WGS data and the reads from the sequencer need to be assembled (to put back together to create a representation of the original chromosomes from which the DNA originated) before cgMLST comparison. Genome assembly is the computational process and reads are either single ends or paired ends. Single reads are simply the short sequenced fragments themselves; they can be joined up through overlapping regions into a continuous sequence called contig. Paired-end sequencing produces a sequence from both ends of a DNA fragment and can help link contigs into scaffolds, order assemblies of contigs with gaps in between, indicate the size of repetitive regions and how far apart the contigs are. Paired-end sequencing enables more accurate read alignment than single-read data. (Armstrong et al., 2019)

There are two common ways to assemble the sequence reads: 1) de novo; reads are assembled to create full-length sequences, without using a template and 2) mapping; reads are assembled against an existing backbone sequence, building a sequence that is similar to the backbone sequence. De novo genome assembly is often used for species with high genomic variability, because it enables detecting previously unknown regions of the genome or regions that vary a lot from previously known genomes. De novo genome assembly has become dominant in cgMLST-based surveillance among bacteria. (Bankevich et al., 2012)

Threshold

cgMLST comparison needs defined thresholds for distinguishing epidemiologically related isolates. These genetic thresholds that distinguish epidemiologically related from unrelated isolates can vary between species. Estimates of the mutation rate in *Enterobacteriaceae* range from 0 to 10 mutations per genome per year and *E. coli* has the highest threshold (Friedrich et al., 2016). The genetic distance between

genomes indicates the phylogenetic relationship, but these may not directly reflect the order and timing of transmission events from donor to recipient (Didelot et al., 2016). Kluytmans-van den Bergh et al. conducted a study where they defined thresholds for genetic distance for many clinically important Enterobacterales in cgMLST (Friedrich et al., 2016).

Nevertheless, the main challenges with all WGS-based typing methods are to define thresholds for genetic distance that can be used to identify an outbreak caused by a single clone. Threshold parameters should be applied with attention and should be used in combination with clinical epidemiological background information and population and species characteristics. (Miro et al., 2020)

2.7.5 Resistance gene detection

AMR gene detection is important in treatment of patients and in implementation of control measures. It can be used both for surveillance and clinical purposes. In addition to AST molecular AMR gene detection can predict more reliably if the bacterial isolate has hospital hygiene relevance. PCR-based methods, commercial or in-house, can be used to detect resistance genes. PCR-based methods have been used before the WGS era and are still feasible because of low cost and fast performance time. In Finland, the recommendation is that the carbapenemase PCR confirmatory test in clinical microbiology laboratory needs to cover at least KPC, OXA-48 type and NDM genes (THL, 2020a).

More accurate AMR gene characterization can be done using WGS data. WGS surveillance makes it possible to define MDR with tremendous precision compared to phenotypic tests (Hendriksen et al., 2019). Bioinformatic analysis can reveal the co-carriage of specific genes underlying different MDR patterns (Hendriksen et al., 2019). The demands on databases are to provide high-quality, curated, and validated reference data. In addition, databases should be updated regularly. At least 47 freely available databases exist for *in silico* AMR prediction, such as, CARD (The Comprehensive Antibiotic Resistance Database, https://card.mcmaster.ca/), ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), KmerResistance (https://cge.cbs.dtu.dk/services/KmerResistance/), and SRST2 (https://github.com/katholt/srst2) (Hendriksen et al., 2019). Resistance gene detection methods can be divided as follows: (1) assembling raw reads to contigs before comparing with a reference database or (2) mapping reads directly to reference sequences (Clausen et al., 2016). When (1) assembling first reads some information can be lost during assembly, for example a gene may be missed if it is divided in two or more contigs (Clausen et al., 2016). This (1) method is highly dependent on the assembly quality. (2) Mapping reads directly has shown to be superior when identifying resistance genes. The sensitivity and accuracy are high.

The use of genotypic AMR in the prediction of phenotypic AST

NGS technologies have shown good concordance with traditional phenotypic susceptibility testing, suggesting that they could be used in AMR surveillance (Clausen et al., 2016). Phenotypic AST detects the arrest of bacterial growth in the presence of antimicrobials, whereas genotypic AST attempts to identify specific resistance genes of genetic mutations using molecular or genomic methods (van Belkum et al., 2019). The EUCAST subcommittee stated in 2017 that for most bacterial species there is currently a lack of evidence to support the use of genotypic AST to guide clinical decision making (Ellington et al., 2017). However, a few years later in 2020 the ResFinder 4.0 was launched, detecting the AMR genes and chromosomal gene mutations and generating *in silico* antibiograms (Bortolaia et al., 2020). The authors behind the ResFinder 4.0 stated that genotypic AST is as reliable as a phenotypic AST for several antimicrobial/bacterial species combination and could be used at least for surveillance (Bortolaia et al., 2020). For example, genotype-phenotype concordance was ≥95% in 46/51 antimicrobial/species combinations evaluated for gram-negative bacteria (Bortolaia et al., 2020).

The limitations of genotypic AST are their inadequate overall sensitivity and the fact that only known AMR mechanisms can be detected. Usually when evaluating WGS AST the phenotypic AST has been considered the correct result, even though, phenotypic testing has important limitations in accuracy and reproducibility (Bortolaia et al., 2020). Tthe advantages of WGS AST are that data can be analyzed using the same bioinformatics pipeline and with 100% reproducibility between laboratories, and that data can be easily stored and re-analyzed. Generally, WGS is currently not suitable for routine or predictive AST in clinical settings and therefore cannot replace phenotypic methods. There are plans in the near future for the use of WGS data for surveillance purposes in international organizations like the WHO and the ECDC. At the moment, decision No 1082/2013/EU of the European Parliament and of the council on serious cross-border threats to health obligates member states in Europe to monitor AMR phenotypically but WGS-based methods could be used to complement the phenotypic methods.

3 Aims

Up-to-date information of the CPE situation in Finland helps evaluate and guide the prevention and control strategies of MDR microbes. Detailed information on whether the CPE cases are sporadic or imported from abroad is important. In many resistance-related aspects Finland is in a similar situation as the other Nordic countries, and during recent years the other Nordic countries were reporting an increasing number of CPE cases. This thesis was set out to investigate the molecular epidemiology and transmission chains of CPE by WGS combined with epidemiological background information from Finland, 2012–2020.

The specific aims:

1. To analyze the molecular epidemiology of CPE and CPE strains causing clusters in Finland during 2012–2018 (I).
2. To trace back the transmission chains in two clusters caused by *K. pneumoniae* KPC-3 ST512 in Finland during 2013–2018 (II).
3. To describe the first three clusters caused by CP *C. freundii* in Finland during 2016–2020 (III).
4. To study the mechanism behind CAZ-AVI resistance in *K. pneumoniae* KPC-2 after CAZ-AVI treatment (IV).

4 Materials and Methods

4.1 CRE and CPE surveillance in Finland

In Finland, all clinical microbiology laboratories electronically notify *E. cloacae, E. coli*, and *K. pneumoniae* isolates with reduced susceptibility to carbapenems (CRE) to the National Infectious Disease Registry (NIDR) and send bacterial isolates with a carbapenemase gene (CPE) to the expert microbiology unit of the Finnish Institute for Health and Welfare (THL). During 2012–2015, THL advised the clinical microbiology laboratories to send CPE or CRE isolates for further characterization and thereafter CPE surveillance has been based on communicable diseases act (1227/2016). Clinical microbiology laboratories may also send other CPE species for further characterization, for example *C. freundii* is not included in the notifiable species to be sent to the NIDR; nevertheless, THL has instructed clinical microbiology laboratories to send CP *C. freundii* isolates for further characterization and clonality analyses by WGS when an outbreak is suspected.

The national guidelines for control of MDR microbes (THL, 2020a) describes specific measures for CPE control in HCFs. Guidelines give instructions for patient´s screening, contact precautions, isolation and cleaning concerning CPE. In addition, it includes the guidelines for laboratory diagnostics of MDR microbes. It defines the minimum level for diagnostics of MDR microbes in the laboratory and gives instructions for screening and genotypic methods. The laboratory part of the national guideline is based on EUCAST Guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance (version 2.0) (Skov & Skov, 2012a), acknowledging also the Public Health England guidelines "Detection of bacteria with carbapenem-hydrolysing β-lactamases (carbapenemases)", latest studies and the Finnish practices. The latest, revised version include also CP *C. freundii*.

The ECDC published a rapid risk assessment of the emergence of CAZ-AVI resistance in carbapenem-resistant Enterobacterales (CRE) in Europe in June 2018 and consequently THL advised the Finnish clinical microbiological laboratories to notify when CAZ-AVI resistance is detected (European Centre for Disease Prevention and Control, 2018).

4.2 Quality of the data

Around 20 clinical microbiology laboratories took part in the surveillance. The annual number of laboratories varied as laboratories merged. Eleven laboratories were accredited by the Finnish Accreditation Service (situation in 3.11.2022). The laboratories performed the AST and the genotypic carbapenemase gene detection when appropriate before sending CPE isolates to the THL. We compared the number of CRE cases notified to the NIDR and the number of CPE isolates sent for further characterization yearly (surveillance described in the chapter 4.1) and estimated that it is unlikely that many CPE isolates were missing. The following information was sent to the THL along with the isolates: species, carbapenemase gene, date of specimen, specimen type, indication (clinical/screening), AST results and background information of the patient (date of birth, gender, place of treatment, information about travelling or hospitalization abroad). We did not have comprehensive data on not having preceding travelling or hospitalization abroad and in couple of cases the indication was missing.

The expert microbiology unit of the THL was also accredited, but not specifically CPE diagnostics. The expert microbiology unit followed the quality system and participated regularly in the internal and external quality assessments.

The Finnish public health microbiology system capabilities and capacities were evaluated with the EULabCap index and received a high score (ECDC, 2018).

4.3 Bacterial isolates

Study I included 231 CPE isolates identified in Finland during 2012–2018, one isolate per patient per species per year or more than one isolate per patient when the patient had isolates with different carbapenemase genes or sequence types (ST) (Table 1).

Table 1. Overview of the carbapenemase-producing Enterobacterales isolates in Studies I-IV.

Study	Time period	Species (No. Of isolates)	No. of CPE isolates/ patients	Specimen type, proportion of clinical specimen (%)	No. of blood specimens/ cultures	Proportion of male (%)	Median age, range (years)
I	01/2012–12/2018	*C. freundii (14), Citrobacter sp. (1), E. aerogenes (2), E. cloacae (9), E. coli (92), E. kobei (1), K. oxytoca (2), K. pneumoniae (105), M. morganii (1), P. mirabilis (3), S. marcescens (1)*	231/202	32	7	57	56, 1–98
II	08/2013–05/2018	*K. pneumoniae (*24)	24/20	79	1	75	69, 30–89
III	09/2016–04/2020	*C. freundii* (21)	21/20	67	3	70	68, 40–94
IV	10/2018–12/2018	*K. pneumoniae* (3)	3/1	100	3	data not shown	data not shown

Study II included 24 CPE isolates obtained from 20 cases. Here, the case was defined as a patient with a *K. pneumoniae* KPC-3 sequence type (ST)512 strain detected in Finland from August 2013 to May 2018. These isolates were also partly included in Study I (molecular epidemiology of CPE in Finland, 2012–2018). In addition to patient isolates, seven environmental isolates of *K. pneumoniae* KPC-3 ST512 were studied (unpublished data, not included in the Table 1).

Study III included CPE isolates obtained from 20 cases. Here, the case was definedas a patient with a CP *C. freundii* positive specimen belonging to one of the three clusters detected by WGS-based cluster analysis between September 2016 and April 2020. These isolates were also partly included in Study I (molecular epidemiology of CPE in Finland, 2012–2018). In addition to the patient isolates, 59 environmental isolates from the three clusters were studied (unpublished data not included in the Table 1).

Study IV included three *K. pneumoniae* KPC-2 ST39 isolates obtained from one single patient during 2018. One of the three isolates was also included in Study I (molecular epidemiology of CPE in Finland, 2012–2018).

4.4 Phenotypic analysis and CPE screening

The species identification was done in the clinical microbiology laboratories by matrix-assisted laser desorption/ionization time-offlight (MALDI-TOF) mass spectrometry (VITEK MS, bioMeriéux, Marcy-L'Etoile, France or Bruker Biotyper, Becton, Dickinson and Company, New Jersey, US) and antimicrobial susceptibilities were assessed by disk diffusion method or by gradient minimum inhibitory concentration (MIC) determination test (ETEST, bioMeriéux, MarcyL'Etoile, France) and interpreted according to the clinical breakpoints as published by EUCAST (versions 2.0–8.1).

CPE screening is directed by the national guidelines for control of MDR microbes (THL, 2020a) and the screening breakpoints for carbapenems are the same for *E. coli, E. cloacae*, and *K. pneumoniae* as published in the EUCAST guidelines (Skov & Skov, 2012b) except for meropenem for which the screening breakpoint is > 0.12 mg/L (zone diameter < 25 mm). For other Enterobacterales, the breakpoints are the same as the EUCAST clinical breakpoints, ≥ 2 mg/L (zone diameter ≤ 22 mm).

4.5 Molecular analysis

Generally, the clinical microbiological laboratories performed the carbapenemase gene confirmation before sending the isolates, with reduced susceptibility to any carbapenem, to THL. Various commercial or in-house molecular amplification

techniques have been used during the study period. However, as they are based on the national guidelines, these methods detect at least *bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ gene (THL, 2020a).

4.5.1 Whole Genome Sequencing

WGS was performed on all CPE isolates at the THL. The isolates from years 2012–2014 were sequenced retrospectively and thereafter, all isolates have routinely been sequenced using WGS. WGS was implemented with a MiSeq instrument (Illumina, San Diego, California, US). For the library preparation 1ng purified DNA was used with a NexteraXT V2 DNA sample preparation kit (Illumina) and paired-end sequenced with a 2×150bp kit (Illumina). Libraries were scaled to reach 100-fold coverage.

Read mapping analysis was performed from the FASTQ files. Files were transferred to the Centre for Scientific Computing environment where sequences were trimmed with Trimmomatic version 0.33, the quality was verified with a FastQC version 0.11.6, resistance genes and MLST were analyzed with an SRST2 version 0.2.0 (Inouye et al., 2014). The commercial software SeqSphere+ (Ridom GmbH, Münster, Germany) was used in cgMLST (Mellmann et al., 2016). cgMLST was performed on *K. pneumoniae* and *E. coli* using the available cgMLST scheme from Ridom and *C. freundii* and *K. oxytoca* cgMLST schemes were made in house having 2007 and 2947 targets, respectively. A cut-off of 10 allele differences was used to define clusters in the minimum spanning tree prepared from the cgMLST schemes. This cut-off has been experimentally determined and used in a previously published study (Friedrich et al., 2016). cgMLST analysis was combined with epidemiological background information. A Geneious version R10.2.6 (Biomatters, Ltd., Auckland, New Zealand) was used to examine the amino acids differences in Study IV (development of CAZ-AVI resistance in *K. pneumoniae* during treatment in Finland, 2018).

4.6 Ethical aspects

CPE surveillance in Finland is based on the communicable diseases act (1227/2016) which obliges the clinical microbiology laboratories to notify *E. cloacae, E. coli* and *K. pneumoniae* with reduced susceptibility to carbapenems. The act also requires comprehensive AMR surveillance. In addition, the act defines that outbreak investigations are conducted on a local level with communicable disease doctors in charge as a part of the infection control activities in the HCF. When the outbreak is caused by an extremely resistant microbe or spread to more than one region the THL guides and supports the local authorities. Studies I and IV were conducted as part of a CPE surveillance and Studies II and III as a part of the outbreak investigation, thus no ethics committee approval was required for these studies.

5 Results

5.1 Molecular epidemiology of CPE in Finland during 2012–2018 (Study I)

The annual number of CPE isolates increased from 9 in 2012 to 70 in 2018 (Figure 5) and, the number of different STs increased from 7 in 2012 to 33 in 2018. The most common species were *K. pneumoniae* (45%), *E. coli* (40%), *C. freundii* (6%), and *E. cloacae* (4%). The proportions of other species ranged between 0-1%. Dominant carbapenemase genes were $bla_{\text{NDM-like}}$ (35%, 80/231), $bla_{\text{OXA-48-like}}$ gene group (33%, 76/231), and $bla_{\text{KPC-like}}$ (31%, 71/231) and these were mostly detected from *E. coli* (n=50), *E. coli* (n=38) and *K. pneumoniae* (n=55), accordingly. Five isolates had double carbapenemases. Of the individual carbapenemase genes, $bla_{\text{KPC-3}}$ was the most common, followed by $bla_{\text{OXA-48}}$, $bla_{\text{NDM-5}}$, and $bla_{\text{NDM-1}}$, respectively.

The clusters detected during the study period were mainly caused by *K. pneumoniae.* We reanalyzed the data by excluding the effect of clusters. In the re-analyses we only included the first isolate from a patient and the first isolate from a cluster during the study period and obtained slightly different results. The most common species were *E. coli* (50%, 82/163), *K. pneumoniae* (34%, 56/163), *E. cloacae* (5%, 8/163) and *C. freundii* (4%, 7/163). The dominant genes were in the same order with different proportions $bla_{\text{NDM-like}}$ (42%), $bla_{\text{OXA-48-like}}$ gene group (37%), and $bla_{\text{KPC-like}}$ (17%) and these were detected from *E. coli* (n=41), *E. coli* (n=33) and *K. pneumoniae* (n=20), accordingly. Four isolates had double carbapenemases. When the effect of clusters was excluded the most common individual carbapenemase genes were $bla_{\text{NDM-5,}}$ followed by $bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$ and $bla_{\text{KPC-3}}$, respectively.

ST distribution was diversified among *E. coli* isolates (92) having 37 different STs compared to *K. pneumoniae* isolates (105) with only 23 different STs. The most prevalent STs among *K. pneumoniae* were ST512 (n=39), ST258 (n=8), ST11 (n=7), and ST395 (n=7) and among *E. coli*, ST167 (n=11), ST38 (n=9), ST405 (n=9), and ST410 (n=7). When excluding the effect of the clusters and multiple isolates of one patient the most prevalent STs among *K. pneumoniae* were ST258, ST512, ST147 and ST395 and among *E. coli* there were no changes. During the study period there were no alteration in ST distribution. Carbapenemase gene distribution also

remained stable during the years, when the number of detected isolates increased the number of dominating STs also increased.

Information on travel history was available for 149/202 (74%) patients: 58 (39%) patients had no links abroad, whereas 91 (61%) patients had a link: 51 (34%) patients had travelled abroad and 40 (27%) patients had been hospitalized abroad. The most common foreign countries linked to the CPE isolates were India (n=29 strains), Greece (n=11), Thailand (n=9), Spain (n=7) and Turkey (n=7).

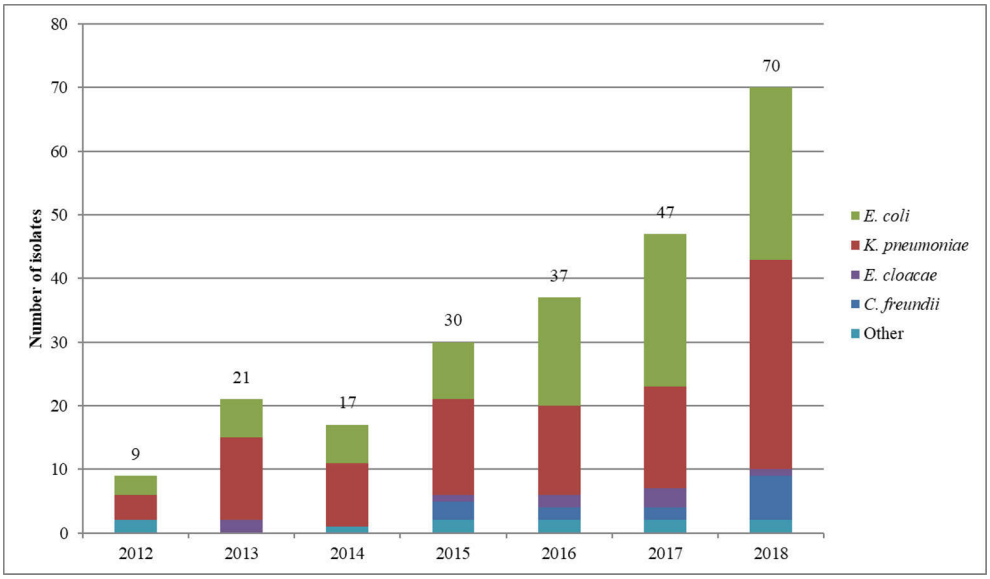

Figure 5. Annual number of carbapenemase-producing Enterobacterales isolates and species distribution detected in Finland, 2012-2018 (modified from Original publication I).

We divided patients with CPE by travel or hospitalization history to different geographic regions according to WHO definitions (https://www.who.int/occupational_health/regions/regionaloffices/en/). When the patient had a travel or hospitalization history in a European region, the most common carbapenemase genes belonged to *bla*$_{\text{OXA-48-like}}$ gene group (18/37); in South-East Asia, Western Pacific, and Africa regions, it belonged to *bla*$_{\text{NDM-like}}$ (34/40, 3/5, and 2/3 respectively); in Eastern Mediterranean region, it belonged to *bla*$_{\text{OXA-48-like}}$ gene group or *bla*$_{\text{NDM-like}}$ (10/16 and 7/16 respectively); and in the US, it belonged to *bla*$_{\text{KPC-like}}$ (3/4) (Figure 6).

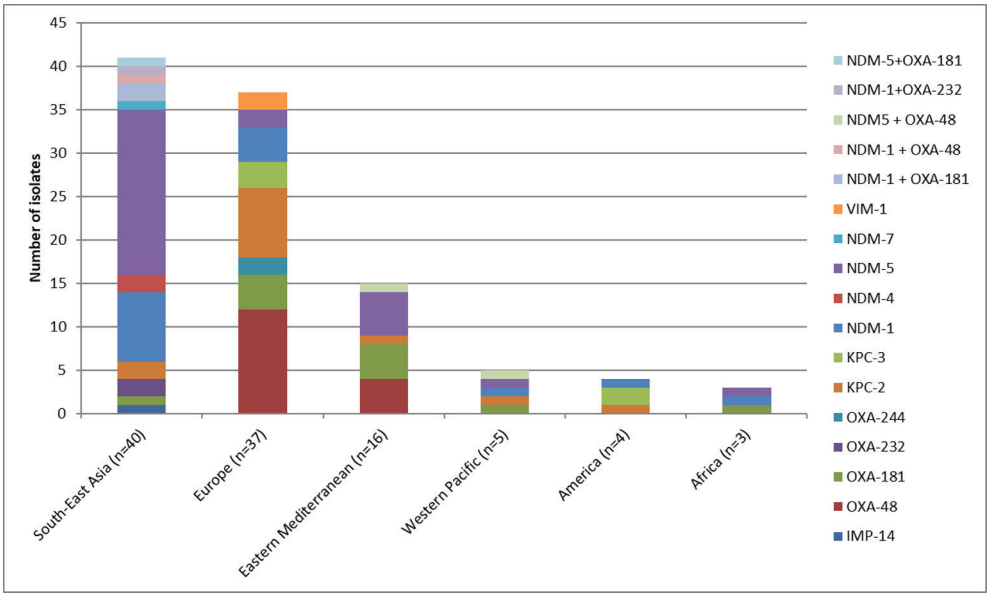

Figure 6. Gene distribution and known travel or hospitalization history abroad of carbapenemase-producing Enterobacterales isolates detected in Finland, 2012–2018 (modified from Original publication I).

Overview of the CPE clusters

In total, eight CPE clusters were detected during 2012–2018 (Study I) and two during 2018–2020 (Study III) (Table 2). An experimentally defined cut-off, 10 allele differences, was used when the clusters were defined, excluding the *C. freundii* cluster in which the first two isolates had 17 allele differences. This isolate was included in the cluster, since the cases were hospitalized in the same HCF less than eight months apart and we later found even more closely related isolates (13 allele difference). *K. pneumoniae* ST512 with KPC-3 gene caused three clusters; two large ones with 9–23 cases and one small with two patients. *C. freundii* ST18 with KPC-2 gene caused one cluster with eight patients and *K. pneumoniae* ST11 with KPC-2 gene one cluster with three patients. Furthermore, there have been three small clusters caused by OXA-48 positive *K. pneumoniae* strain with different STs. Four of the eight clusters were caused by *K. pneumoniae* strains belonging to the clonal complex (CC) 258, including ST258, ST11, ST340, ST437 and ST512. In three clusters, the link abroad was identified.

Table 2. Clusters caused by carbapenemase-producing Enterobacterales in Finland, 2012-2020, (Studies I-III). (Partly modified from Original publication I.)

Year(s)	No. of isolates/ cases or patients	Species	Sequence type	Gene(s)	Link abroad, country
2013	9/9	*K. pneumoniae*	ST512	bla_{KPC-3}	No
2013–2018	26/23	*K. pneumoniae*	ST512	bla_{KPC-3}	No
2015–2016	2/2	*K. pneumoniae*	ST512	bla_{KPC-3}	Hospital transfer, Italy
2016–2020	16/16	*C. freundii*	ST18	bla_{KPC-2}	No
2016–2018	3/3	*K. pneumoniae*	ST11/NF*	bla_{KPC-3}	Yes, Colombia
2018	2/2	*K. pneumoniae*	ST395	bla_{OXA-48}	Hospitalization, Russia
2018	2/2	*K. pneumoniae*	ST307	bla_{OXA-48}	No
2018	2/2	*K. pneumoniae*	ST273	bla_{OXA-48}	No
2018–2019	3/2	*C. freundii*	ST604	$bla_{OXA-181}$, bla_{GES-5}	No
2020	2/2	*C. freundii*	ST116	bla_{KPC-3}	No

Meropenem resistance among the three most common species, *K. pneumoniae*, *E. coli* and *C. freundii*, was 69% (101/147) and non-susceptibility (intermediate and resistant) was 77% (113/147) of the CPE isolates (Table 3).

Table 3. Meropenem susceptibility categorization of *Klebsiella pneumoniae*, *Escherichia coli* and *Citrobacter freundii* isolates in Finland, 2012-2018.

		Meropenem SIR			
Species	**Carbapenemase**	**S**	**I**	**R**	**Total number**
K. pneumoniae	KPC	2	0	38	40
	NDM	0	3	12	15
	OXA-48-like	5	2	8	15
	NDM+OXA-48-like	0	0	4	4
E. coli	KPC	1	1	0	2
	NDM	0	2	32	34
	OXA-48-like	22	1	2	25
C. freundii	KPC	0	3	4	7
	NDM	0	0	1	1

5.2 Two clusters of *K. pneumoniae* ST512 producing KPC-3 in Finland, 2013–2018 (Study II)

Two epidemiologically unrelated clusters with *K. pneumoniae* KPC-3 ST512 were recognized in a cgMLST analysis of the national surveillance data (Figure 7). The first cluster was during regional outbreak with 18 *K. pneumoniae* KPC-3 sequence type (ST)512 cases in five HCFs in Northern Finland between August 2013 and May 2018, and the second cluster occurred in a single hospital with two *K. pneumoniae* KPC-3 ST512 cases in Western Finland between July 2015 and April 2016. The strain in the first cluster was only susceptible to CAZ-AVI, colistin and gentamicin and the strain in the second cluster to gentamicin and colistin (CAZ-AVI not tested).

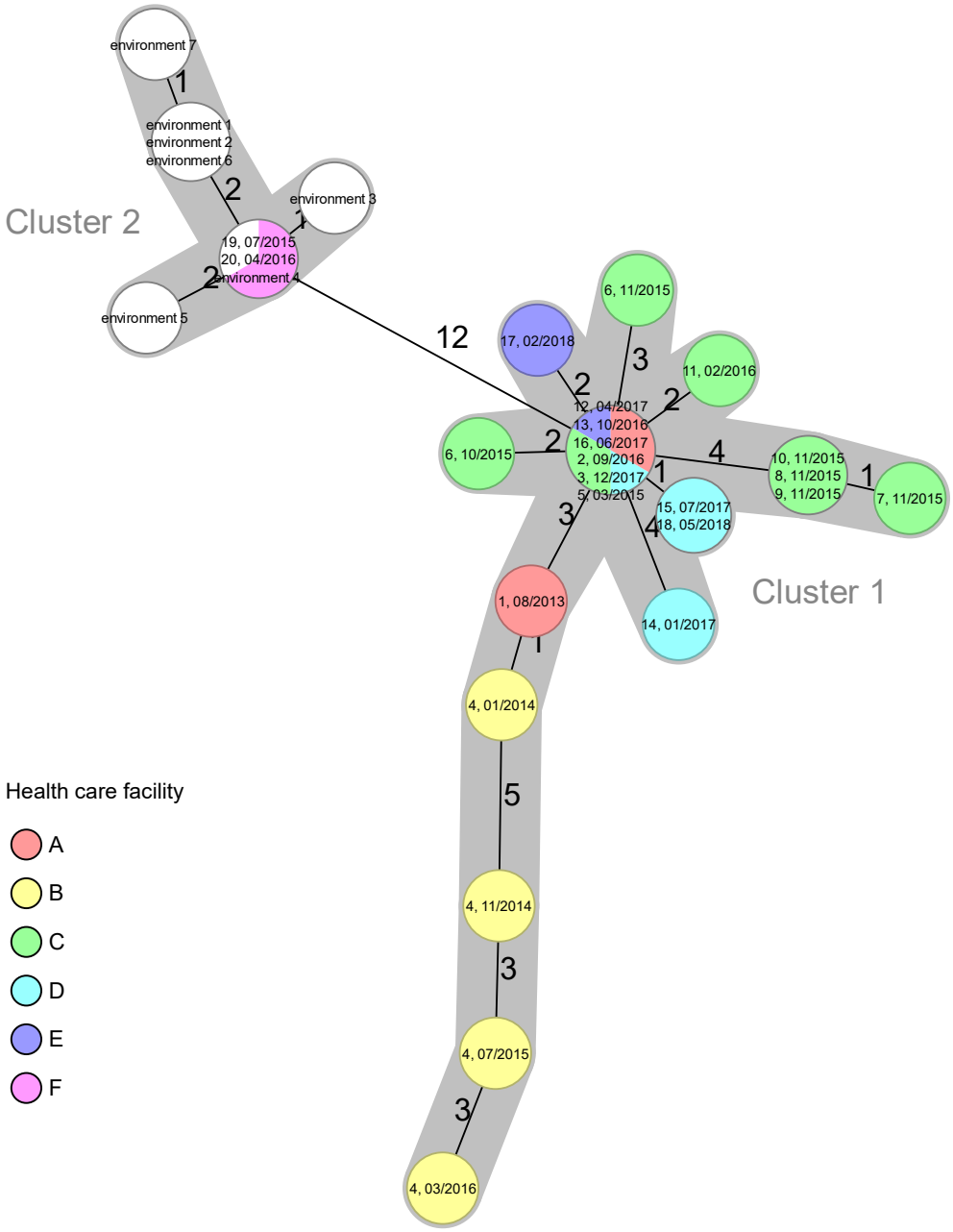

Figure 7. Minimum spanning tree of 27 *K. pneumoniae* KPC-3 producing ST512 isolates (total 20 patient isolates and 7 environmental isolates) on core genome multilocus sequence typing based 2358 columns. Each circle represents one or multiple identical sequences. Numbers between the circles indicate the number of allele differences between connected sequences. Text in the circle indicates the case number, sample month/year, and colors indicate the health care facility. Environmental isolates are uncolored. (Modified from Original publication II.)

Description of the clusters

Cluster 1 and 2 have 12 allele differences with each other, when comparing the closest isolates.

Cluster 1

In cluster 1 the link abroad was unclear; 6/18 cases occupied the same room without an overlapping stay in one of the five HCFs. The index case 1 in HCF A was treated in same room as the later cases 2, 3 and 4. Case 4 was detected in HCF B. Case 5 was detected in HCF C without a known link to previous cases. Case 6 was treated in the same ward as case 5 without an overlapping stay. Case 7 shared a room with cases 9 and 10 without an overlapping stay. Cases 8, 9 and 10 were found by ward screening and case 8 was treated on the same ward as case 5. Case 11 was detected without a previous link to the other cases. Case 12 had an identical strain with case 5 and they both had attended the same treatment weekly in HCF C. Cases 13 and 14 were detected in HCF D and had both stayed in the same room as case 1 in HCF A. Case 15 had stayed in the same ward as case 13 in HCF D. Case 16 had stayed in the same ward and room as case 15 in HCF D. Case 17 was identified in HCF E and had been treated previously in HCF D, in the same room as case 13. Case 18 had been on the same ward and room as case 17. The median time interval between the first positive specimens of all cases in cluster 1 was three months (range: 0-15 months). In addition, to enhanced patient screening the environment was also screened. All tested environmental specimens from HCF A, C, D and E were negative. Later on, when new cases were detected the environment was also screened again and positive specimens were detected.

Cluster 2

In cluster 2 the first case (case 19) had been transferred from an Italian hospital and case 20 occupied the same room although more than eight months apart in HCF F. Environmental screening in HCF F found seven positive specimens for *K. pneumoniae* KPC-3 ST512, and these were either identical or had a maximum of three alleles differences compared with the isolates from the cases. The positive sites were the patient´s desk, windowsill, floor drain, toilet seat, and inner toilet surface near to the water´s edge. The toilet remained repeatedly positive and required 2,000 ppm chlorine treatment.

5.3 Three clusters of CP *C. freundii* in Finland, 2016–2020 (Study III)

We detected three CP *C. freundii* clusters, which were genetically unrelated. Within the clusters and among the closest isolates the distance varied between 0-13 alleles in the largest cluster and between 1–6 alleles in the two smaller clusters. Cluster 1

included 16 cases in five HCFs (HCF A-E) in South-Eastern and Southern Finland between September 2016 and April 2020, cluster 2 two cases (three isolates) in two HCFs (HCF F and G), in Southwestern Finland between January 2018 and March 2019, and cluster 3 two cases in one HCF H in Northern Finland in January 2020 (Figure 8). None of the cases had reported a travel or hospitalization history abroad in the preceding year.

The strain in cluster 1 had KPC-2 carbapenemase and sequence type (ST)18. Cluster 2 strain had OXA-181/GES-5 carbapenemases and ST604 and cluster 3 strain had KPC-3 carbapenemase and ST116. In addition, environmental screening found genetically related isolate/s and one environmental isolate for each cluster is shown in Figure 8.

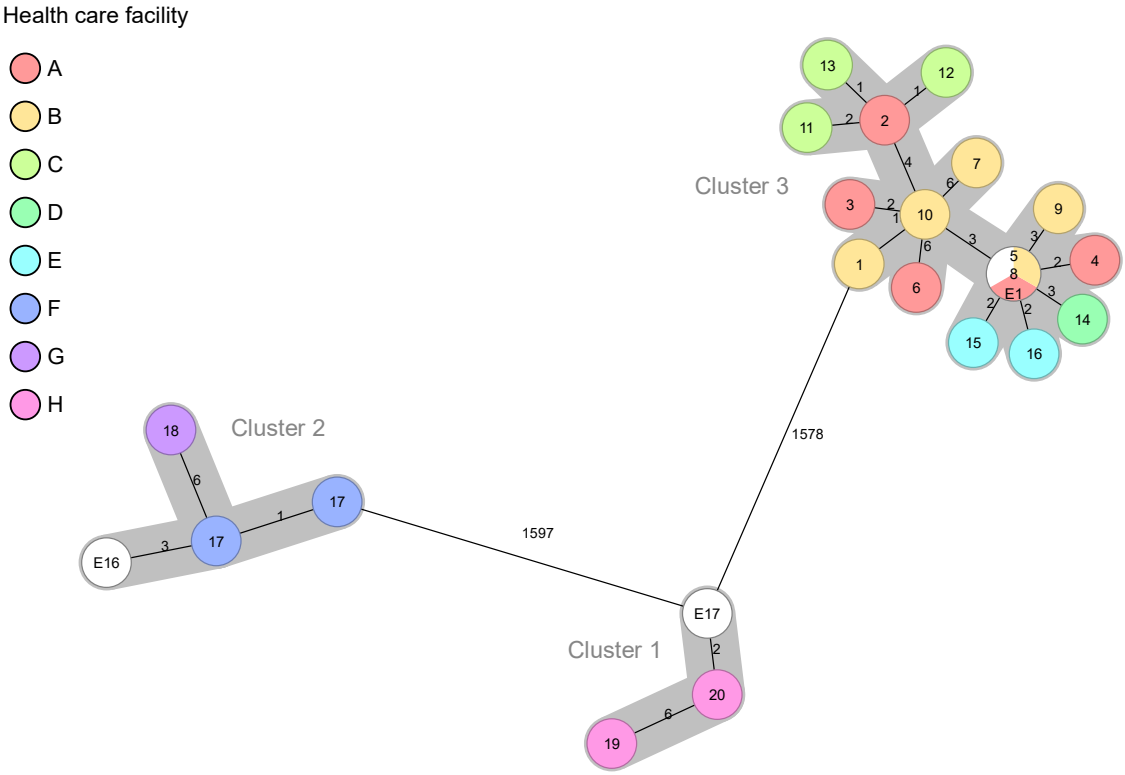

Figure 8. Minimum spanning tree of 24 CP *C. freundii* isolates belonging to clusters 1, 2 and 3 (total 20 cases, 21 patient isolates and 3 environmental isolates) on core genome multilocus sequence typing based 2007 columns. Each circle represents one or multiple identical sequences. Numbers between the circles indicate the number of allele differences between connected sequences. The text in the circle indicates the case number, and the colours indicate the health care facility (A–H). Environmental (E) isolates are uncoloured. (Modified from Original publication III).

Description of the clusters

Cluster 1

The first case in cluster 1 was detected in HCF A in September 2016 and later during 2017-2019 there were five more cases. During 2016-2020 there were also four cases in HCF B. The links between HCFs A and B were cases 2 and 5, who were treated in both HFCs. Cluster 1 was detected in HCF C with three cases. Case 8 had previously been hospitalized in HFC A before admission to HCF C. A screening specimen had been taken from Case 18 in HCF D December 2019 and the patient had had a preceding stay in HCF B. Cluster 1 was presumably transferred from HCF B to HCF E via case 14. In HCF E there were two cases. After detecting CPE-cases, screening was enhanced in HCFs A and B, but no new cases were found. The median time interval between the first positive specimens of all cases in cluster 1 was three months (range: 0–15 months). Environmental screening in HCF A did not find any CP *C. freundii* isolates, but in HCF B the screening revealed several isolates genetically related to cluster 1.

Cluster 2

The first case (17) in cluster 2 was a patient with two CP *C. freundii* ST604 isolates in HCF F. The first isolate was from a clinical urine specimen with OXA-181 carbapenemase and the second from blood with OXA-181 and in addition GES-5 carbapenemases in January 2018. The second case (18) in cluster 2 had a preceding stay in HCF F before HCF G, and a *C. freundii* strain with OXA-181 carbapenemase was observed from blood specimen in March 2019. Environmental screening in HCF F revealed isolates genetically related to cluster 2.

Cluster 3

While conducting enhanced MDR screening because of *K. pneumoniae* ST512 producing KPC-3 outbreak in HCF H, two related CP *C. freundii* ST116 cases were found in January 2020 (Study II). The first case (19) initially had a urine infection caused by a *K. pneumoniae* outbreak strain and five days later *C. freundii* with KPC-3 carbapenemase and CTX-M-15 β-lactamase were found in a screening specimen obtained from urine. The following day, Case 20 with *C. freundii* having KPC-3 carbapenemase was found in a screening specimen. Environmental screening in HCF H found genetically related isolates to cluster 3.

5.4 Development of CAZ-AVI resistance in *K. pneumoniae* during treatment in Finland, 2018 (Study IV)

In total, three *K. pneumoniae* isolates were isolated from a patient transferred from a hospital in Greece. When the patient was admitted to a Finnish hospital, the screening specimens for MDR bacteria were obtained at admission, according to the national guidelines for control of MDR microbes (THL, 2020a). *K. pneumoniae* KPC-2 ST39 was isolated from sacrum decubitus, isolate 1, susceptible to CAZ-AVI and resistant to carbapenems and several other antimicrobials in addition isolate had the bla_{KPC-2} gene. Later, the patient developed fever and *K. pneumoniae* KPC-2 ST39 was isolated from the blood culture, isolate 2, susceptible to CAZ-AVI and resistant to carbapenems and having the bla_{KPC-2} gene. The treatment with tigecyclin and CAZ-AVI was given for two weeks. After two days without antimicrobials, the patient developed fever again and the second infection was treated with fosfomycin and CAZ-AVI for 19 days. Ten days after the antimicrobial treatment was stopped, after a total of 34 days of the CAZ-AVI treatment, the patient developed fever once more. This time the blood culture was positive for *K. pneumoniae* KPC-2-variant ST39, isolate 3, resistant to both CAZ-AVI and carbapenem, but susceptible to colistin and sulfamethoxazole-trimethoprim. The patient recovered from the infection.

We analyzed in detail the isolate 3 mutated bla_{KPC-2} gene sequence and found 45 nucleotide insertion. Gene bla_{KPC-2} encodes KPC-2 protein and in isolate 3 45 nucleotide insertion corresponds to 15 amino acid insertion (AVYTRAPNKDDKHSE) after position 259 of the KPC-2 protein (Figure 9).

Figure 9. Nucleic acid sequences of bla_{KPC-2} gene of the three studied isolates in the mutated area.

Comparative protein modelling based on an inhibitor-blocked KPC-2 crystal structure (PDB ID: 5UJ4) showed that the 15 amino acid insertion add to a loop region connecting the central beta-sheet to the carboxyl-terminal alpha-helix proximal to the KPC-2 active site (Figure 10).

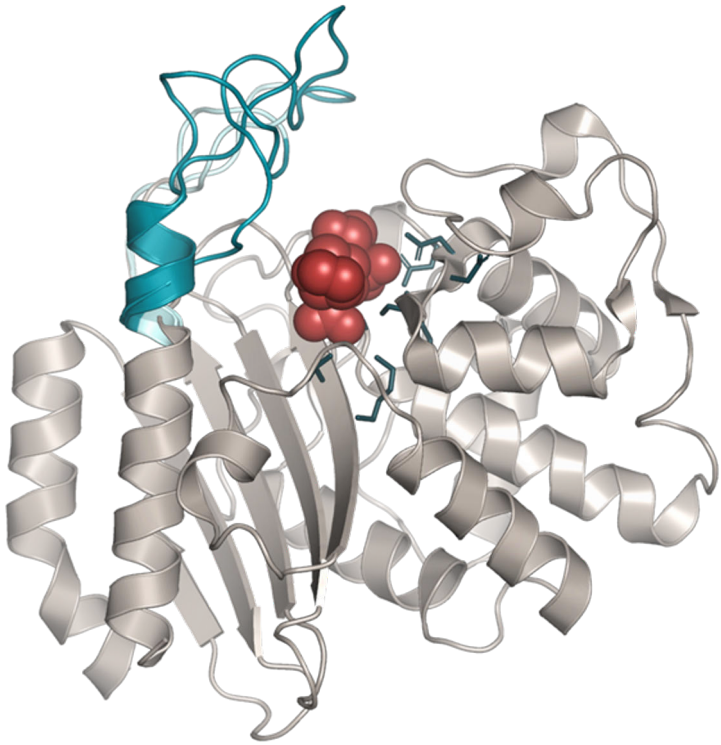

Figure 10. Illustration/Comparative protein modelling of 15 amino acid insertion in mutated protein KPC-2. Turquoise color is the inserted loop region and red shape in the middle is avibactam. Picture credits to Santeri Puranen.

6 Discussion

6.1 Benefits of nationwide WGS surveillance

We detected or confirmed clusters during routine CPE surveillance (Studies I, II and III). All CPE isolates were sequenced and the gained genomic data was analyzed with cgMLST in Ridom SeqSphere+ (Ridom GmbH, Münster, Germany) to enable continuous molecular epidemiology surveillance (Mellmann et al., 2016). Ridom SeqSphere+ software has the graphical user interface and it is easy to use even without wide bioinformatic knowledge and it was since suitable for our usage. WGS-based surveillance produces such comprehensive data from the bacterial genome that according to Snitkin et al. the transmission route can be traced back quite reliably using different analyses and it can reveal unknown transmission links within clusters (Snitkin et al., 2012). Our mighty advantage in Finland was that we were able to perform WGS on all CPE isolates nationwide. The ECDC report found that only seven of the 30 surveyed countries stated that they were either using or planning to start using routine first line genomic surveillance and outbreak investigations by 2019 (ECDC, 2018). The low number of CPE per year in Finland was one factor that enabled the usage of WGS compared to some endemic countries where CPE numbers per year can be manifold.

Centralized CPE control has a high value in identifying local transmission through prospective genomic and epidemiological surveillance (Lane et al., 2020). Close collaboration and coordination with hospitals or HCFs infection control teams and having epidemiological background information comprehensively available was important. Nationwide surveillance with patient data enabled us to detect clusters at an early stage and start interventions to stop them spreading. Some of the clusters or cases (Studies I and III) were suspected in advance but not in all cases. Both *K. pneumoniae* ST512 producing KPC-3 clusters (Study II) were recognized by routine nationwide WGS surveillance. A study from Australia even suggests that this kind of, or even more rapid, surveillance could help maintain low CPE prevalence (Lane et al., 2020).

We detected several clusters because surveillance was conducted using WGS and it enabled precise molecular cluster analysis. In many clusters the first finding was from clinical specimens from patients without a link abroad. This phenomenon

was alarming and indicated hidden transmission. Another worrying observation was that after detecting a cluster, spreading was not halted every time. WGS data could be utilized more on an international level and now Europe is making coordinated efforts. The timing is optimal to foster the use of WGS because currently sequencing accuracy has increased, and costs decreased. Hopefully in the future WGS could be used routinely to investigate CPE outbreaks internationally as well. There are already some examples of how international collaboration of monitoring the spread of CPE can be implemented via coordination of the ECDC (Ludden et al., 2020). In addition, the WHO plans to use WGS data for AMR surveillance in the future. WGS data is digital, and it provides greater inter-laboratory comparability than phenotypic testing. WGS could be used on an international level for monitoring pathogen populations, detecting high-risk AMR clones, identifying sources of transmission and assessing the impact of interventions.

Defined thresholds for distinguishing unrelated isolates

The threshold or cut off in cgMLST cluster analysis is important because it guides decisions about having a cluster or not and provides warning signals when attention is required. Variables like species, sequence type, assembly quality, analysis schema, number and diversity of isolates analyzed, time between samples all affect the cut off value (Sherry et al., 2019). For many years, we have used a ten allele cut off based on literature where cut offs were calculated from the threshold for genetic distance (Friedrich et al., 2016). In addition, epidemiological background information was included, and it supported mostly the cluster findings. However, the first case in CP *C. freundii* in Finland (Study III), in cluster 1 had 17 allele differences to the second isolate (and 13 allele differences to the closest isolate) as described in the results. This relatively long exceeding of the genetic distance was not common compared to the previous CPE clusters in Finland (Studies I and II), however, the same phenomenon found in *C. freundii* which has a lengthier genetic relation, has been previously described (Marmor et al., 2020). Long-term carriage of resistant bacteria can accelerate the genetic changes and can be a confounder (Conlan et al., 2016; E. Ruppé et al., 2017). This one case was an exception in our material. The cut off used in these studies was applied to our data set and workflow.

An Australian study observed that genomic data, such as the use of a genomic cluster definition alone, may merge epidemiologically dispersed cases, reducing the ability to identify risk factors and the geographical or temporal focus of transmission (Nicole Stoesser et al., 2020). Moreover, spreading can be other than clonal and plasmid IncX3-mediated spread of KPC-3 carbapenemase has been shown to occur among CP. *C. freundii* (Venditti et al., 2017; Lane et al., 2020). The WGS method we used produced short read sequences and for a proper plasmid analysis more long

read sequences are necessary. On the other hand, it is possible that an existing epidemiological link was not found despite epidemiological investigation. In three clusters of CP *C. freundii* in Finland (Study III) cluster 3 was detected when HCF H had an ongoing *K. pneumoniae* KPC-3 ST512 outbreak (Study II) and enhanced screening of patients and environment. An interesting finding was that the outbreak strain *K. pneumoniae* and *C. freundii* had the same KPC-3 carbapenemase. We did some preliminary plasmid profile analysis and it showed that the strains shared two similar plasmids IncFIB(pQil) and IncFII(K) (data not shown). These plasmids are known to carry the bla_{KPC} gene and this discovery indicates possible plasmid transfer between species occurred.

6.2 Molecular epidemiology of CPE in Finland during 2012–2018

Species and genes

Molecular epidemiology of CPE in Finland, 2012-2018 (Study I) indicated that CPE isolates were increasingly more prevalent in Finland and have caused several clusters during the study period 2013-2018. This same rise in CPE cases has also been seen in other Nordic countries in the same time period (information from the NSCMID 2021 presentation by Arnfinn Sundsfjord). The three dominant CPE species were *K. pneumoniae, E. coli* and *C. freundii*. The most common carbapenemase was KPC-3 and the most frequent CPE finding in our material was KPC-producing *K. pneumoniae,* which caused most of the clusters. In Sweden, Norway, Denmark and the Netherlands *E. coli* and *K. pneumoniae* were also the dominating species among CPE (*Årsrapport*, 2020; Attauabi et al., 2021; Samuelsen et al., 2017; van der Zwaluw et al., 2020). KPC was often detected in *K. pneumoniae* and seldom in *E. coli;* a similar trend was observed elsewhere in Europe (*Årsrapport*, 2020; Grundmann et al., 2017). If the clusters were excluded the most common carbapenemase types were NDM, OXA-48-like group, and KPC, accordingly (molecular epidemiology of CPE in Finland, 2012-2018, Study I). In Sweden and Denmark OXA-48 and NDM have been the two most common carbapenemase types over many years (2013–2020) (*Årsrapport*, 2020; Attauabi et al., 2021). In Norway KPC, NDM and OXA-48 were the most common carbapenemase types between 2007 and 2014 (Samuelsen et al., 2017) and from 2015 to 2020 OXA-48 and NDM were also clearly dominant in Norway (Resistance, 2020). In Finland, the clusters caused by KPC-producing *K. pneumoniae* had notable effects on prevalence but when the clusters were excluded the data indicated a greater similarity to other Nordic countries.

Sequence types

E. coli had more STs than *K. pneumoniae,* showing less clonal population structure. Successful clone STs predominated in our material: *K. pneumoniae* ST512, ST258, ST11 and *E. coli* ST38, ST410, ST167. In Europe *K. pneumoniae* clonal complex (CC) 258 was spread comprehensively (including the ST258, ST512, ST340, ST437 and ST11) (Pitout et al., 2015; Wyres & Holt, 2016) and it has been shown that *K. pneumoniae* strains with carbapenemase genes spread more aggressively than less resistant strains (David et al., 2019). Our species, ST range and carried gene results were very similar to the data from Norway (Samuelsen et al., 2017). When analyzing all the isolates the *K. pneumoniae* ST512 that exclusively had the KPC-3 gene was the most prevalent, but after excluding the clusters the *K. pneumoniae* ST258 with the KPC-2 or KPC-3 gene was the most prevalent. In the data from Norway the *K. pneumoniae* ST258 with the KPC-2 or KPC-3 gene was also the most prevalent (Samuelsen et al., 2017). The third most common ST in Finland for *K. pneumoniae* was ST11 with a more wide range of carbapenemase types (KPC-2, KPC-3, OXA-48 and NDM-1) and this ST11, in the data from Norway, also had two different carbapenemase gene types (Samuelsen et al., 2017). *K. pneumoniae* ST147 and ST395 were associated mostly with the OXA-48-like gene group and to a lesser extent the NDM-1 gene. These sequence types have been described to be the most dominant among CPE producing OXA-48 in France (Liapis et al., 2014).

E. coli ST167 were the dominant ST and in 10 cases of 11 it had an NDM-5 gene (one was double carbapenemase with OXA-181) and one isolate had an OXA-181 gene. Nine of these cases had a travel history abroad and ST167 has been described to be the dominating ST among NDM-positive *E. coli* and has been detected in multiple countries (Wu, 2019). The second most prevalent STs among *E. coli* were ST38 and ST405. ST38 carried only OXA-48-like genes and this has been associated with global dispersion of OXA-48-like genes (Pitout et al., 2020). ST405 was mostly associated with NDM-genes and to a lesser extent with OXA-48-like gene group genes. Typically ST405 *E. coli* has been associated with a global distribution with ESBLs but associations with the NDM-gene has been documented in Europe with previous connections to Asia (Zhang et al., 2018). *E. coli* ST410 was the fourth most common ST and had a wide selection of carbapenemase types; NDM-1, NDM-5, OXA-48 and OXA-181. *E. coli* ST410 has also been associated with the spread of OXA-48 like genes (Pitout et al., 2020), has caused several hospital outbreaks in several countries and is proposed to be considered as a high-risk clone (Roer et al., 2018). *E. coli* ST38, ST410 and ST405 were found in Norway as well, and ST38 and ST410 were the only *E. coli* STs having genetically related isolates (Samuelsen et al., 2017).

Traveling and importation of CPE

The most of CPE cases detected in Finland were still related to traveling or hospitalization abroad as in the previous report from Finland 2008–2011 where 18 of the 25 patients with CPE had a known link abroad (Österblad et al., 2012). The latest reports from Sweden, Norway and Denmark also showed that most CPE cases are imported (Attauabi et al., 2021; Löfmark et al., 2015; Samuelsen et al., 2017). The carrier rate of resistant bacteria among the healthy population in Finland was low: ESBL-producing *E. coli* prevalence was 6.6% and no CPE were identified between 2015 and 2017 (Ny et al., 2018). International traveling is closely related to acquisition of MDR organisms and three major risk factors are the travel destination, antimicrobial usage and traveler´s diarrhea (Arcilla et al., 2017). ESBL-producing Enterobacteriaceae colonization during travel to high-endemic country can be even 100% when sampling is done daily (Kantele et al., 2021). Compared to CPE, ESBLs are more common and colonization can be acquired through direct transmission or common exposure such as food or water consumption (Kantele et al., 2021). CPE acquisition has long been strongly associated with contact to healthcare during traveling, which is still one important aspect (Kajova et al., 2021). However, recent data shows that international traveling without any connection to healthcare is enough to gain CPE (Mellon et al., 2020; Van Hattem et al., 2016). After traveling a colonized person has the potential to spread the bacteria in a household (Van Hattem et al., 2016). The influence of the endemicity of a certain carbapenemase gene can be seen in our material when examining the areas visited and the genes imported. Strains isolated, especially those found after a patient´s hospital transfer from a foreign country were probably imported; however isolates isolated after a patient had been traveling were also in concordance with the global trend: for example in South-East Asia the NDM-gene is endemic, and we found that patients who had traveled in those areas had most often imported NDM-genes (Logan & Weinstein, 2017). Furthermore, the broad selection of species, sequence types and different carbapenemase genes detected supports the discovery that most cases were imported.

CPE clusters

CPE clusters in Finland were caused by *K. pneumoniae* or *C. freundii (*molecular epidemiology of CPE in Finland, 2012–2018, Study I). An interesting finding was that CP *E. coli* did not cause any clusters in Finland during the study period, although it was the second prevalent species found. When all the globally documented CPE clusters in acute hospital settings for 2000–2015 were reviewed by French et al. The results showed that more than 80% were caused by *K. pneumoniae* (French et al., 2017). In contrast, in Sweden, ten small CPE clusters were reported in 2020 and

nine of those were caused by *E. coli* and only one by *K. pneumoniae* (*Årsrapport*, 2020). In Denmark they had a similar situation as in Sweden that CPE clusters were often caused by *E. coli* or *Enterobacter hormaechei* (Attauabi et al., 2021). CPE clusters caused by *K. pneumoniae* in Sweden and Denmark were not caused by KPC-producers, as for the most part was also the case in Finland (*Årsrapport*, 2020; Attauabi et al., 2021). All CP *K. pneumoniae* clusters we detected belonged to international high-risk clones; 4/7 were part of CC258 (Wyres & Holt, 2016), one cluster had ST395 (Dogan et al., 2021), one ST307 (Pitout et al., 2020) and one ST273 (Chou et al., 2016).

CP *C. freundii* was not a notifiable species in Finland but all CP species were characterized if there was a suspicion of an outbreak. In recent years, clusters caused by CP *C. freundii* have been described in numerous countries and their number among other CP species is increasing (Arana et al., 2017; Faccone et al., 2019; Hammerum et al., 2016; Jiménez et al., 2017; Marmor et al., 2020; Pletz et al., 2018; Rödel et al., 2019; Schweizer et al., 2019; Venditti et al., 2017; Yao et al., 2021).

We described three clusters of CP *C. freundii* in Finland, 2016-2020 (Study III). The largest of these totaled 16 cases and was the second largest cluster detected during 2012–2020. In Denmark six CP *C. freundii* clusters have been reported betweeen 2012–2020 (Attauabi et al., 2021). Two of these Danish CP *C. freundii* clusters were the same STs, ST18, as the largest cluster in Finland, but the carbapenemase genes were different (NDM-1 and OXA-48). One of these ST18 clusters in Denmark was also the second biggest during 2012-2020 (Attauabi et al., 2021; Hammerum et al., 2016). In Germany they have also detected CP *C. freundii* ST18 cluster but the gene was KPC-3, whereas in Finland it was KPC-2 (Yao et al., 2021). Among detected CP *C. freundii* clusters no links abroad were discovered in Finland.

6.3 Phenotypic profile of CPE isolates according to species and carbapenemase variant

Our results show that CP *K. pneumoniae* (88%) was more often non-susceptible to meropenem than CP *E. coli* (62%) and bacteria having carbapenemase gene types KPC, NDM and OXA were non-susceptible 94%, 100% and 33%, respectively. The susceptibility results, as a whole, were in concordance with the results from Canada, where a similar CPE screening practice to the one we used in Finland was employed using meropenem, and it seemed to optimally also find carbapenem susceptible CPE isolates (Fattouh et al., 2016). A study from the Netherlands with more isolates showed that isolates with different carbapenemase gene alleles also had different meropenem susceptibility levels (van der Zwaluw et al., 2020).

The sample size of CP *C. freundii* was small, 10 strains, but among these, 8/10 were non-susceptible to meropenem. Among all CPE isolates, 23% were meropenem susceptible, but still detected if our national guidelines for screening breakpoint were used.

6.4 Tracing back transmission routes of two *CP K. pneumoniae* clusters, Finland, 2013–2018

When tracing back transmission routes of two *K. pneumoniae* KPC-3 ST512 clusters (Study II), we noticed two factors that caused the spread of the CPE clone: unknown carriers transferred between the HCFs and environmental contamination. Most cases in these two clusters were detected by clinical specimens. Even though screening was boosted in all wards where CPE cases were detected, only a few cases were identified by screening. For instance, the index case patient had negative surveillance cultures for MDR microbes before the positive clinical specimen. Long intervals between the cases were a challenge for epidemiological investigation and even more challenging was having to conduct a multi-institutional outbreak investigation compared to an outbreak investigation done in only one institution.

An epidemiological link was found in 16/20 of the cases and only two cases had overlapping stays in a common room. This also supports the idea that dissemination was conducted via environmental contamination. The environment has been reported many times as prolonging the outbreak or being the source of it (Jolivet et al., 2021; Kotsanas et al., 2013; Leitner et al., 2015; Snitkin et al., 2012; Tofteland et al., 2013; Vergara-López et al., 2013). It is speculated that particularly in prolonged outbreaks environmental reservoirs are underestimated (French et al., 2017). Several Enterobacterales are shown to survive long periods in the environment, and after starting environmental screening it was noticed that the disinfection control methods used were not sufficient to destroy the MDR bacteria and stop the spread. In addition, long intervals between new cases in cluster detection support that the environment had a role in dissemination. Consequently, we updated our national guidelines for control of MDR microbes concerning CPE. Changes were made to terminal cleaning following the patient´s discharge and screening strategies. The updated guidelines highlighted that hospital environment needs be taken into account during CPE outbreaks and sanitary facilities needs to be cleaned with chlorine. In CPE outbreaks screening was suggested to continue longer than in outbreaks caused by other MDR microbes.

It is still unclear where the index case in cluster 1 initially got the strain, but index case (19) in cluster 2 had a preceding hospital stay in Italy. It is noteworthy that this clone has been described in several reports in Italy (Migliavacca et al., 2013; Piccirilli et al., 2020).

Four studies analyzing outbreaks caused by the ST512 clone with WGS were found in Italy and one in Spain (Table 4). The clusters in the studies included 4–19 isolates confirmed by WGS, but usually only a part of the isolates was analyzed by WGS and the clusters were probably larger. Among these studies, more than one cluster was often detected, and other successful clones were found causing clusters too. The first outbreak of KPC-producing the *K. pneumoniae* strain ST512 in Finland was in 2013 and affected nine patients in a primary care hospital (Kanerva et al., 2015). Two *K. pneumoniae* KPC-3 ST512 clusters (Study II) were sequential and this time in the second cluster the spreading was regional. The core genome (cg)MLST analysis revealed two clusters with 12 allele differences. We did not find any epidemiological link between these two clusters and the allele differences were over the cluster threshold, and therefore we estimated that these clusters were separate. On the other hand, Case 4 had several specimens during a period of two years and three months and the allele differences were 11. Long-term carriage of resistant bacteria can accelerate the genetic changes and lead to misinterpretations of strain relationships (Conlan et al., 2016; E. Ruppé et al., 2017). When analyzing WGS data strict cut-offs are problematic and epidemiological data is needed for better interpretation.

Table 4. Summary of carbapenemase-producing *K. pneumoniae* ST512 clusters analyzed with whole genome sequencing, 2012-2020.

Country, Time period	No. Of involved HCFs	Speciality	No. of isolates	Proportion of clinical specimen (%)	No. of blood specimens	No. of isolates investigated by WGS	No. of isolates belonging to cluster confirmed by WGS	Epidemiologial background information	Environmental source	Reference
Spain, 2012–2014	7*	Several	2443, 81 were KPC-producing *K. pneumoniae*	Not specified	Not specified	3	1	No	No	(Oteo et al., 2016)
Italy, 2015–2016	1	Intensive care	144 (23 patients)	32	16	32	5 and 4 (two clusters)	Comprehensive	Probable	(Ferrari et al., 2019)
Italy, 2015–2016	1	Several	147	100	147	35	7, 9, and 4 (three clusters)	Comprehensive on 86% of the patients	Possible	(Fontana et al., 2020)
Italy, 2016	1	Neuro-surgery	38	0	0	38	16	Comprehensive	Possible	(Arena et al., 2020)
Italy, 2019–2020	6	Several	39	100	Not specified	39	19 and 6 (two clusters)	Limited	No	(Venditti et al., 2021)
Finland, 2013–2018	5 and 1	Several	24	79	1	24	22 and 2 (two clusters)	Comprehensive	Possible	(van Beek et al., 2019)

* Totally there were 29 HCFs, but the clone was found in 7.

HCF Health care facility

WGS Whole Genome Sequencing

6.5 Description of three CP *C. freundii* clusters detected in Finland during 2016–2020

The Finnish national CPE surveillance by WGS and cgMLST analysis revealed three unrelated CP *C. freundii* clusters with 21 isolates (20 cases) between September 2016 and May 2020 (Study III). For the same time period a total of 23 CP *C. freundii* isolates from Finnish clinical microbiology laboratories were received, meaning that 91% of the isolates belonged to the detected clusters. This can be explained by previous CPE directions in Finland where only isolates causing the suspicion of an outbreak were sent for further characterization. Among CP *C. freundii* clusters, the majority 67% (14/21) of the isolates were from clinical specimens, despite enhanced screening. The same was discovered in two *K. pneumoniae* KPC-3 ST512 clusters (Study II) and among CP *C. freundii* in the Danish study (Hammerum et al., 2016) and the explanation could be that the screening method was not sensitive enough to detect CP *C. freundii* in the patient or the environment (Jiménez et al., 2017; Pletz et al., 2018).

The role of the hospital environment as a reservoir of the CP *C. freundii* was also strongly suspected in this study. The epidemiological link was usually a stay on the same ward with a previously detected CP *C. freundii* case, but only a minority of the stays overlapped. CPE have been previously described as being found in drains, sinks and faucets (Jolivet et al., 2021; Kizny Gordon et al., 2017) and we found genetically related environmental CP *C. freundii* isolates in all of the three detected clusters. However, the environment was not sampled in a systematic manner after appropriate cleaning and the interpretation of the findings was difficult as to which of the contaminates were transient or long term.

Based on our previous experience, in Study II (two *K. pneumoniae* KPC-3 ST512 clusters), through monitoring the clusters and the environmental findings, HCF B changed four toilet seats and three sink drains in the wards where the cases had mainly been hospitalized and the new cases stopped appearing; whereas in HCF A and C this was not done it, and cases continued to appear sporadically. In the French study they also replaced the CPE-positive toilet bowls and tanks by rimless toilets in the ward where the CPE outbreak was ongoing, and the replacement successfully ended the outbreak (Jolivet et al., 2021). In addition to the environment, CP *C. freundii* has been connected to the food chain, with findings from fresh vegetables in China and with a large nosocomial foodborne outbreak in Germany (Liu et al., 2018; Pletz et al., 2018).

We had an interesting link between CP *K. pneumoniae* and CP *C. freundii* clusters sharing the same carbapenemase gene when one of the patients had both outbreak strains. A similar multi-species outbreak was described in France (Jolivet et al., 2021). In addition, polymicrobial cultures from patients have been described where *C. freundii* shared the same carbapenemase gene with another bacterial

species (Yao et al., 2021). Advanced analyzing methods can partly explain this observation when interspecies spreading of resistance genes can be explored, but it seems that *C. freundii* is effectively collecting resistance genes and these are almost always located on plasmids (Yao et al., 2021). Moreover, cluster reports of CP *C. freundii* from different countries show that carbapenemase genes are same as abundant ones among other species (Yao et al., 2021). We detected some *C. freundii* isolates from the same location and with the same carbapenemase gene as the outbreak strain but they were genetically unrelated (data not shown). In Germany they had a similar situation and they did detailed plasmid analysis, which revealed that genetically unrelated CP *C. freundii* isolates shared a genetically related plasmid encoding KPC-2 gene (Yao et al., 2021).

In recent years, clusters caused by CP *C. freundii* have increasingly been reported, but the usage of WGS in previous investigations of CP *C. freundii* clusters has mostly been restricted to one hospital only (Table 5). To our knowledge inter-hospital spread detected by WGS has been reported only twice before, one minor cluster with two cases in Germany and another larger cluster with 77 cases in Canada (Paré et al., 2020; Schweizer et al., 2019). In addition, epidemiological background information was scarce in many studies.

Table 5. Summary of studies of carbapenemase-producing *C. freundii* clusters analyzed with whole genome sequencing, 2012-2020.

Country, Time period	No. of involved HCFs	Speciality	No. of isolates analyzed by WGS/ no. of isolates total*	No. of *C. freundii isolates* belonging to cluster confirmed by WGS	Multiple species	Sequence type	Carbapenemase genes	Epidemiological background information	Suspected source, other info	Reference
Denmark, 2012–2015	1	Several	24/45 (7 patients)	13	Yes	ST18	bla_{NDM-1}	Comprehensive	Unknown origin, environmental screening without findings.	(Hammerum et al., 2016)
Australia, 2012–2016	1	Haemato-logy and intensive care	59/72 (63 patients)	10	Yes	ST8	bla_{IMP-4}	Comprehensive	Environment and unrecognized carriers, environmental sampling CP-*C. freundii* negative but other species causing outbreak was found. Retrospective analyze.	(Marmor et al., 2020)
Spain, 2013–2015	1	Haemato-logy	3/12 (9 patients)	3	No	ST169	bla_{OXA-48} and bla_{VIM-1}	Limited		(Lalaoui et al., 2019)
United States, 2014	1	Acute care	5/6	3	No	ST102	bla_{KPC-3}	Comprehensive	Environment, cleaning was enhanced but environment was not sampled.	(Jiménez et al., 2017)
Germany, 2016	1	Several	11/76	11	No	Not specified	bla_{VIM-1}	Comprehensive	Food, positive food and environmental specimens.	(Pletz et al., 2018)
Germany, 2016	1	Several	42/56	13	Yes	Not specified	bla_{VIM-1}	Limited	Food, two positive food specimens. Focus was using MALDI-TOF mass spectrometry detecting outbreaks.	(Rödel et al., 2019)

Country, Time period	No. of involved HCFs	Speciality	No. of isolates analyzed by WGS/ no. of isolates total*	No. of *C. freundii isolates* belonging to cluster confirmed by WGS	Multiple species	Sequence type	Carbapenemase genes	Epidemiological background information	Suspected source, other info	Reference
Canada, 2016–2018	4	Several	82/82 (65 patients)	77	Yes	ST22	$bla_{OXA-204}$	Comprehensive	Patient to patient and environment, three positive environmental specimens found in comprehensive sampling.	(Paré et al., 2020)
France, 2016–2018	1	Haemato-logy	Not specified/ 37	19	Yes	ST22	bla_{OXA-48}	Comprehensive	Environment, environmental sampling was comprehensive and especially toilets were found to be colonized.	(Jolivet et al., 2021)
Finland, 2016–2020	5, 2 and 1	Several	21/21	16, 3 and 2 (three clusters)	Yes	ST18, ST604 and ST116	bla_{KPC-2}, $bla_{OXA-181}$+ bla_{GES-5} and bla_{KPC-3}	Comprehensive	Patient to patient and environment, genetically related environmental strains were found in all of the three clusters.	(Räisänen et al., 2021)

*May include other species than *C. freundii*, only strains isolated from human

6.6 New mechanism causing CAZ-AVI resistance in KPC-producing *K. pneumoniae* during CAZ-AVI treatment

We reported the first CAZ-AVI resistant *K. pneumoniae* isolate with bla_{KPC} gene isolated in Finland (Study IV) and this also seemed to be the second reported case in Europe. The patient was colonized with KPC-2 producing *K. pneumoniae* ST39 and later developed a bloodstream infection. CAZ-AVI resistance was detected after 34 days of CAZ-AVI treatment.

The CAZ-AVI resistant isolate had a mutated bla_{KPC-2} gene encoding KPC-2 protein with 15 amino acid insertion. The mutation in bla_{KPC-2} gene detected in our study has not previously been reported. Comparative protein modelling (LLC, n.d.; Sali & Blundell, 1993) based on an inhibitor-blocked KPC-2 crystal structure (PDB ID: 5UJ4) showed that the 15 amino acid insertion added a loop region connecting the central beta-sheet to the carboxyl-terminal alpha-helix proximal to the KPC-2 active site.

We hypothesize that the insertion caused a structural change that weakened the inhibitory activity of avibactam by preventing its binding to the active site. This inability of avibactam to bind to the active site was suspected to be the cause of the resistance. Prior to our study there have been very few published studies showing mutations in bla_{KPC} genes connected to development of CAZ-AVI resistance during/after CAZ-AVI treatment.

CAZ-AVI was launched in the US in 2015 and in Europe in 2016. The first reports of resistance during treatment were published in the US, from 2016 onwards. In the first report, *K. pneumoniae* ST258 isolates with three mutations in KPC-3 (D179Y/T243M double substitution, D179Y and V240G) were discovered after 10–19 days of CAZ-AVI treatment (Haidar et al., 2017; Shields et al., 2017). Later, in Italy, the D179Y mutation in KPC-3 was also described in the *K. pneumoniae* ST1519 isolate after 17 days of CAZ-AVI treatment (Gaibani et al., 2018). In the second report from the US, the same D179Y mutation was described in KPC-2 in *K. pneumoniae* ST258 isolate after 12 days of CAZ-AVI treatment (Giddins et al., 2018). Interestingly, these earlier reports indicated that during CAZ-AVI resistance, meropenem MICs decreased and some strains even became susceptible to meropenem. We observed the same decrease in meropenem MICs (from < 32 to 16), but our strain remained resistant.

6.7 Limitations of the studies

This thesis was based on results from routine surveillance data as opposed to allocated research. Surveillance in Finland is focused on CPE and not all CRE are analyzed. When focusing on CPE isolates with laboratory confirmation in clinical

microbiology laboratories, we might have missed some CRE isolates with carbapenemase genes which were not included in the methods used. Moreover, susceptibility results from CPE strains were deficient and dependent on the data the clinical microbiology laboratory provided with the isolates. Susceptibility results collected in Study I (molecular epidemiology of CPE in Finland, 2012–2018) was available in 64% of the studied isolates. The carbapenem resistance situation in Finland was followed separately in the Finres report which is published annually.

CPE species instructed to be sent to THL affected the species spectrum of CPE in our studies. For example, after detecting the clusters caused by CP *C. freundii* this species was added to the list and its proportion increased among CPE collected in Finland.

In general, in routine surveillance some information is always missing to a certain extent. In Study I (molecular epidemiology of CPE in Finland, 2012–2018), information on prior traveling or hospitalization abroad was interesting, but it was not systematically collected. We attempted to complete the information but nevertheless 26% of this information remained missing.

Focusing only on clonal spread and not on plasmid-mediated spread can also be counted as a limitation of the thesis. Clusters described in this thesis result from clonal spreading and are relatively easy to detect from the short-read sequencing that we use in our routine surveillance. Short-read sequencing can be misleading if studying plasmid-mediated outbreaks with a broad host-range where plasmid is moving between different species within or between patients or environmental organisms.

One of the weaknesses in the study was that β-lactamases or virulence factors were not analyzed in detail. Furthermore, fluoroquinolone resistance would be interesting to study carefully. Our resistance gene detection method with SRST2 could not find penicillin binding proteins or point mutations.

When detecting new mechanism causing CAZ-AVI resistance in KPC-producing *K. pneumoniae* during CAZ-AVI treatment (Study IV), our hypothesis could have been validated with a recombinant experiment. Plasmids from a CAZ-AVI resistant *K. pneumoniae* isolate with mutated $bla_{\text{KPC-2}}$ gene and non-mutated $bla_{\text{KPC-2}}$ gene could have been isolated and transferred to donor bacteria. The susceptibility results of these donor bacteria could have proved that the mutated gene was causing the resistance. With unlimited research capacity, the mutated KPC-2 protein could have been modelled with crystallography.

7 Conclusions

- The yearly detected cases of CPE showed an increasing trend in Finland from 2012 to 2018. Most of the CPE cases were related to travel or hospitalization abroad.
- As we showed CPE clusters and outbreaks are common even in low-prevalence countries, like Finland, due to their high transmissibility. This can have a global impact. *K. pneumoniae* CC258 includes successful and high-risk clones which were shown to cause the majority of the outbreaks in Finland.
- When the number of CP *C. freundii* isolates were seen to have increased and CP *C. freundii* was identified as causing the outbreaks, the species was added to our national guidelines for control of MDR microbes. Based on our studies CP *C. freundii* should also be added to the notifiable species together with CP *E. cloacae, E. coli* and *K. pneumoniae*.
- The hospital environment can be a reservoir of CPE and prolong the length of the outbreak. The national guidelines for control of MDR microbes concerning CPE were updated regarding terminal cleaning and screening strategies.
- AMR exists even for new drugs and the development of attentive monitoring of resistance using bacterial cultures and subsequent susceptibility testing during treatment and further characterization with WGS is important.
- When analyzing WGS data it is essential to include the epidemiological background information in the transmission analysis. Real-time CPE surveillance using WGS, collaboration between hospital infection control teams and coordinated outbreak investigations are crucial to rapidly identify clusters and to trace and control transmission chains nationally.

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