CHARACTERIZATION OF BACTERIA CAUSING OUTBREAKS BY USING MOLECULAR METHODS IN A CLINICAL MICROBIOLOGY LABORATORY

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ACADEMIC DISSERTATION

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Success consists of going from failure to failure without loss of enthusiasm.
–Winston Churchill

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ABBREVIATIONS

AFLP	Amplified fragment lenght polymorphism
CA	Community-acquired
CCFA	Cycloserine cefoxitin fructose agar
CDI	*Clostridium difficile*-infection
CDT	*Clostridium difficile* binary toxin
CdtLoc	CDT gene locus
CPE	Carbapenemase producing *Enterobacteriaceae*
CRE	Carbapenem-resistant *Enterobacteriaceae*
DL	Diversilab type
ECDC	European Centre of Disease Prevention and Control
ECDIS	European *Clostridium difficile* infection survey
EIA	Enzyme immunoassay
ERIC	Enterobacterial repetitive intergenic consensus
ESGCD	European study group of *Clostridium difficile*
ESBL	Extended spectrum β-lactamase
ESBL-PE	Extended spectrum β-lactamase-producing *Enterobacteriaceae*
GDH	Glutamate dehydrogenase
GES	Guiana extended-spectrum
HA	Hospital-acquired
HAI	Healthcare-associated infections
KPC	*Klebsiella pneumoniae* carbapenemase
LA	Livestock-associated
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
MDRO	Multidrug-resistant organisms
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multilocus variant-repeats analysis
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NAAT	Nucleic acid amplification test
NGS	Next-generation sequencing
NDM	New Delhi metallo-β-lactamase
ORF	Open reading frame
PaLoc	Pathogenicity Locus
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine leukocidin
REA	Restriction endonuclease analysis
REP	Repetitive extragenic palindromic
Rep-PCR	Repetitive PCR
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid

SIRO	Finnish hospital infection programme
spa	Staphylococcal protein A gene
SLST	Single locus sequence typing
SNP	Single nucleotide polymorphism
SCC	Staphylococcal cassette chromosome
ST	Sequence type
TC	Toxigenic culture
THL	National Institute for Health and Welfare (Finland)
VIM	Verona-integron-encoded metallo-β-lactamase
VNTR	Variable number of tandem repeats
WGS	Whole genome sequencing

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following publications, which are referred in the text by their Roman numerals I-VI.

I **Pasanen T**, Koskela S, Mero S, Tarkka E, Tissari P, Vaara M, Kirveskari J., 2014. Rapid Molecular Characterization of Acinetobacter baumannii Clones with rep-PCR and Evaluation of Carbapenemase Genes by New Multiplex PCR in Hospital District of Helsinki and Uusimaa. PLoS One. 9:e85854.

II **Pasanen T**, Jalava J, Horsma J, Salo E, Pakarinen M, Tarkka E, Vaara M, Tissari P. 2014. An outbreak of CTX-M-15 -producing Escherichia coli, Enterobacter cloacae, and Klebsiella in a children's hospital in Finland. Scand J Infect Dis. 46:225-30.

III Hirvonen JJ, **Pasanen T,** Tissari P, Salmenlinna S, Vuopio J, Kaukoranta SS. 2012. Outbreak analysis and typing of MRSA isolates by automated repetitive-sequence-based PCR in a region with multiple strain types causing epidemics. Eur J Clin Microbiol Infect Dis. 31:2935-42.

IV **Pasanen T**, Kotila SM, Horsma J, Virolainen A, Jalava J, Ibrahem S, Antikainen J, Mero S, Tarkka E, Vaara M, Tissari P. 2011. Comparison of repetitive extragenic palindromic sequence-based PCR with PCR ribotyping and pulsed-field gel electrophoresis in studying the clonality of Clostridium difficile. Clin Microbiol Infect. 17:166-75.

V **Pasanen T**, Korkeila M, Mero S, Tarkka E, Piiparinen H, Vuopio-Varkila J, Vaara M, Tissari P. 2010. A selective broth enrichment combined to real-time nuc-mecA-PCR in the exclusion of MRSA. APMIS. 118:74-80.

VI Antikainen J, **Pasanen T**, Mero S, Tarkka E, Kirveskari J, Kotila S, Mentula S, Könönen E, Virolainen-Julkunen AR, Vaara M, Tissari P. 2009. Detection of virulence genes of Clostridium difficile by multiplex PCR. APMIS. 117:607-13.

ABSTRACT

Outbreaks of bacteria causing hospital- and community-acquired infections have dramatically increased in number over the recent years. For example, travelers become colonized by resistant bacteria and may transmit the strains to other people and to medical care settings when they return home**.** The challenge is new and problematic for laboratories performing bacterial diagnostics. Fast and highly specific tools are needed to identify and characterize resistant bacterial strains in order to prevent outbreaks in local hospitals.

This thesis studies the fast identification of virulence and resistance genes of the most important hospital-acquired (HA) bacteria. In addition, the applicability of rapid outbreak analysis for HA-bacteria using molecular methods outside of the national reference laboratory was studied. The aim of the study was to establish sensitive, reliable multiplex-PCR methods suitable for daily use in a microbiological diagnostic laboratory and to speed up the reporting of bacteria causing outbreaks. Another aim was to study the usefulness and functionality of a commercial repetitive PCR (DiversiLab) and compare it with reference molecular typing methods. The thesis also gives an overall view of the occurrence of HA-bacteria witnessed in the district of Helsinki and Uusimaa over the recent years.

The thesis consists of six studies on four different bacterial species/types causing outbreaks: MRSA (methicillin-resistant *Staphylococcus aureus*), ESBL (Extended Spectrum Beta-Lactamase)-producing *Enterobacteriaceae*, *Acinetobacter baumannii* and *Clostridium difficile*. Consecutive and retrospective bacterial isolates of the each species/type were collected and examined. The methods used were conventional multiplex PCR and real-time multiplex PCR. The typing methods were repetitive PCR (DiversiLab), PFGE (pulsed field gel electrophoresis), PCR ribotyping, *spa* typing, and sequencing-based methods.

During this thesis three usable in-house multiplex PCRs were established for the detection of MRSA, carbapenemase genes, and virulence genes in *C. difficile*. The repetitive PCR method, DiversiLab, was found to be a fast and proprietary typing method, very beneficial in the first-line identification of outbreaks. In addition, the Diversilab system may be used in the comparison of resistant bacterial isolates. The method produced better results with Gram-negatives (ESBL-producing *Enterobacteriaceae* and *A. baumannii*). However, the reference methods still serve their purpose in global isolate comparison. In the future, whole genome sequencing will, however, most likely replace contemporary typing methods.

TIIVISTELMÄ

Sairaalainfektioita aiheuttavat sairaalabakteerit ovat lisääntyneet viime aikoina. Matkailijat tuovat mukanaan entistä resistentimpiä bakteerikantoja omiin sairaaloihimme sairaalasiirtojen yhteydessä. Tämä haaste on uusi ja ongelmallinen myös bakteereita tunnistaville laboratoriolle. Tarvitaan tarpeeksi spesifisiä ja nopeita työkaluja tunnistamaan ja kartoittamaan nämä mikrobilääkkeille resistentit bakteerikannat, jotta estetään näiden kantojen leviäminen omissa sairaaloissamme mahdollisimman tehokkaasti.

Tämä väitöskirjatutkimus käsittelee tärkeimpien sairaalabakteerien entistä nopeampaa virulenssi- ja resistenssigeenien tunnistamista, sekä bakteerikantojen molekyylibiologista tyypittämistä keskussairaalatasolla. Tutkimuksen tarkoituksena oli pystyttää rutiinikäyttöön sairaalabakteerien tutkimiseen tarkoitettuja nopeampia virulenssi- ja resistenssigeenejä tunnistavia ns. multiplex-PCR-menetelmiä. Lisäksi tarkoituksena oli tutkia uusien ja nopeampien tyypittämismenetelmien toimivuutta ja käytettävyyttä vanhoihin standardimenetelmiin verrattuna. Samalla tutkimus antaa kuvan viime vuosien tilanteesta Helsingin ja Uudenmaan sairaanhoitopiirin alueella esiintyvistä sairaalabakteerikannoista.

Tutkimus koostuu kuudesta osajulkaisusta, joissa käsitellään neljän eri bakteerilajin tai -tyypin aineistoja. Bakteerit ovat MRSA (metisilliinille resistentti *Staphylococcus aureus*), enterobakteerien kannat, jotka muodostavat ESBL-entsyymejä (Extended Spectrum Beta-Lactamase), *Acinetobacter baumannii* ja *Clostridium difficile*. Kustakin lajista ja tyypistä oli kerätty peräkkäisiä (konsekutiivisiä) sekä takautuvia sairaalabakteerikantoja. Tutkimuksessa käytettiin erilaisia PCR-menetelmiä, kuten konventionaalinen ja reaaliaikainen multiplex-PCR. Tyypitysmenetelminä käytettiin kaupallista DiversiLab-menetelmää (repetitiivinen PCR), PFGE (pulssikenttäelektroforeesi)-menetelmää, PCR-ribotyypitystä, *spa*-tyypitystä ja erilaisia sekvensointiin perustuvia tyypitysmenetelmiä.

Väitöskirjatyön aikana pystytettiin kolme erilaista multiplex-PCR menetelmää, joiden avulla edelleen tunnistetaan MRSA, karbapenemaasien tuottogeenejä sekä *C. difficile*n virulenssigeenejä. Repetetiivisellä PCR menetelmällä tehdyissä tutkimuksissa havaintomme oli, että kaupallinen nopeampi bakteerien tyypitysmenetelmä, DiversiLab, voisi olla hyödyksi kantojen vertailussa. Näin voitaisiin saada lisäarvoa epidemioiden ja niitä aiheuttavien bakteerikloonien nopeaan ensilinjan tunnistamiseen, varsinkin gramnegatiivisten bakteerien, kuten ESBL:n ja *A. baumannii*n kohdalla. Perinteisemmillä menetelmillä, kuten PFGE, on vielä toistaiseksi paikkansa kansainvälisessä kantavertailussa. Tulevaisuudessa kantojen kokogenomisekvensointi tullee kuitenkin syrjäyttämään tämänhetkisiä tyypitysmenetelmiä.

1. REVIEW OF THE LITERATURE

1.1 Healthcare-associated infections and outbreaks

The microbial pathogens that cause healthcare-associated infections (HAIs) have two special properties: firstly, they are recognized as hospital pathogens, secondly, they have an innate ability to survive on surfaces in the hospital environment for long periods of time [1]. The hospital environment provides an important ecological niche for organisms that could have clinical significance. Hospitals house large numbers of severely ill patients, often with impaired immunity. Patients usually undergo many medical procedures bypassing the body´s natural protective barriers (e.g. surgery, catheter placement). Medical staff are moving from patient to patient providing the way for pathogens to spread [2]. Hospital organisms also tend to be more resistant to antibiotics [3]. Bacteria may manifest resistance to antibacterial drugs through a vast variety of mechanisms. Some species are innately resistant to one or more classes of antimicrobial agents. On the other hand, the acquired resistance occurs via several different mechanisms. The organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent, or efflux pumps that extrude the antibacterial agent from the cell. Bacteria may also acquire several genes for a metabolic pathway which produces an altered bacterial cell wall, or another target, that no longer contains the binding site for the antimicrobial agent. Bacteria also undergo mutations that limit the access of antimicrobial agents to the intracellular target site. Bacterial resistance may develop vertically as a result of chromosomal mutation followed by selection under antibiotic selective pressure. Bacterial resistance may also develop horizontally when a susceptible bacterial cell acquires resistance factors from a resistant strain through genetic exchange, like conjugation, transformation or transduction. This horizontal evolution can occur between strains of the same species, or between different bacterial species or even genera [4]. It has long been known that there is a relationship between antibiotic consumption and resistance, but antibiotic usage is also associated with resistance profiles of organisms isolated from floors and other surfaces within a defined local environment. Wards or units that are constantly exposed to antibiotics are more likely to harbor resistant micro-organisms [3]. Resistant bacteria are spreading and becoming severe problems to control, not only within healthcare institutions, but in the community as well.

HAIs and the worldwide increasing prevalence of multidrug-resistant organisms (MDROs) pose an extraordinary challenge to healthcare systems. The role of screening should be better assessed and, in many instances, acknowledged, regarding the burden and financial cost for healthcare institutions [5]. Infectious disease surveillance data are principally used to establish a baseline or reference point for the detection of outbreaks requiring immediate investigation and intervention. The aim of surveillance is to identify problems on an endemic level and surveillance produces baseline data that are useful in confirming the presence of outbreak. The purpose of outbreak investigations is to track a common source and identify specific clusters and risk factors [2]. Increasing numbers of outbreaks caused by HAIs have generated much attention over the last decade. In the future, the utilization of whole-genome sequencing in outbreak analysis might facilitate the rapid and accurate identification of virulence factors of the pathogen and be used to identify the path of disease transmission within the population and provide information on the probable source [6].

The public has associated the visual experience of dirty hospitals with the risk of HAI, but there is little evidence to support this at present [1]. Hand hygiene and, more specifically, use of hand rubs (today generally alcohol-based), is the most important means of standard precautions to prevent HAIs and transmission of multidrug-resistant bacteria [7, 8]. The ubiquitous environmental contamination by micro-organisms poses an additional risk, however, since the personal belongings of the patient and nearby surfaces are often contaminated with pathogens, including MDROs. When these surfaces are touched by healthcare staff, pathogens may be transmitted to patients. This is more likely, if the hand hygiene compliance is low [9]. The impact of environmental contamination on HAI and the cost-effectiveness of surface disinfection, as opposed to detergent-based cleaning, remains a scientifically unresolved issue, despite a growing body of literature [1, 10, 11].

Since no scientific standards have been presented to measure the effect of an individual cleaner, or to assess environmental cleanliness, finding the evidence for a beneficial outcome in the control of infection is further complicated. The organisms that cause infection are invisible to the naked eye and their existence is not necessarily associated with the presence of visual dirt. Recently, tests for detecting the presence of adenosine triphosphate (ATP) as a proxy of microbial contamination have been increasing in popularity [12]. Sites that are frequently touched by hand are thought to provide the greatest risk for patients, and the people in the immediate vicinity of the patients provide the most prominent risk. The responsibility for cleaning the sites near the patient which are touched by hand does not always rest with the ward cleaner, however, since beds, drip stands, lockers and over bed tables may also be cleaned by nurses. This overlapping responsibility may create confusion and it may lead to cleaning opportunities of some items being missed or even abandonend [1, 13].

A broad consensus exists that high standards are essential in hospital cleaning. It is apparent that the interior and equipment in patient rooms differ greatly between European countries. Further investment is necessary to engage more well-trained staff and to improve hospital architecture in many countries, but as this is cost consuming, it is unlikely to be accomplished in many hospitals. However, as expected, the savings gained by low prevalence of HAI caused by MDROs may well outweigh such costs [11].

Surveillance, including feedback, is a crucial part of infection control activities. The centers for disease control and prevention (CDC) plays a pivotal role in ensuring that state and local public health systems are prepared for public health emergencies due to its unique abilities to respond to infectious, occupational, or environmental incidents that affect the health of the public. The Finnish National Institute for Health and Welfare (THL) has made out the standard precautions for preventing the speed of multidrug-resistant (MDR) bacteria in Finland [14]. The Finnish Hospital Infection Programme (SIRO, established in 1999), has been focusing on the prevention and surveillance of healthcare-associated infections and provides services for the investigation of hospital outbreaks [15].

1.1.1 Persistence of HAI pathogens

Inanimate surfaces have often been described as the source for outbreaks of nosocomial infections. Most Gram-positive bacteria, such as *Staphylococcus aureus*, including methicillin-

resistant *Staphylococcus aureus* (MRSA), survive months on dry surfaces (7 days to 7 months). Many Gram-negative species, such as *Acinetobacter* spp. (3 days to 5 months), *Escherichia coli* (1.5 hours to 16 months) and *Klebsiella* spp. (2 hours to >30 months), are survivors, too. In addition, spore-forming bacteria, including *Clostridium difficile*, can survive up to five months. Humid conditions improve persistence for most types of bacteria. Only *S. aureus* was found to persist longer at low humidity [16]. In hospitals, surfaces experiencing hand contact are often contaminated with nosocomial pathogens and may serve as vectors for cross transmission [17-19]. Transmission to hands is most successful with *E. coli* and *S. aureus* [20] and the main route of transmission is via the transiently contaminated hands of healthcare workers. An outbreak of HAIs caused by *Acinetobacter baumannii* in an intensive care unit may serve as an example [21-23]. During outbreaks, the environment may play a significant role in the transmission of nosocomial pathogens, as suggested by observational evidence [13, 16]. This has been described for various types of microorganisms, such as *A. baumannii*, *C. difficile* and MRSA. However, the evidence to support a role of environmental correlation is not equally strong for all types of nosocomial pathogens. For *C. difficile* and MRSA correlation is stronger than for other pathogens such as *A. baumannii* [24]. The role of surface disinfection for the control of nosocomial pathogens has been a contentious issue for some time. Disinfection of surfaces, however, has been described to reduce the acquistion of nosocomial pathogens, such as *A. baumannii*. It is therefore advisable to control the spread of nosocomial pathogens at least in the direct inanimate environment of the patient by routine surface disinfection [13, 16].

Multi-resistant Gram-positive bacteria have been reported to be detected in the inanimate environment significantly more frequently than Gram-negative pathogens [24]. Traditional sites for Gram-negative microbes in hospitals have been those constantly or intermittently exposed to water. These include hand-wash basins, sinks, sluices, showers, baths and toilets. Bacterial biofilm builds up in plumbing components including taps, water filters and sink traps underneath water outlets. In addition, bacteria within the biofilm may display greater capacity for antimicrobial resistance, and tolerate chlorine or other disinfectants [25].

For patients harboring multi-resistant Gram-positive bacteria, strict contact isolation in a single room as outlined in the guidelines should be performed. For patients with multi-resistant Gram-negative pathogens, barrier precautions are necessary only for close contact, and a single room does not seem to be of benefit [24].

1.1.2 Costs and benefits of prevention of HAIs

HAIs account for a large proportion of the impairments caused by health care and are associated with high costs. In the USA, additional annual costs associated with infections caused by resistant organisms, as compared to susceptible organisms, are estimated between 21 billion and 34 billion dollars and more than 8 million additional hospital days [26]. In Europe health care and productivity costs are estimated to be more than 1,5 billion euros per year, while costs in the USA are estimated to be 55 billion dollars [27]. In the EU, MRSA infections have been estimated to result in 1 million extra days of hospitalization and an attributable additional hospital cost of 380 million euros annually [28]. Better evaluation of the costs of these infections could motivate health care administrators and payers to justify investing in prevention and in necessary systems to decrease these infections [29]. For example, MRSA search and destroy policy in a country with

low endemic MRSA incidence saves money and lives. In countries that did not implement the proposed policy, the number of cases of MRSA bacteremia increased [11]. Among *C. difficile*, elimination of within-ward transmission by improving sanitation and reducing the average length of stay (from six to three days) resulted in the most potent symptomatic infection control combination, cutting rates down from three to less than one per 1000 hospital bed days [30]. It has been estimated that the rate of HAI per 1000 patient days has increased by 36 % from 1975 to 1995 [31]. Estimating the economic burden of antibiotic-resistant bacterial infections still remains a challenge, due to variability in different studies [27].

The more recent concern is the carbapenem-resistant *Enterobacteriaceae*, which are increasingly reported worldwide and have gained substantionally more attention to prevention around the world [32-34]. With rapid diagnostic techniques and strict implementation of hygiene measures it is possible to control their spread in hospital settings [35].

1.2 Methicillin-resistant *Staphylococcus aureus* (MRSA)

S. aureus is a human pathogen, but also found among animals such as livestock, wildlife and companion animals, and it causes both nosocomial and community-acquired (CA) infections. *S. aureus* can cause a wide variety of diseases, such as pneumonia, mastitis, osteomyelitis, endocarditis, skin infections, abscesses, food poisoning, toxic shock syndrome and septicemia [36]. *S. aureus* is a leading cause of bacteremia and endocarditis and is associated with high morbidity and mortality [37]. *S. aureus* can express an extensive number of different virulence factors, playing a role in the pathogenesis of infection. A virulence factor may have several functions in pathogenesis, and multiple virulence factors may perform the same function. Three classes of factors are involved in pathogenesis: surface proteins (e.g. staphylococcal protein A, Spa), secreted proteins, including superantigens (e.g. toxic shock syndrome toxin TSST-1), cytotoxins (e.g. Panton-Valentine leukocidin, PVL), and cell-surface-bound proteins [38]. MRSA is epidemiologically the most important resistant Gram-positive pathogen. It can cause fatal infections especially to patients of old age and/or underlying disease [39-41]. HA- CA-, and LA-MRSA has spread worldwide during the last 70 years and continues to be a growing burden for human and animal healthcare systems as well [42].

In 1942, 2 years after the introduction of penicillin for medical use, the first penicillin-resistant *S. aureus* was observed. In addition, *S. aureus* developed methicillin-resistance due to the acquisition of the *mecA* gene in 1961, two years after the introduction of methicillin. The *mecA* gene, coding for the 78-kDA penicillin-binding protein (PBP) 2a (or PBP2´), causes resistance to methicillin and most other β-lactam antibiotics with only a few exceptions (e.g. ceftobiprole and ceftaroline). In methicillin-susceptible *S. aureus* (MSSA) strains the β-lactam antibiotics bind to the native PBPs that are present in the cell wall and inhibit the synthesis of the peptidoglycan layer. This results in the death of the bacterium. In MRSA the β-lactam antibiotics cannot bind to its target [43]. The *mecA* gene is regulated by the repressor MecI and the trans-membrane β-lactam-sensing signal-transducer MecR1 [44]. The *mecA* gene (2,1 kb) is located on a mobile genomic island called staphylococcal cassette chromosome *mec* (SCC*mec*) [45]. The origin of SCC*mec* is not known. It is considered to have originated from other bacterial species, and subsequently integrated into the chromosome of *S. aureus* through the horizontal transfer of the *mecA* gene [43].

SCC*mec* is a 21-67 kb fragment present at a specific attachment site (*att*B*scc*) in the chromosome of *S. aureus*. The site *att*B*scc* is found in an open reading frame (ORF) of unknown function, designated *orf*X. In addition, SCC*mec* consists of the mec complex, cassette chromosome recombinase (*ccr*) complex, joining regions (J), as well as of repeated 15 bp core sequences at both ends [45, 46]. To date, eleven different types of SCC*mec*s have been identified in *S. aureus*, all defined by the combination of the *ccr* gene complex and the class of *mec* gene complex [47]. SCC*mec* types I, IV, V, VI, and VII mainly cause β-lactam resistance, while SCC*mec* types II and III cause multiresistance due to additional drug resistance genes integrated into SCC*mec* [43]. In general, SCC*mec* types I, II and III are involved in hospital-acquired MRSA (HA-MRSA) isolates and SCC*mec* types IV, V and VII in community-acquired MRSA (CA-MRSA) isolates [48]. In addition, a strong epidemiological association has been found between PVL and the emergence of CA-MRSA. PVL strains seem to be particularly prone to cause disease in the community, in association with the methicillin chromosomal gene cassette SCC*mec* type IVa [49–51]. However, the distinction between CA-MRSA and HA-MRSA is becoming increasingly blurred and the typical patient at risk for carrying MRSA is more and more difficult to define. It also seems that CA-MRSA is spreading from closed risk communities into the general population [52].

In 2007 the novel *mecA* gene ($mecA_{LGA251}$) was found in England during an epidemiological study of bovine mastitis. Similarly to conventional *mecA*, $mecA_{LGA251}$ is located within a SCC*mec* element inserted into the 3´ region of *orf*X. This novel SCC*mec* was given the designation type XI SCC*mec*. In 2012 it was renamed *mecC*. The *mecC* gene encodes the expression of protein PBP2c [53–55]. *Mec*C MRSA strains have now been isolated in small numbers from humans and a wide range of other host species in several European countries [53, 54]. The first report of bovine MRSA carrying *mecC* in Finland was in 2013 [56], and in same year three MRSA strains with the *mecC* gene were isolated from clinical samples in the hospital district of Helsinki and Uusimaa, Pirkanmaa and North Savo. Two of the strains were of the *spa* type t843 and one of the *spa* type t742 [57].

In 1997, the first clinical strain of MRSA with reduced susceptibility to vancomycin (MIC 8 mg/L) was found in Japan [58]. The mechanism of vancomycin intermediate-resistant *S. aureus* (VISA) strains is mediated by mutations and altered expression of genes resulting in a thickened cell wall that prevents the functioning of vancomycin. More strains with similar levels of vancomycin resistance have subsequently also been isolated in other countries [59]. The first case of vancomycin-resistant *S. aureus* (VRSA) was found in 2002 in Michigan, USA [60]. Since then only few isolates have been encountered.

1.2.1 MRSA carriage and prevalence

S. aureus is both a pathogen and a commensal organism. *S. aureus* colonizes the skin and mucosal surfaces of humans. Three types of *S. aureus* nasal carriers have been historically distinguished: persistent carriers (20 %), intermittent carriers (30 %) and non-carriers (50 %) [61]. Later it was proposed that there are only two types of nasal carriers: persistant carriers and others [62]. The most common *S. aureus* carriage sites are the nose, skin, throat and, possibly, the gastro-intestinal tract. Colonization provides a reservoir from which bacteria can be introduced when host defenses are breached and it clearly increases the risk for subsequent infection. Colonization also allows *S. aureus* to be transmitted among individuals in both health care and community settings [61].

MRSA is one of the most frequent causative agents of antibiotic-resistant HAIs worldwide. Intensive care units and burn units characteristically have higher rates, because of high antibiotic usage and a high number of vulnerable patients [39, 63]. MRSA prevalence varies strongly between countries and between different regions and hospitals. In Europe, MRSA percentages range from under 1 % (Norway) to over 60 % (Romania). Fortunately, the percentage of invasive MRSA isolates in Finland in 2013 was only 1,7 % [64, 65]. Finland has been a country of low MRSA incidence for a long time [65, 66] even though isolated MRSA outbreaks have been published [67].

The major HA-MRSA clones are the clonal complex CC5, CC8, CC22, CC30 CC45 or the sequence type ST239 [68]. The distribution of HA-MRSA clones varies geographically and often respects geographical borders. ST239 is successful in Asia and South America and has caused hospital outbreaks for a short period in the UK [69], but did not become established and had almost disappeared by 2006. CC22 has become the dominant HA-MRSA clone in the UK [70]. Invasive MRSA clones in Europe display a typical epidemic behavior and have a predominantly regional distribution. The most frequent *spa* types in Europe are t032 (UK, Ireland, Portugal, Germany and Hungary), t008 (Belgium and France), t041 (Croatia, Slovenia and Italy), t003 (Czech Republic), t002 (Greece) and t067 (Spain). The most frequent sequence type is ST22 [71]. ST22 has a broad host range and behaves as a nosocomial pathogen within a veterinary health-care setting just as it does within human hospitals [72]. USA300 (ST8) is a virulent and easily transmissible strain of MRSA. It is the predominant community-associated MRSA clone in the United States [73, 74]. It is also increasingly seen in Europe [75]. MRSA belonging to ST398 has emerged in several European countries and is regarded as being a livestock-associated (LA) MRSA [71, 76]. According to the data from THL, 1417 MRSA cases were found in Finland in 2014 and 35 % of them were from the district of Helsinki and Uusimaa (Figure 1) [77]. In 2013, the most common *spa* types in Finland were t172, t067, t008, t032 and t044 [57].

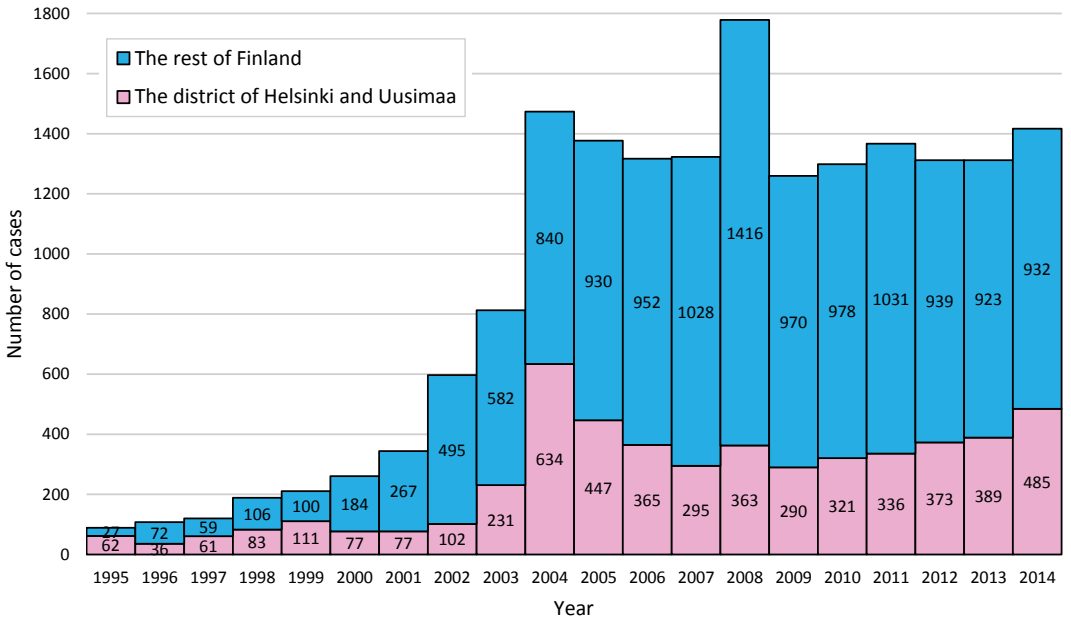

Figure 1. MRSA cases in Finland between 1995 and 2014.
Source: THL [77].

1.2.2 Identification of MRSA

Although methicillin is no longer produced as an antibiotic for human therapy, the name MRSA has persisted and can be regarded as referring to resistance to virtually all β-lactam antibiotics. Susceptibility testing now typically uses oxacillin and/or cefoxitin. The minimum inhibitory concentrations (MICs) of oxacillin for MRSA strains vary from apparently sensitive levels to those of >256 μg/ml. Cefoxitin has generally been accepted as a potent up-regulating agent of *mecA* expression and has been used in selective media for the isolation of MRSA, as well as in screening methicillin-resistance in staphylococci [44, 78].

Several rapid MRSA-PCR-methods, both in-house and commercial, targeting *S. aureus*-specific genes e.g. *femB*, *femA*, *nuc* or *coa* in combination with *mecA* have been described [79-85]. To facilitate the rapid detection of colonized patients, commercial real-time PCR assays have been developed [85]. The first method to directly detect MRSA from clinical specimens was developed by Huletsky et al. [86] and it became commercially available as the IDI-MRSA™ assay. This method was based on specific amplification products at the junction of the right extremity sequence of the staphylococcal cassette chromosome (SCC*mec*) and the chromosomal *orfX* gene sequences of *S. aureus* located to the right of the SCC*mec* integration site [86]. The principle of this method is used in two commercially available tests, the BD GeneOhm MRSA assay (BDGO) (BD, San Diego, CA, USA) and the Xpert MRSA assay (Cepheid, Inc., Sunnyvale, CA, USA). These tests are very effective in identifying the negative samples, but for PCR positive samples back-up cultures may be useful [85, 87].

1.3 Extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* strains

Members belonging to the family *Enterobacteriaceae* are widely distributed throughout the environment and many species are commonly recognized pathogens and consistently seen in health care-associated infections [88]. *Enterobacteriaceae* is one of the most important groups causing a wide range of diseases. These bacteria infect the urinary tract, respiratory tract, and can cause bloodstream infections. They form a large potential reservoir for resistant genes between different family members. *Escherichia coli* and *Klebsiella pneumoniae* are the clinically most important Gram-negative bacteria that cause serious infections. From all Gram-negative bacteremias the proportion of *E. coli* and *K. pneumoniae* is 75-87 % in community-acquired and 62-64 % in health care-associated infections [89, 90].

Many resistant genes are shared by different species of *Enterobacteriaceae*, but also a multitude of different resistance genes or variations of existing features have developed [91]. Potential interconnections exist between the resistomes of soil, the human gastrointestinal tract, and clinical pathogens. Transmission from soil to clinic establishes soil as a direct source of pathogenic resistance genes [92]. This increasing resistance has culminated with the emergence of panresistant strains for which there are no therapeutic options [88]. ESBL production is the major cause of clinical cephalosporin resistance in *Enterobacteriaceae* and has become particularly prominent, not only in nosocomial but also in community-onset *Enterobacteriaceae* infections worldwide [93]. ESBLs have been reported most frequently in *Escherichia coli* and *Klebsiella* species. They have been found in other bacterial species as well, including *Salmonella enterica*, *Proteus mirabilis* and *Serratia marcescens* [94].

There are two major strategies which *Enterobacteriaceae* have adopted. The acquisition and evolution of ESBLs: 1) single or multiple amino-acid substitution at a critical position causing extended substrate specificity of existing β-lactamases, and 2) capture, stabilization, and spread of new genetic element encoding new enzymes with ESBL activity [95]. As mentioned before, ESBLs are found both in the nosocomial settings and in the community, and *Escherichia coli* has become dominant among the ESBL-producing enterobacterial species. This is especially disturbing as the human gut carriage of *E. coli* favors the dissemination of ESBLs in the community [96]. Also of concern is that ESBLs often show multiple coresistance [97]. In hospitals, patients with serious underlying diseases, prolonged stay, prior antibiotic use and invasive medical devices are at high risk for ESBL colonization and infection [94, 98].

1.3.1 β-lactamases

The production of β-lactamases is the predominant cause of resistance to β-lactam antibiotics in Gram-negative bacteria. These enzymes cleave the amide bond in the β-lactam ring, rendering β-lactam antibiotics, including third generation cephalosporins. Generally ESBLs are inhibited by clavulanic acid, sulbactam and tazobactam [98, 99].

There are different schemes for the classification of β-lactamases, like Ambler Classes [100] and Bush Groups [101] (Table 1). The Bush Groups 1 through 3, base on the substrate and inhibitor profile. The Ambler classification divides enzymes into four classes (A to D) on the basis

Table 1. Classification schemes for bacterial β-lactamases. Updated and modified from various sources [103-107].

Molecular class	Bush-Jacoby group (2009)	Distinctive substrate(s)	Inhibited by		Representative enzyme(s)
			CA / TZB	EDTA	
C	1	Cephalosporins	No	No	E. coli AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1, DHA-1, ACC-1
C	1e	Cephalosporins	No	No	GC1, CMY-37
A	2a	Penicillins	Yes	No	PC1
A	2b	Penicillins, early cephalosporins	Yes	No	TEM-1, TEM-2, SHV-1
A	2be	Extended-spectrum cephalosporins, monobactams	Yes	No	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1, GES-1
A	2br	Penicillins	No	No	TEM-30, SHV-10
A	2ber	Extended-spectrum cephalosporins, monobactams	No	No	TEM-50
A	2c	Carbenicillin	Yes	No	PSE-1, CARB-3
A	2ce	Carbenicillin, cefepime	Yes	No	RTG-4
D	2d	Cloxacillin	Variable	No	OXA-1, OXA-10
D	2de	Extended-spectrum cephalosporins	Variable	No	OXA-11, OXA-15
D	2df	Carbapenems	Variable	No	OXA-23, OXA-48, OXA-181, OXA-24/40, OXA-58
A	2e	Extended-spectrum cephalosporins	Yes	No	CepA
A	2f	Carbapenems	Variable	No	KPC-2, IMI-1, SME-1, GES-2
B (B1)	3a	Carbapenems	No	Yes	IMP-1, VIM-1, CcrA, IND-1, SPM-1, GIM-1, AIM-1, SIM-1, NDM-1, DIM-1
B (B3)					L1, CAU-1, GOB- 1, FEZ-1
B (B2)	3b	Carbapenems	No	Yes	CphA, Sfh-1

CA, Clavulanic acid
TZB, Tazobactam

of their amino acid sequences. Classes A and C are the most frequent [102]. Classes A, C and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site (serine). They are known as serine β-lactamases. Class B β-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis. These are known as metallo-β-lactamases (MBLs) [103].

1.3.2 ESBL enzymes

The spread of extended spectrum β-lactamase-producing *Enterobacteriaceae* strains is an ongoing concern in health care [93, 98], and more than 350 different (at 2010) ESBLs have been identified [95, 107]. The classification of ESBLs is complex, as so many enzymes are responsible, the most prevalent being CTX-M [25]. In addition, as new ESBL enzymes are being found, the redefinition of extended spectrum β-lactamase has been suggested in the Nordic countries [108, 109].

The majority of ESBLs are from the TEM, SHV and CTX-M families and are classified into class A. The first TEM-1 enzyme was identified in 1965 in Greece. For decades TEM (TEM-1 and TEM-2) and SHV used to be the main enzymes in class A [110]. These enzymes are usually located in a plasmid. Plasmids can easily be transmitted into different bacterial cells, causing rapid resistance. Some of the class A enzymes hydrolyze penicillins, first, second and third generation cephalosporins, as well as monobactams [111].

The CTX-M enzymes (belonging to class A) arose by plasmid transfer from preexisting chromosomal ESBL genes from *Kluyvera* spp. by mobile elements (such as insertion sequence IS*Ecp1*). ISEcp1 plays an important role in the expression and continuous spread of these β-lactamases. Today the CTX-M enzymes have superseded the TEM and SHV ESBLs [102, 112]. Currently as many as 172 CTX-M alleles have been identified [107, 113]. The CTX-M β-lactamase carries 291 amino acid residues and the change in any one of them results in a new CTX-M variant [95]. The first CTX-M was detected in a clinical *E. coli* isolate in 1990, and was named after its higher hydrolytic activity against cefotaxime than ceftazidime, and the place of first isolation (Munich, Germany) [114]. Since 2000, CTX-M-producing *Enterobacteriaceae* have been globally detected. CTX-M-genes have become widespread and represent a threat to the treatment of infections in the community as well as in hospitals [93, 95, 115].

The CTX-M types belong to the molecular class A or functional group 2be-lactamases and include at least six major lineages (i.e., CTX-M-1-like, CTX-M-2-like, CTX-M-8-like, CTX-M-9-like, CTX-M-25-like, and KLUC-like) based on amino acid sequence similarities [91, 102]. CTX-M-15 is the most widely distributed CTX-M. It was first detected in *E. coli* isolated from India in 2001. CTX-M-15 belongs to the CTX-M-1 cluster and has often been associated with co-production of other β-lactamases. The successful spread of *E. coli* producing CTX-M-15 is due to the spread of an epidemic clone with selective advantages (such as multiple antibiotic resistance and enhanced virulence factors) between hospitals, long-term care facilities and the community, or the horizontal transfer of plasmids or genes that carry $bla_{\text{CTX-M-15}}$ alleles [112]. The worldwide spread of the CTX-M-15 is mainly driven by two endemic *E. coli* strains belonging to phylogroups B2 and D, which are known to be associated with the hospital settings and extraintestinally pathogenic *E. coli*. The dissemination of *E. coli* CTX-M-15 phylogroup B2 strain corresponds to the sequence type 131 (ST131) that was responsible for several clonal

outbreaks [116]. The multidrug resistant clone ST131 (serogroup O25b) is a pandemic clone, predominantly causing community-related infections and rapidly emerging all over the globe [117]. The understanding from the various reports is that CTX-M-15 and CTX-M-14 (in the Western Pacific region) are the most important types posing a threat to human and animal health [113].

ß-lactamase inhibitors are effective in vitro against class A β-lactamases including CTX-M, TEM and SHV. They are the agents that inhibit the β-lactamase enzyme by irreversible binding to its active site, thus rendering it permanently inactive. The first clinically used β-lactamase inhibitor was clavulanic acid (isolated from *Streptomyces clavuligaris*). It has a weak antimicrobial activity, but if combined with amoxicillin, it significantly increases the antimicrobial activity of amoxicillin. The other ß-Lactamase inhibitors such as sulbactam, tazobactam or avibactam are combined with other β-lactamases, such as ampicillin, piperacillin, ceftazidime, and ceftaroline [111].

1.3.3 ESBL carriage and prevalence

The digestive tract is the main reservoir from which enterobacteria originate both in community-acquired and hospital-acquired infections. The first faecal carriage of an ESBL- *Enterobacteriaceae* strain in the community was reported in Spain in 2001 [118]. Before 2008, the reported rates of ESBL carriage in the community were almost always under 10 %, but after 2008 carriage rates increased in some parts of Southeast Asia exceeding 50 % [113]. Traveling plays an important role in the disseminating ESBL strains. *E. coli* CTX-M-15 producing ST131 clone is dominant in persons returning from the Indian subcontinent, Europe, and the Middle East, CTX-M-14 producing strains in persons returning from East Asia [95, 119]. Such acquisition can be achieved even without hospitalization or contact with the health care system in the visited country [119]. Traveling in highly endemic areas, such as India, has been associated with colonization [120, 121]. In a recent Finnish study where 430 travelers were examined after traveling outside Scandinavia, it was found out that 21 % of the travelers became colonized with ESBL. The occurrence of traveler´s diarrhea and the use of antimicrobials raised the risk up to 80 %, but the risk factors proved to be independent. In this study, the area of highest risk was South Asia [122]. The acquisition rate of multidrug-resistant *Enterobacteriaceae* is higher in Asia than in sub-Saharan Africa or Latin America, and carriage lasts longer in travelers returning from Asia [123].

In the recent years we have found approximately one thousand new clinical cases per year in the district of Helsinki and Uusimaa (Figure 2) when screening samples have been excluded. According to data from the National Institute for Health and Welfare in 2014 approx. 4500 ESBL carriers were found in Finland and more than 90 % of them were *E. coli* strains [77]. One third of all cases were from the district of Helsinki and Uusimaa.

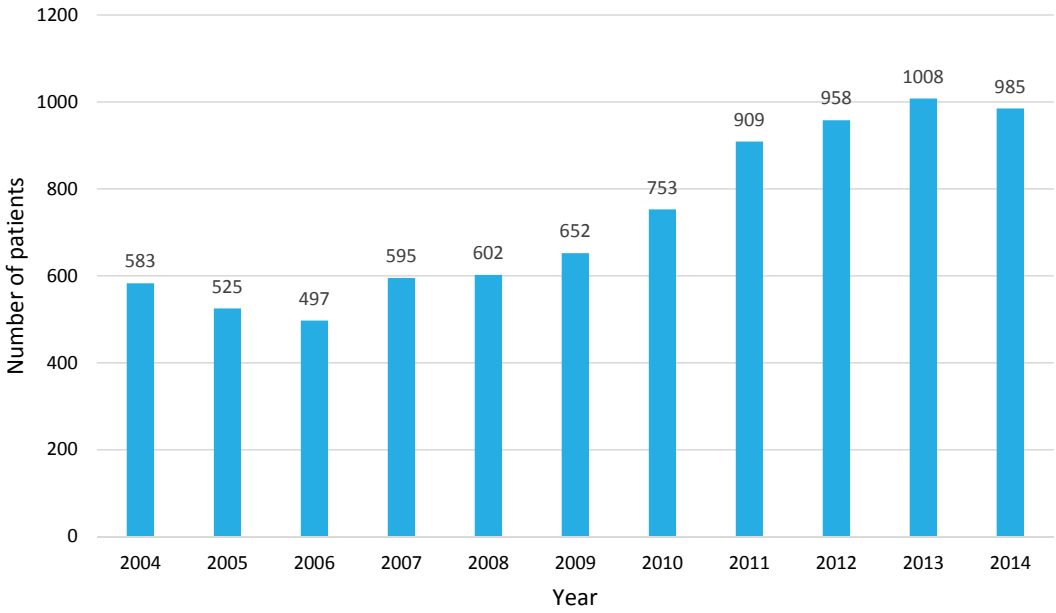

Figure 2. ESBL producing *Enterobacteriaceae* isolates in the district of Helsinki and Uusimaa (HUSLAB) material between 2004 and 2014. (The first isolate only, screening excluded)

1.3.4 Identification of ESBLs

The identification of ESBL-producing *Enterobacteriaceae* is challenging, and there are several methods for the laboratory to choose from. Both phenotypic and genotypic methods are available for the detection of ESBLs [94, 98].

Several selective culture media agar, supplemented with cefotaxime and/or ceftazidime at various concentrations, have been proposed for the detection of ESBL producers. Also a commercial selective chromogenic agar medium (e.g. chromID ESBL) has been developed for the detection of ESBLs. The chromID ESBL uses cefpodoxime as a substrate contrary to the previously used ceftazidime or cefotaxime. This was noted to increase sensitivity [124]. ESBL screening and detection can be performed by double disk approximation or the double disk synergy method, using combination disks or microdilution. Confirmation can be achieved using MIC broth dilution, the Etest or an automated instrument (e.g. Vitek). Molecular methods are available as well, such as PCR, DNA probes and restriction fragment-length polymorphism (RFLP) [94].

Several typing methodologies are available in characterizing *E. coli* and *K. pneumoniae* in epidemic situations, the most widely used of which is PFGE. Other methods include several repPCRs and MLST [125]. Today the accessibility of whole genome sequencing (WGS) presents the opportunity for national reference laboratories to provide understanding of the epidemiology and pathogenicity of bacteria that are a threat to public health. WGS has a huge potential in both diagnostics and routine surveillance [126].

Screening for ESBL carriage is systematic in many hospitals. The cornerstone of diagnosing ESBL is a reliable phenotypic screening and confirmatory method. In addition, a reliable and rapid typing method for the detection of internationally spread ESBL clones, as well as for the identification of outbreaks, is needed [93]. Once identified, ESBL carriers are in most cases isolated in single rooms, where contact precautions are recommended to prevent cross-transmission. However there is wide variation, even within a single geographical area, in infection control strategies to contain or control ESBL-PE and carbapenemase producing *Enterobacteriaceae* (CPE) [127] and the effectiveness is debatable [128]. At present, systematic screening for ESBL carriage upon admission to hospital is not recommended, because it is costly and its efficiency has not been demonstrated [113].

Infections, such as blood stream infections due to *Escherichia coli* and other *Enterobacteriaceae* have become more prevalent, along with increasing antibiotic resistance. Traditionally, β-lactam antibiotics have been used, but currently more strains demonstrate the presence of extended-spectrum beta-lactamases (ESBLs) which confer resistance to penicillins, as well as to most cephalosporins, with the exception of cephamycins. The genes responsible for the production of these enzymes among *Enterobacteriaceae* are often accompanied by genes mediating resistance to other groups of antibiotics, e.g. aminoglycosides and fluoroquinolones [25]. Carbapenems are the drugs of choice for life-threatening and severe infections caused by ESBL-producing organisms [129].

Colistin, tigecycline and fosfomycin are effective drugs against carbapenem-resistant *Enterobacteriaceae*. Colistin has become the backbone agent in their treatment, typically in combination with other antibiotics, despite its nephrotoxicity [130].

1.4 Carbapenemase producing *Enterobacteriaceae* (CPE)

Carbapenem-resistant *Enterobacteriaceae* (CREs) or carbapenemase-producing *Enterobacteriaceae* (CPEs) are quite recently encountered challenges. Their evolution and spread indicate a complex and sophisticated genetic evolution involving transcontinental spread [131]. The first carbapenemase producer in *Enterobacteriaceae* (NmcA, class A serine carbapenemase) was identified in 1993 [132]. Since then, a large variety of carbapenemases have been found, mostly belonging to Ambler classes A and D (serine carbapenemases) as well as class B (metallo-β-lactamases). In addition, chromosomally encoded Ambler class C (cephalosporinases) may possess notable activity toward carbapenems [133]. CPEs have the propensity to spread easily between humans via hand carriage or contaminated food and water, and acquired genetic material spreads through horizontal gene transfer mediated mostly by plasmids and transposons [131, 133]. The emergence of CPE has been described in a number of countries and there are limited options for treatment of infections caused by these organisms, hence the importance of infection prevention and control measures. Acquisition of drug-resistant Gram-negative bacilli is usually associated with admission to a tertiary hospital, but both ESBLs and CPEs can be found outside acute hospitals, due to patient carriage to long-term care facilities. The international spread of these bacteria and their presence in the food chain implies that their prevention and control is a greater challenge than that presented by MRSA and *C. difficile* [131].

1.4.1 Class A serine carbapenemases

Class A serine carbapenemases include the non-metallocarbapenemase of class A (NmcA). Some are chromosome-encoded, such as NmcA, IMI-1, SME and SFC-1 and others are plasmid-encoded such as KPC, IMI-2 and GES [133]. Members of this group hydrolyze carbapenems as well as cephalosporins, penicillins and aztreonam, and are partially inhibited by clavulanic acid in vitro [134]. Primarily these carbapenemases have been identified in *Enterobacter cloacae*, *Serratia marcescens* and *Klebsiella pneumoniae* [111]. *Klebsiella pneumoniae* carbapenemase (KPC) is the clinically most common enzyme and KPC producers have spread around the world and caused major clinical and public health concerns. The first KPC (KPC-2) producer was identified in 1996, in the United States [135]. Up to now there are 24 different KPC variants, all being point-mutant derivatives of the common amino acid sequence [35, 107]. Several hospital outbreaks, most often due to *K. pneumoniae* KPC isoenzymes KPC-2 and KPC-3, have been reported, and the most predominant clone is *K. pneumoniae* ST258 [91].

1.4.2 Class B metallo-β-lactamases (MBLs)

Class B enzymes are Zn^{2+} -dependent β-lactamases. The hydrolytic mechanism differs from serine β-lactamases, which have serine at their active site. Organisms producing these enzymes hydrolyze all β-lactams except monobactams, such as aztreonam. They are usually resistant to all penicillins, cephalosporins, carbapenems and the clinically available β-lactamase inhibitors. Their activity is inhibited by EDTA but not by clavulanic acid. MBL genes are located in the chromosome, plasmid or integrons. Typically *Pseudomonas aeruginosa*, *K. pneumonia*e and *Acinetobacter baumannii* produce these metallo β-lactamases encoded by mobile genetic elements [111]. The most common MBLs are VIM (Verona-integron-encoded metallo-β-lactamase), IMP and more recently NDM (New Delhi metallo-β-lactamase-1), described first in 2009 in a Swedish patient returning from New Delhi [136]. The first MBL, IMP, (IMP-1) was identified in Japan in 1991 in *Serratia marcescens* [137]. Since then MBLs have been reported worldwide, VIM being the most common. NDM positive *Enterobacteriaceae* are now the focus of global attention, and NDM producers are being reported in the environment and in the community. NDM-1 has been identified mostly in non-clonal *E. coli* and *K. pneumoniae*, and many NDM producing strains remain susceptible only to tigecycline, colistin and fosfomycin. Up to now 11 variants of this enzyme have been published and 16 have been assigned, originating mostly from Asia [107, 133, 138, 139]. NDM-1 has also been identified in *E. coli* ST 131, a well-known source of community infections [140].

1.4.3 Class C serine cephalosporinases

Class C AmpC β-lactamases are usually encoded by *bla* genes located in the bacterial chromosome, although plasmid-borne AmpC enzymes are becoming more prevalent. Class C β-lactamases include CMY-2, P99, ACT-1 and DHA-1 (Table 1) [103, 141]. AmpC producers that resist inhibition by clavulanate and sulbactam in vitro, are members of *Enterobacteriaceae*, typically *Enterobacter* spp. and *Citrobacter* spp. Instead, *Klebsiella* spp., *Salmonella* spp. and *Proteus mirabilis* normally do not harbor chromosomal bla_{AmpC} genes. Organisms expressing the AmpC β-lactamase are typically resistant to penicillins, β-lactam-β-lactamase inhibitor combinations, and cephalosporins. Production of chromosomal AmpC can be derepressed by

induction with certain β-lactams, particularly cefoxitin, which can cause dramatic increase in MICs during the course of β-lactam therapy [104, 111].

1.4.4 Class D serine oxacillinases

Class D β-lactamases were initially categorized as "oxacillinases" because of their ability to hydrolyze oxacillin. OXA β-lactamases can also confer resistance to penicillins, cephalosporins, extended spectrum cephalosporins (OXA-type ESBL) and carbapenems (OXA-type carbapenemases). OXA enzymes are typically resistant to inhibition by EDTA as well as by clavulanate, sulbactam, and tazobactam, with some exceptions. Interestingly, sodium chloride at concentrations of >50 to 75 mM inhibits some carbapenem-hydrolyzing oxacillinases [111]. OXA-48 represents the predominant enzyme isolated around the world, displaying thus far six different variants (OXA-48, OXA-162, OXA-163, OXA-181, OXA-204 and OXA-232) [142]. The first OXA-48 (from *K. pneumoniae*) producer was identified in Turkey in 2003 [143]. OXA-48 enzymes are found in multiple species of *Enterobacteriaceae* and are nowadays frequently observed in *E. coli*. These enzymes are not found in nonfermenters and are not related to the OXA-carbapenemases that have been found in *Acinetobacter* [144]. On the other hand, OXA enzymes also include rapidly emerging enzymes in *A. baumannii*, such as OXA-58, OXA-23 and OXA 24/40 [111].

1.4.5 Prevalence of CPE

KPC enzymes are currently clinically the most significant enzymes among the class A carbapenemases. Endemic areas in Europe are Greece and Italy, and probably Poland. Among MBLs, the most common families are VIM and IMP groups together with the emerging NDM group [35]. In certain areas of Pakistan up to 20 % of the population may carry NDM-1 producers, and these organisms have been identified in tap and environmental water of New Delhi. NDM-1 has also been identified in *E. coli* sequence type ST131, which is known to efficiently mobilize the ESBL CTX-M-15 worldwide, as a source of CA-infection [133]. The carbapenem-hydrolyzing class D β-lactamases or oxacillinases, such as OXA-48, are widely disseminated throughout European countries [35]. Until now, only a few cases of carbapenemase producing *Enterobacteriaceae* have been found in Finland [145, 146]. In the HUSLAB material, 53 carbapenemase producing *Enterobacteriaceae* strains were found between 2008 and 2014. The first two *K. pneumoniae* KPC-2 strains carrying ST258 were detected in Finland in 2009 [147, 148].

1.4.6 Identification of CPE

The carbapenemase production cannot simply be inferred from the resistance profile. Criteria must be established to discern which isolates should be suspected and screened for carbapenemase production, and to advise which tests should be adopted for confirmation of the resistance mechanisms. The detection of carbapenemase producers in clinical infections is based firstly on susceptibility testing results obtained by disk diffusion or by automated systems, such as Vitek. However, carbapenem MICs for carbapenemase producers may vary within the susceptibility range, as defined by either the CLSI or the EUCAST clinical breakpoints [149].

A number of simple phenotypic tests, such as the modified Hodge test (MHT) and Carba NP, have been described for the detection of carbapenemase producers. Most of them base on the disc diffusion format. In addition, several inhibitor-based tests have been developed. For the specific detection of MBL producers, the Etest MBL (bioMérieux, Solna, Sweden) is available.

It bases on the inhibition of MBL activity by EDTA. Boronic acid-based inhibition testing has been reported to be specific for KPC detection in *K. pneumoniae* when performed with imipenem or meropenem [133, 150]. Spectrophotometric measurement of carbapenem hydrolysis is needed for detecting carbapenemase activity, and it is considered to be the reference standard method for the detection of carbapenemase producers. Nowadays the standard for identification of carbapenemases is based on the use of molecular techniques, mostly PCR. Real-time PCR methods (simplex and multiplex PCRs) are routinely performed for the detection of the organisms. There are also commercially available kits based on PCR and hybridization [151]. Sequencing of PCR products may be of interest, mostly for epidemiologic purposes.

The prevention of the spread of carbapenemase producers relies on the early detection of carriers. Screening should include patients who were hospitalized while abroad and then transferred to another country, as well as patients at risk, such as immunocompromised patients [133]. Unlike with MRSA and VRE, there is less agreement about who and when to screen and which identification methods to use for multidrug-resistant *Enterobacteriaceae*, due to the diversity of bacteria involved and their resistance mechanisms [25]. There is no universal screening medium able to detect all types of carbapenemase producers with high sensitivity and high specifity.

1.5 Multidrug resistant *Acinetobacter baumannii*

Acinetobacters are typical opportunistic pathogens that usually form a threat to critically ill, hospitalized patients only. Hospital-acquired *Acinetobacter* infections comprise of ventilator-associated pneumonias, bloodstream infections, urinary tract infections, wound infections, skin and soft tissue infections, and secondary meningitis [152]. *Acinetobacter baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis* and *Acinetobacter calcoaceticus* are grouped as the *Acinetobacter calcoaceticus-baumannii* complex (ABC) as they are mutually closely related and are difficult to distinguish from each other by phenotypic properties [153]. While *A. calcoaceticus* is not associated with human infections, *A. baumannii*, *A. pittii* and *A. nosocomialis* have been known to cause both CA and HA infections [154]. Among *Acinetobacter* species, *A. baumannii* is regarded as one of the most common organisms that causes healthcare-associated infections and outbreaks worldwide, especially within intensive care units (ICUs) [152, 155]. Increased risks of having an infection caused by *A. baumannii* are encountered in a trauma unit (especially war trauma), burn unit, and a long-term care facility, particularly a facility caring for ventilator-dependent patients, as well as at the ICU. Risk factors for colonization and infection are recent surgery, central vascular catheterization, tracheostomy, mechanical ventilation, enteral feeding, and treatment with third-generation cephalosporin, fluoroquinolone, or carbapenem antibiotics [156].

A. baumannii has become endemic in hospitals due to its versatile genetic machinery, which allows it to quickly evolve resistance factors, and to its remarkable ability to tolerate harsh environments [157]. Also reports of CA-*Acinetobacter* infections have increased over the past decade [158].

A. baumannii is clonal in nature, and molecular typing has shown the existence of three distinct clusters (EUI, II and III) from various locations in Europe. Global epidemiology performed by using rep-PCR indicates that there are eight worldwide clusters (WW1-8) [155]. KPC- and GES-type

of class A carbapemases have been described [159], as well as four groups of MBLs: IMP-like, VIM-like, SIM-like and recently the NDMs [160]. It has been strongly suggested that *Acinetobacter* spp. has been a reservoir of NDM genes before being hosted by enterobacterial species. Those findings highlight that even though *A. baumannii* is usually recognized as a final acceptor for resistant genes, it may acquire several resistance determinants and transfer them to *Enterobacteriaceae* and *Pseudomonas* spp. [138].

The class D carbapenemases are by far the most prevalent carbapenemases in *A. baumannii* and they can be grouped into six subclasses: intrinsic chromosomal OXA-51-like, the acquired OXA-23-like, OXA-24/40-like, OXA-58-like, OXA 143-like and OXA-235-like [160]. The first OXA-type enzyme (OXA-23) in *A. baumannii* was detected in 1985 in Scotland [161]. The emergence and spread of several outbreaks or sporadic *A. baumannii* strains producing OXA-23-like enzymes have been reported around the world [160].

Acinetobacter is a notorious pathogen of hot and humid climates, where it is a major cause of infections, particularly in intensive care units (ICUs) and within debilitated patients [156]. Töölö hospital, which takes care of patients having suffered an accident, burn unit patients and plastic surgery patients from the district of Helsinki and Uusimaa and partially from other parts of Finland, had a more serious problem with this bacterium in 2005. The tsunami disaster in Southeast Asia brought severe infections caused by multiple-resistant *A. baumannii* to this hospital [162, 163]. However, in the HUSLAB material from the district of Helsinki and Uusimaa the prevalence of multidrug resistant *Acinetobacter* spp. is nowadays very low (Figure 3) [148].

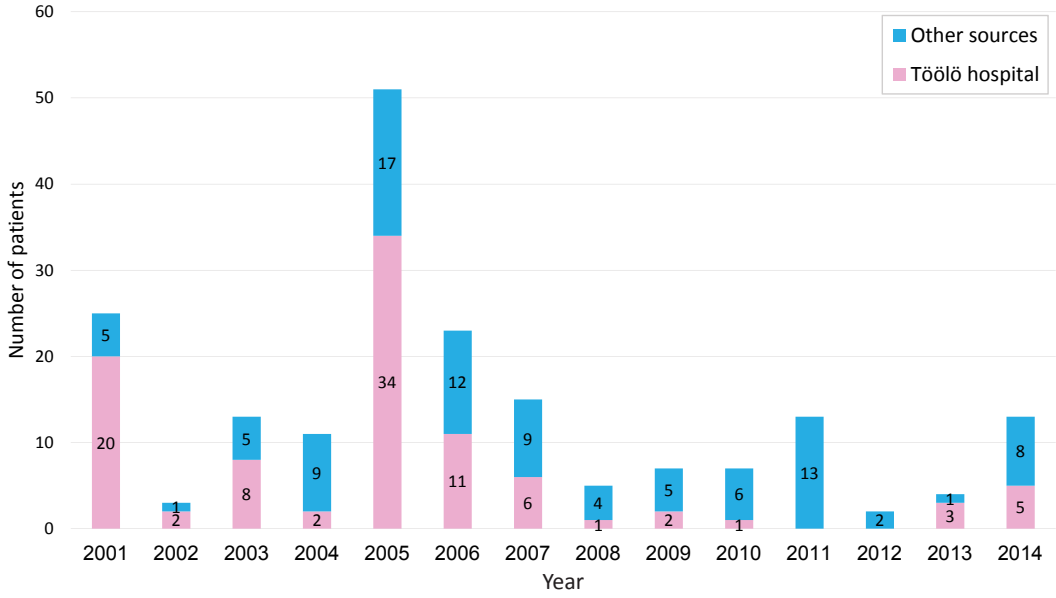

Figure 3. The prevalence of multidrug resistant *Acinetobacter* spp. MDR isolates in HUSLAB between 2001 and 2014. (The first isolate only, screening excluded) MDR definition used in 2001-2010: nonsusceptibility to meropenem, tobramycin and piperacillin-tazobactam. From 2011 onwards: nonsusceptibility to meropenem and nonsusceptibility to at least two of the following: ceftazidime, fluoroquinolone, aminoglycoside, ampicillin / sulbactam.

1.6 *Clostridium difficile*

Clostridium difficile was first identified in 1935 as a component of the normal intestinal flora. Its ability to cause diarrhea and colitis was found in 1978 [164]. Both toxin-producing, toxigenic, and non-toxigenic strains exist naturally, and both can colonize their hosts, humans and mammals (such as horses, dogs and pigs), but only the toxigenic strains are associated with disease [165]. Toxigenic strains have become a leading cause of hospital-acquired infections and are the major cause of antibiotic-associated diarrhoea, responsible for 15-25 % of all cases [166]. Epidemiological reports from the United States have implied that *C. difficile* has replaced MRSA as the most common cause of HAI [167]. *C. difficile* is usually acquired by the ingestion of spores via the fecal-oral route. Spores are infective and highly resistant and facilitate *C. difficile* persistence in aerobic environments. Transmission of *C. difficile* in health care institutions is mediated primarily by spores [168].

C. difficile infection (CDI) can manifest itself as severe disease. The clinical features of *C. difficile* can vary from asymptomatic carriage of infection to fulminant colitis, or pseudomembranous colitis, necessitating colectomy, and in some cases even leading to fatality. The major risk factors for CDI include high age, hospitalization, immuno-compromising conditions and exposure to certain antimicrobial agents, especially clindamycin, cephalosporins, and fluoroquinolones. Furthermore, the combination of multiple antibiotics and longer duration of antibiotic use are associated with increased risk of developing CDI [169]. Another clinically significant property of CDI is its relatively high recurrence rate (5-47 % of cases) [170]. The incidence and the mortality rate of CDI have considerably increased in both hospital and community settings due to the spread of hypervirulent strains and improper administration of antibiotics. One reason for that might be the aging of the population especially in the industrialized world, and it is well known that high age is an increased risk for CDI [171, 172].

1.6.1 Toxins of C. difficile

Toxigenic *C. difficile* encodes one or two glucosyltranferase toxins, toxin A (TcdA) and toxin B (TcdB). TcdA and TcdB belong to the same family of large clostridial glucosylating toxins and are considered to be the main virulence factors of *C. difficile*. These two related toxins are encoded by the 19,6 kb genomic island (five-gene locus) known as the Pathogenicity Locus (PaLoc). These toxins glucosylate and inactivate GTPases, including Rac, Rho and Cdc42 in host epithelial cells leading to signalling alterations and apoptosis [165]. The Pathogenicity Locus includes the genes for three other proteins, TcdR, TcdE and TcdC, as well. *C. difficile* toxin synthesis is negatively regulated by protein TcdC [173, 174].

Some *C. difficile* strains produce a third toxin, binary toxin CDT. Binary toxin positive strains have become increasingly prevalent in the past ten years as a cause of CDI [175]. This toxin is an ADP-ribosylating toxin that consists of two subunits: CDTa, which is the enzymatic component, and CDTb, the binding component. CDT is encoded by two genes *cdtA* and *cdtB*, which are transcriptionally linked and are located on the *C. difficile* chromosome as a part of the CDT locus (CdtLoc) [176].

Toxigenic strains must produce at least one of the three known toxins, and five toxin production patterns can be differentiated (Table 2). The definition of a toxinotype in *C. difficile* is based on the variability of the PaLoc region coding for two toxins, TcdA and TcdB [174].

Table 2. *C. difficile* toxin production types [174, 175, 177].

Toxin production type	Toxinotype	Ribotype
$A^+B^+CDT^+$	**Minor types**	
	XXIV	131
	Major types	
	III	027, 034, 075, 080
	IV	023, 058, 059, 063
	V	078, 126, 045, 066
	VI	045, 063, 066
	VII	063
	IX	019
	XIV	111
	XV	122
	XXII	ND
	XXIII	ND
	XXV	027
	XXVIII	126
$A^+B^+CDT^-$	I	003, 012, 102
	II	103
	XII	056
	XIII	070
	XVIII	014
	XIX-XXI	ND
	XXVI-XXVII	ND
$A^-B^+CDT^+$	X	036
	XVI	078
	XVII	ND
	Some V-like strains	ND
	XXX	280, 281
	XXXI	237
$A^-B^+CDT^-$	VIII	017, 047
$A^-B^-CDT^+$	XIa, XIb	033
	XI, Some strains without PaLoc	ND
$A^-B^-CDT^-$	Nontoxigenic strains	

ND, not detected

1.6.2 Deletions in the tcdC gene of C. difficile

Five lineages have been published that display different types of deletions in the *tcdC* gene (the negative regulator gene of toxin production), such as 18 bp (associated or not associated with a deletion at position 117), 36 bp, 39 bp, and 54 bp [178]. The notorious epidemic which was associated with a *C. difficile* strain was characterized as toxinotype III (Table 2), North American PFGE type 1 (NAP1), and PCR ribotype 027. The strain has a 18 bp deletion in the *tcdC* gene (at position 330-347) [179], and produces the binary toxin (CDT) [180]. Unlike the 18-bp deletion, a single-base-

pair deletion at position 117 in the *tcdC* regulator gene represents a frameshift, leading to a stop codon and a truncated TcdC protein [181]. The excessive toxin production is considered to be due to this defect in the TcdC protein. The normal inhibition of toxin production before the stationary phase is missing in these strains [180]. However, the mechanisms that regulate the levels of toxin synthesis are only slowly being unravelled. Recently, the direct role for alterations in the *tdcC* as a predictor of hyperproduction of toxin A and B has been shown to be misleading [182]. Toxins are positively regulated by *tcdR* and negatively regulated by *tcdC*, but *tcdC* may not be as important in toxin regulation as has previously been thought [183]. The *TcdE* gene encodes a holin-like protein that may facilitate the release of toxins into the extracellular environment. The protein TcdE helps efficient secretion of toxins by a phage type system. Hence, under natural conditions *C. difficile* presumably expresses an amount of TcdE sufficient to form pores that allow release of toxin without causing cell lysis [184].

There are also larger deletions than 18 bp in the *tcdC*-amplification products. These large deletions do not lead to truncations as such, but result in amino acid deletions in the putative protein products [185]. Genotype *tcdC*-A contains a nonsense mutation at position 184 that is predicted to result in a severe truncation of the TcdC protein, and a 39 bp deletion at position 341-379 in the *tcdC* gene. For example PCR ribotype 078, belonging to the toxinotype V (Table 2), has both toxin genes, *tcdA* and *tcdB*, and binary toxin genes as well, and it has a 39 bp deletion in the *tcdC* gene. It has been reported that this strain can also cause severe CDI and increased mortality [186]. There is evidence for clonality of ribotype 078 that is frequently isolated worldwide from human infections and from animals used for food [187]. A nonsense mutation at position 184 and a 54 bp deletion at position 313-366 in the *tcdC* gene is another known large deletion [178, 188]. In this study this deletion is assigned to genotype *tcd-A* variant (*tcd-A*$_{var}$) and these strains belong to the toxinotype IV and ribotype 023 groups (Table 2). *TcdC* gene sequences harboring a 36 bp deletion at position 301-336 have a mutation at position 191 [178]. The *tcdC*-variants with these larger deletions also contain stop codons comparable to the one detected in *C. difficile* 027, and thus these variants may be potential hyperpoducers of toxins A and B [179].

1.6.3 C. difficile carriage and prevalence

C. difficile spores germinate in the gut to produce vegetative cells that express the toxins. While the toxins are responsible for the observed pathogenicity, the colonization of host mucosal surfaces is an essential first step for CDI establishment. Asymptomatic carriage of *C. difficile* affects 10-52 % of the defined populations [189]. More specifically it has been estimated that 3-15 % of healthy adults carry *C. difficile* [190], and for infants the estimate is as high as 60-70 % [191]. After the recognition of a hypervirulent *Clostridium difficile* (ribotype 027) in 2005 in Europe, the ESCMID Study Group on *Clostridium difficile* (ESGCD) contacted the European Centre of Disease Prevention and Control (ECDC) and conducted several actions. The first pan-European surveillance study, the "European Clostridium Infection Survey (ECDIS)", was performed in 2008-2009. The incidence of *C. difficile* varied between European hospitals, and detailed information was obtained from the ECDIS study group for 509 patients in 34 different European countries. In this surveillance 65 different PCR ribotypes were identified, of which 014/020, 001 and 078 were the most prevalent. The prevalence of PCR ribotype 027 was 5 % (Figure 4) [192].

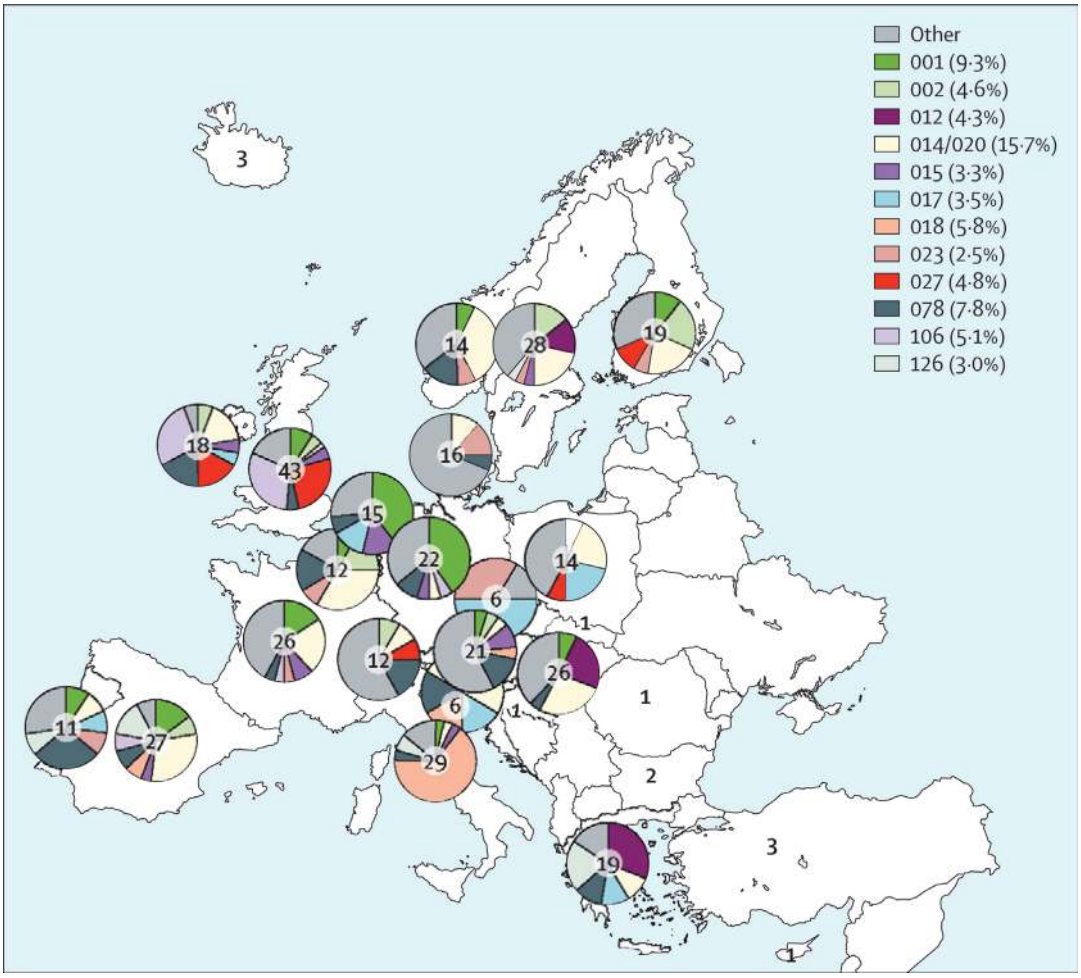

Figure 4. Geographical distribution of *Clostridium difficile* PCR ribotypes in European countries in 2008 with more than five typeable isolates [192], copyright 2015.

In 2008 the most prevalent PCR ribotypes in Finland were 014 and 002, but the proportion of the hypervirulent strain PCR ribotype 027 was significant as well [192]. The first case of fatal *C. difficile* ribotype 027-associated disease was detected in October 2007 [193]. In 2014 5159 toxin producing *C. difficile* cases were found in Finland and more than 20 % (1149 isolates) of them were found in the district of Helsinki and Uusimaa [77].

1.6.4 Identification of C. difficile

The diagnostic tests for *C. difficile* can be divided into tests for *C. difficile* products (glutamate dehydrogenase (GDH), aromatic fatty acids, toxins A and/or B), culture methods for the detection of toxin-producing *C. difficile* (toxigenic culture, TC), and tests for *C. difficile* genes (PCRs for 16S RNA, toxin genes, genes for GDH) [194]. Toxigenic culture consists of culture on selective media followed by toxin detection. The selective and differential agar, such as cycloserine-cefoxitin-fructose agar (CCFA), is the first choice of isolation media for the identification of *C. difficile* from fecal samples. *C. difficile* toxins can be detected by several methods: e.g. by cell culture

cytotoxicity assay (CCA), cell culture neutralization assays (CCNA), immunoassays, like enzyme immune assay (EIA) and immunochromatography, and PCR [194, 195]. GDH is the common antigen of *C. difficile* and it has proved to be commonly produced and conserved by both toxigenic and nontoxigenic isolates [196]. EIA tests available include detection of GDH and toxin A only, or both toxins A and B. A test including both an EIA detecting toxin A and EIA detecting GDH is also available [194].

Strain typing can help with the infection control procedures by tracking the source and spread of *C. difficile* infections. Several molecular typing methods, such as PCR ribotyping, pulsed-field gel electrophoresis (PFGE), and multilocus variable-number of tandem repeat analysis (MLVA) are used as tools with *C. difficile* [197]. PCR ribotyping is the gold standard in typing *C. difficile* in Europe and PFGE in the US.

1.7 Verification and typing of bacteria causing outbreaks

1.7.1 Molecular methods for detecting bacteria causing outbreaks based on in-house PCRs

PCR permits the synthesis of large quantities of a target nucleic acid sequence. PCR methods have been used routinely by reference laboratories as the standard for detecting and verifying microbes causing HAIs. The single PCR is the ancestor for the molecular methods used in diagnostic and epidemiological studies for the detection of antibiotic resistance or virulence genes [198]. Multiple sets of primers can be induced in a single reaction tube (multiplex PCR). This increases the efficiency and reduces reagent costs. In the development of a multiplex PCR, the key strategy is the design of the primers; all of the primers should have very close annealing temperature optima. One must be sure that all primers in the reaction tube are mutually compatible, the formation of primer-dimers and other non-specific products should be avoided or minimized. Different sizes for the intended amplicons allow the direct differentiation e.g. when PCR products are run in the agarose gel electrophoresis [199].

The real-time PCR consists of an amplification reaction coupled with simultaneous detection of the exponentially amplified target and visualization of the reaction phases. The number of cycles necessary to reach the exponential phase often depends on the initial quantity of target present in the reaction, allowing the quantification of the DNA. The visualization is based on the fluorescence emission occurring during the amplification. The fluorescence emission can be obtained using a generic dye (e.g SYBR green), which fluoresces when introduced in the double-strand DNA or using specific probes, such as hydrolysis probes (e.g. TaqMan®) harboring a fluorophore at the 5′ end and a quencher (preventing fluorescence emission) at the 3′ end. Hybridization/FRET probes harbor two different fluorescent labels. The molecular beacons consisting of the probe flanked by two complementary sequences carrying a fluorophore and a quencher at their endings, can be used as well [200]. The analysis of melting temperature (Tm) curves allows a specific and easy discrimination of amplified products obtained in a multiplex real-time PCR. Tm analyses provide discrimination between genes and allow the detection of gene variants [198].

Multiple free software programs and websites are available to design primers and probes for PCR. These tools often take into consideration the default requirements for primers and probes,

although new research advances in primer and probe design should be progressively added to different algorithm programs. After a proper design, a precise validation of the primers and probes is necessary [201].

Multiplex PCR methods, conventional and real-time, have been used to detect virulence factors and resistance genes from a variety of bacterial isolates and clinical specimens. Real-time PCR avoids the time-consuming steps (e.g., electrophoresis analysis) and in many cases does not require DNA sequencing of the obtained products [198]. Nowadays, there are many commercially available real-time PCR systems which are valuable for surveillance studies and rapid identification of carriers [202-205].

1.7.2 Bacterial typing methods

The main role of microbial typing is to assess the relationships between microbial isolates. Typing enables to determine the source and routes of infections, confirm or rule out outbreaks, trace cross-transmission of HAI, recognize virulent strains, and evaluate the effectiveness of control measures. Outbreaks of infectious diseases often result from exposure to a common source of the etiologic agent. Clonally related organisms are members of the same species that share virulence factors, biochemical traits, and genomic characteristics. However, there is sufficient diversity at the species level for organisms isolated from different sources at different times and in different geographical regions to be differentiated or classified into subtypes or strains [206].

Typing of bacteria can be useful at different levels, such as locally (hospitals), regionally and nationally (in reference laboratories or research centers) and globally (through dedicated networks). The choice of typing methods depends on the level at which it is meaningful to perform typing. The process of subtyping is epidemiologically important for recognizing outbreaks of infection, detecting the cross-transmission of nosocomial pathogens, determining the source of infection, recognizing particularly virulent strains, and monitoring vaccination programs [207, 208].

Any subtyping method must have high differentiation power and be reproducible. It must be able to clearly differentiate unrelated strains, such as those that are geographically distinct from the source organism, and at the same time to demonstrate the relationship of all organisms isolated from individuals infected through the same source. Molecular methods differ widely in their ability to differentiate strains. Reproducibility refers to the ability of a technique to yield the same result when a particular strain is repeatedly tested. Reproducibility is especially important for the construction of reliable databases containing all known strains within a species to which unknown organisms can be compared for classification. Variable expression of phenotypic characteristics, such as sporadic expression of virulence genes or antigens, can contribute to problems with reproducibility [207, 209].

Typing methods can be classified into phenotyping and genotyping methods. Phenotyping techniques detect the characteristics expressed by bacteria, while genotyping methods assess the variation in the chromosomal or extrachromosomal nucleic acid composition of bacterial isolates. The shortcomings of phenotypically based typing methods, such as antimicrobial susceptibility testing, serotyping and phage typing, multi locus enzyme electrophoresis (MLEE), or mass spectrometry have led to the development of typing methods based on the microbial

genotype or DNA sequence. Genotyping minimizes problems with typeability and reproducibility and, in some cases, enables the establishment of large databases of characterized organisms. Bacterial typing contributes to increase the effectiveness of surveillance systems and provides significant clues to public health control strategies. Typing defines the extent of epidemic spread of bacterial clones and the number of clones involved in transmission and infection, monitoring the reservoirs of epidemic clones. Some molecular typing methods have been applied to study bacterial populations, evolution and the phylogenetic relationships between species [208, 209].

The molecular typing methods most commonly used are the DNA-based methods. Genotypic typing methods assess variation in the genomes of bacterial isolates with respect to composition, overall structure, or precise nucleotide sequence. Different genotyping methods can be classified into three main categories: hybridization-mediated, fragment-based (fingerprint methods) and sequence based methods [208]. Several methods have been introduced and developed for the investigation of the molecular epidemiology of microbial pathogens, such as restriction endonuclease analysis of genomic and plasmid DNA, southern hybridization analysis with the use of specific DNA probes, plasmid profiling, chromosomal DNA-profiling using either pulse-field gel electrophoresis or PCR-based methods. Each method has advantages and limitations.

Several criteria should be considered when evaluating, validating and comparing typing methods [210]. For performance, the following criteria should be taken into account: stability, typeability, reproducibility, discrimination power, Wallace coefficient (a value indicating the probability that two strains classified as the same type by one method are also classified as the same by another method [211]), concordance and test population. Other criteria include flexibility, rapidity, accessibility, ease of use, cost, computerized analysis and data storage (Table 3) [208, 209, 212, 213].

Table 3. Characteristics of some main molecular typing systems. Adapted and modified from Ranjbar et al., 2014 [209].

Feature	Pasmid analysis	Ribotyping	PFGE	Rep-PCR	MLST
Typeability	Many	All	All	All	All
Repeatability	Moderate	Excellent	Excellent	Good to excellent	Excellent
Reproducibility	Moderate	Excellent	Excellent	Good to excellent	Excellent
Discriminatory power	Poor	Moderate to excellent	Excellent	Moderate to excellent	Good to excellent
Stability	Moderate	Good	Good	Good	Good
Ease of interpretation of data generated	Moderate	Moderate to good	Moderate	Moderate to good	Good
Ease of use	Moderate	Poor to moderate	Poor	Good	Moderate
High throughput	No	No	No	Yes	Yes
Cost	Low	High	Moderate	Low to moderate	Moderate
Time required (days)	1	1 to 3-5	3	1	>3

Whole genome sequencing (WGS) is becoming increasingly competitive with any diagnostic technology, including traditional methods of culturing bacteria, and in a few years WGS could become the sole diagnostic and molecular epidemiological tool, involving identification, genetic characterization and drug susceptibility testing [209].

1.7.2.1 Sequence based typing

Amplification-based typing methods are also identified as PCR-based methods. The PCR method, which is widely known as the most frequently applied nucleic acid amplification and detection method, has had a substantial impact on the diagnosis and epidemiological investigation of infectious diseases. Specific microbial DNA sequencing has made it a powerful molecular tool. DNA sequencing-based genotyping of bacteria has significantly contributed to many aspects of genotyping by identifying single nucleotide polymorphisms (SNPs), sequence deletions or insertions (including sequence duplications, such as VNTRs), and genes under positive selection [209, 214]. For the past 30 years, the Sanger method has been the dominant approach and gold standard for DNA sequencing [215]. It involves creating different sizes of fluorescently end-labelled PCR products in the same reaction tube. DNA fragments of varying lengths are synthesized by incorporating both nucleotides and dideoxy terminators, which are separated by agarose gel electrophoresis. Pyrosequencing is designated as a sequence-by-synthesis technique because DNA synthesis is monitored in real time. It is rapid and useful for genotyping (e.g for typing beta-lactamase genes [216]) and organism identification. Pyrosequencing relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides [217]. The technique was first demonstrated in 1987 by Pål Nyrén who showed that DNA polymerization can be monitored by measuring pyrophosphate production, which can be detected by light [218].

In 1995, the first whole bacterial genome was sequenced using conventional Sanger sequencing [219], and a decade later the advent of the first high-throughput or next-generation sequencing (NGS) technologies became available. These techniques deliver bacterial genome sequences in hours or days rather than in months or years, like before. The sequence data is also thousands of times cheaper than with Sanger sequencing. Nowadays there are many different bench-top instruments (e.g. 454 GS Junior, Roche, Ion Proton, Life technologies, MiSeq, Illumina) that use different kinds of sequencing technologies to produce sequence data. They all are capable of sequencing a whole bacterial genome in one day [220].

Sequence data for specific loci (genes for virulence, drug resistance, etc.) from different strains of the same species have revealed variability in specific genes, such as single nucleotide polymorphisms and areas with repetitive sequence that demonstrate a potential for epidemiological application. The single locus sequence typing (SLST) is a term for a variety of typing methods in which the sequencing of a single genetic locus provides a useful typing result. The target locus for SLST must be highly variable to be able to provide sufficient discriminatory power [208]. For example, DNA sequencing of the polymorphic X region or the short sequence repeat (SSR) region [221] of protein A gene (*spa*) is the most popular technique for the typing of MRSA [222, 223]. The polymorphic X region consists of 24 bp repeats and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence. The existence

of well-conserved regions in the *spa* gene allows the use of primers for PCR amplification and direct sequence typing [224].

Multilocus sequence typing (MLST) compares the nucleotide sequences of internal 400- to 500 bp regions of a series of housekeeping genes, which are present in all isolates of a particular species. MLST assesses DNA sequence variation among the alleles (usually five to ten) of housekeeping genes [208].

Single nucleotide polymorphism (SNP) genotyping involves the determination of the nucleotide base that is present in a given isolate at defined nucleotide positions known to be variable within the population. SNP genotyping methods are primarily applied to define the relationships among isolates of homogenous pathogens [208].

1.7.2.2 PCR ribotyping

Ribotyping is a classic variant of a southern hybridization mediated assay that estimates the number of ribosomal gene loci and their position in the chromosome. It is reproducible, but usually has lower discriminatory power than PFGE, for example [212]. Each ribosomal operon typically consists of three genes encoding the structural rRNA molecules, 16S, 23S, and 5S. The copy number, overall ribosomal operon sizes, nucleotide sequences, and secondary structures of the three rRNA genes are highly conserved within bacterial species. The 16S rRNA gene is the most conserved of the three rRNA genes, and 16S rRNA sequencing has been established as the gold standard for the identification and taxonomic classification of bacterial species. Knowledge of intraspecies conservation of the 16S rRNA gene and basic 16S-23S-5S ribosomal operon structure led to the first insights into its usefulness in developing ribotyping for bacterial classification [225, 226]. PCR ribotyping was developed in 1990 for clinical microbiology laboratory purposes to discriminate pathogenic microorganisms without using probes, rendering the analysis more widely applicable. The method uses primers complementary to the 3´end of the 16S rRNA gene and the 5´end of the 23 rRNA gene. PCR ribotyping reveals length heterogeneity of the PCR amplified internal spacer regions located between the 16S and 23S genes [226]. One species for which this method is widely used is *C. difficile*, having a total of 11 ribosomal operons. Variation in the spacer region occurs not only between different strains, but also between different copies of the operon on the same chromosome [227].

1.7.2.3 Chromosomal DNA-profiling based typing

Fragment-based methods include plasmid typing, restriction analysis, such as restriction endonuclease analysis (REA), pulsed field gel electrophoresis (PFGE) and PCR fingerprinting methods, such as PCR restriction fragment length polymorphism (PCR-RFLP), amplified fragment length polymorphism (AFLP), multilocus variable number tandem repeat analysis (MLVA) and repetitive PCR (rep-PCR) [208, 228].

1.7.2.4 PFGE

Restriction endonuclease analysis (REA) was the first widely used method among restriction fragment length polymorphism (RFLP). In this method the chromosome was digested by frequently cutting restriction enzymes into several hundred small fragments, which were separated by horizontal gel electrophoresis into complex patterns. The pulsed-field gel electrophoresis

(PFGE) technique made it possible to separate large DNA fragments in agarose gels by periodic alternation of the angle of the electric field´s direction [214, 229]. The bacterial chromosome is digested by spesific cutting enzymes which recognize specific DNA sequences of 6-8 bases, yielding a low to moderate number of fragments. This approach can resolve DNA fragments up to 800 kb in size. Point mutations, deletions, insertions and loss or acquisition of plasmids might account for minor differences in profiles within subtype or among epidemiologically related strains. These changes usually result in two to three fragment differences in PFGE banding patterns. The generally accepted interpretation rule is that one isolate is closely related to another when the difference is around two to three fragments, possibly related when it is four to six, and unrelated when the difference is seven or more fragments [230]. A visual comparison of profiles with usually only 10 to 20 bands is relatively easy, and computer-based analysis with the possibility of creating database libraries is available [231]. PFGE has been the gold standard for typing and successfully used in short-term and long-term epidemiological investigations for many bacterial pathogens (e.g. *C. difficile*, *S. aureus*) due to its high discriminatory power and reproducibility [210, 214, 223]. Modified PFGE protocols for subtyping previously non-PFGE typeable isolates have been developed e.g for *C. difficile* [232]. However, there are some shortcomings: PFGE is technically demanding, slow, and expressing low throughput, also the interpretation of band profiles is subjective and interlaboratory comparison of strain profiles is difficult [214, 223].

1.7.2.5 Plasmid typing

The study of plasmids is important in medical microbiology because plasmids can encode genes for antibiotic resistance or virulence factors. As many species of bacteria contain plasmids, plasmid profile typing has been used to investigate outbreaks of many bacterial diseases and to trace the inter- and intra-species spread of antibiotic resistance. Plasmid typing assesses the molecular size, numbers and restriction endonuclease digestion profiles of these bacterial extra chromosomal genetic elements, after agarose gel electrophoresis. This method has been used for the typing of many bacterial species, but typeability and discrimination are variable, depending on the bacterial species [233].

1.7.2.6 Repetitive PCR (Rep-PCR) and DiversiLab

A series of naturally occurring repetitive DNA sequences are dispersed in multiple copies throughout bacterial genomes. The PCR fingerprinting technique consists of PCR amplification of spacer fragments lying between repeat motifs of the genome using consensus primers designed to be directed outwards from repetitive elements [208]. Three families of repetitive sequences have frequently been used in REP-PCR assays: the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element sequence [214]. REP elements are 38 bp sequences consisting of six degenerate positions and a 5 bp variable loop between each side of a conserved palindromic stem in the bacterial genome. Multiple functions have been proposed for these highly conserved, dispersed REP elements, including roles in transcription termination, mRNA stability and chromosomal domain organization [234]. Detection of DNA fragments is obtained when amplicons are separated by agarose gel electrophoresis of capillary electrophoresis to generate migration patterns that are compared to one another to determine genetic relatedness. Banding patterns differ as a result of the number of repetitive elements and their relative position within the bacterial genome [209]. Because of the low cost of materials, rapidity, ease of use and

low labor intensity, rep-PCR may be a valuable tool for bacterial strain typing. On the other hand, reproducibility can be affected by variability in PCR reagents, thermal cycling and gel electrophoresis conditions.

Healy and coworkers developed a semi-automated rep-PCR in 2004 [228, 235]. This was commercialized and integrated to bioMérieux (DiversiLab, bioMérieux, Marcy l´Étoile, France) for application to clinical laboratories. The DiversiLab method uses primers targeting noncoding repetitive sequences interspersed throughout the bacterial genome [234, 236]. The modifications for automation include changes in rep-PCR chemistry and thermal cycling parameters. The method uses microfluidic chip-based DNA fragment separation (Agilent 2100 Bioanalyzer, Agilent Technologies, Inc. Paloalto, CA, USA) unlike the traditional gel electrophoresis. The system also provides data analysis software to compare the banding patterns and to generate dendrograms and an online database for strain identification and typing [228]. The DiversiLab software allows multiple report options to make interpretation easier. In order to compare the rep-PCR fingerprints, the electropherograms, or sample graphs are analyzed. For the calculation of percent similarities three options exist: Pearsson correlation (PC), the modified Kullback-Leibler (KL) and extended Jackard (XJ) coefficients to determine the distance matrices. All methods calculate similarity based on the relative intensities of the sample pair at each datapoint; however, they each place a different emphasis on peak intensity and presence. The unweighted pair group method with an arithmetic mean (UPGMA) is used to create dendograms and scatter plots. More detailed clustering and outbreak analysis can be performed by using the pattern overlay analysis (DiversiLab analysis software, version 3.4).

The method has been successfully used to distinguish several different bacteria, fungi and archaea [237]. At first, the method was used to differentiate *Aspergillus* isolates in 2004 [235]. In 2005 it was used for the typing of *S. aureus* [238] and MRSA [239], and for the identification of dermatophytes [240]. In 2006 the method was applied for the identification of *Coccidioides* species, *Blastomyces dermatitidis* and *Histoplasma capsulatum* [241], as well as

for typing vancomycin-resistant enterococci [242]. For the identification and differentiation of *Candida* spp. it was employed in 2007 [243]. After 2008 DiversiLab has been used increasingly (Figure 5) and mostly for the typing of Gram-negative rods, such as *Acinetobacter* [155, 244-247], ESBL producers [248-253], carbapenemase producers [254-256], *Serratia marcescens* [257], *Salmonella* spp. [258-260], and *Pseudomonas aeruginosa* [261]. The DiversiLab method has also proved to be suitable for typing *C. difficile* isolates as demonstrated for the first time in this study (Study IV), and subsequently also by other authors [262-265]. Accordig to bioMérieux, there are currently approx. one hundred DiversiLab users in Europe.

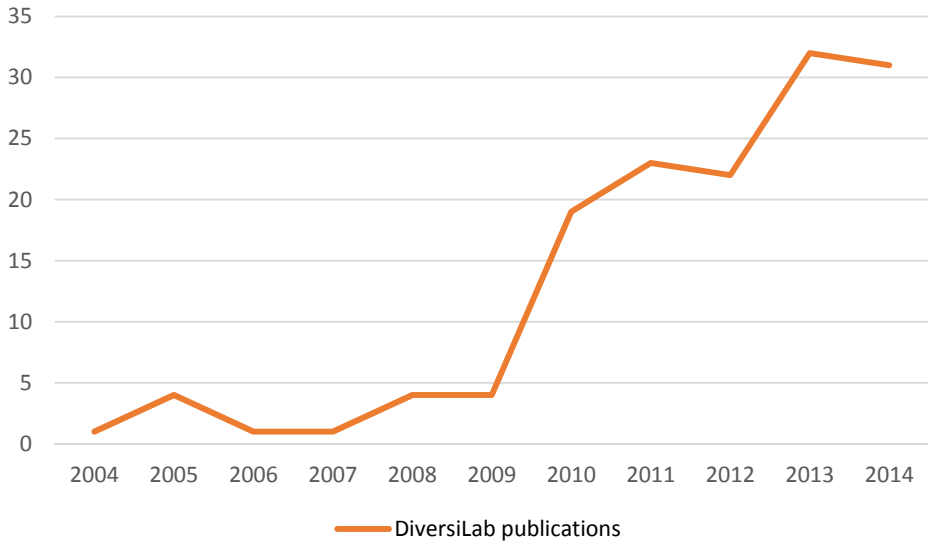

Figure 5. Usage of the DiversiLab method according to the publications in PubMed.

2. THE AIMS OF THE STUDY

I Rapid identification of hospital-acquired infections by establishing new molecular methods (multiplex PCRs)

MRSA-PCR: To assess a sensitive, reliable MRSA-PCR method suitable for daily use in a microbiological diagnostic laboratory enabling faster reporting of negative MRSA screening results.

Carbapenemase gene-PCR: Real-time multiplex PCR for detecting and verifying carbapenemase genes.

C. difficile-PCR: Conventional multiplex PCR for detecting and screening hypervirulent *C. difficile* and other potentials toxin hyperproducer strains.

II Rapid outbreak analysis for hospital-acquired bacteria outside of the national reference laboratory

III Comparison of commercial repetitive PCR (DiversiLab) with other molecular typing methods

3. MATERIALS AND METHODS

3.1 Bacterial isolates

Finland is divided into 20 hospital districts of which five are university hospital districts, of these the Hospital District of Helsinki and Uusimaa (HUS) is the largest. HUSLAB, the laboratory in the Helsinki and Uusimaa region analyzes the microbiological samples from 24 municipalities and 21 hospitals throughout the province of Uusimaa (Figure 6). The Joint Authority is made up of five hospital areas: the Helsinki University Central Hospital (HUCH), Hyvinkää, Lohja, Porvoo and Länsi-Uusimaa Hospital areas. This area is responsible for the secondary and tertiary care of approximately 1,5 million people and has approximately 2800 hospital beds. In 2013 HUS was treating more than 500 000 patients and carrying out 87 000 surgical operations per year [266].

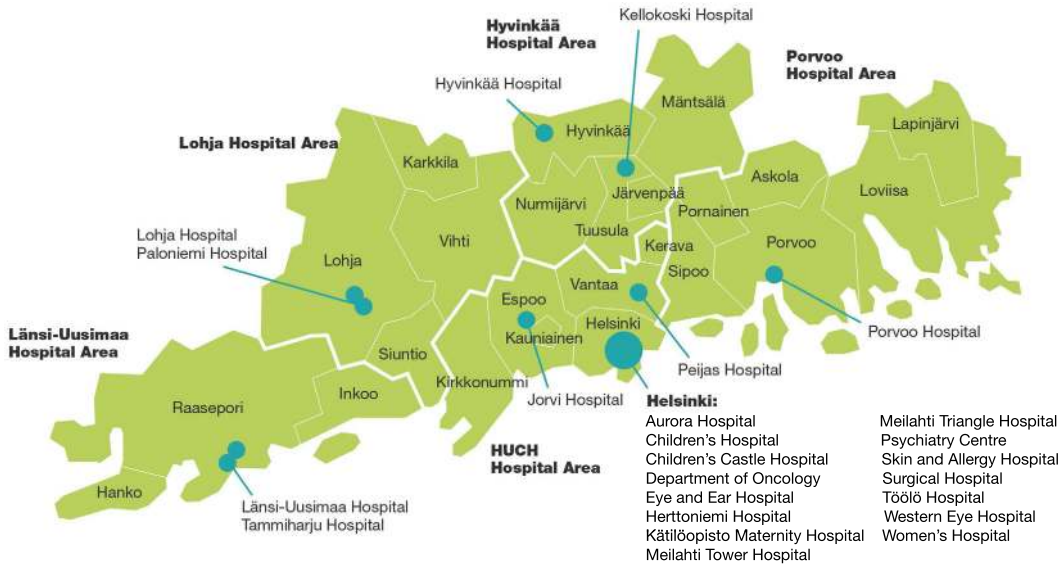

Figure 6. The province of Helsinki and Uusimaa, copyright 2015 [267].

The bacterial isolates used in the different studies are presented in Table 4. Two kinds of sets of bacterial isolates were used: consecutive screening samples (MRSA and *C. difficile*), and clinical isolates collected retrospectively (MRSA, ESBL and *A. baumannii*).

Table 4. Strain settings used in this study.

BACTERIUM	NUMBER OF ISOLATES	DESCRIPTION OF ISOLATES	REFERENCE	COLLECTION YEAR
MRSA	1250	Clinacal screening samples	V	Sep-Nov 2005
	69	Vaasa outbreak	III	2003-2010
	132	HUS culture collection samples ("reference strains")	III	
ESBL strains of *Enterobacteriaceae:*	39	Isolates from 20 patients of the pediatric surgery ward	II	2004-2008
(*E. coli, K. pneumoniae, K. oxytoca, E. cloacae)*	30	Clinical samples, negative controls		
Acinetobacter baumannii	51	Clinical isolates with reduced susceptibility	I	1993-2008
	4	Clinical samples, negative controls		
Clostridium difficile	730	Concecutive clinical screening samples	VI	2007-2008
	205	Two sets of concecutive clinical isolates from THL and HUSLAB	IV	2007-2008

HUSLAB received a total of 68873 MRSA screening samples in 2005 from health care centers, nursing homes, secondary-care and tertiary-care hospitals, and 1250 of them were analyzed in the validation of multiplex PCR for excluding MRSA. Today we receive approximately 82000 screening samples yearly. In the comparison of DiversiLab with other typing methods Vaasa outbreak isolates (n=69) and culture collection isolates (n=132) of HUSLAB were used.

In autumn 2007 an increasing number of ESBL-producing *E. coli*, *K. pneumoniae*, and surprisingly *E. cloacae* isolates were detected in one ward of our University Children's Hospital. All identified ESBL isolates (n=39) from this ward were studied using the DiversiLab system.

Carbapenemase PCR was validated from clinical samples using 710 isolates of *A. baumannii*, *P. aeruginosa* and *Enterobacteriaceae* with reduced susceptibility during 2008-2011. In the characterization of *A. baumannii* clones using the DiversiLab system 55 *Acinetobacter* isolates were analyzed.

In 2007 the first hypervirulent *C. difficile* isolate was identified and an epidemic situation started in Finland. The multiplex PCR was applied on a total of 730 toxin positive C. *difficile* isolates from consecutive clinical screening samples. In the comparison of DiversiLab with other typing methods two sets of consecutive isolates (n=205) from THL and HUSLAB were used.

3.2 Classical methods

All bacterial isolates were cultured on a suitable agar (Table 5) and incubated in suitable conditions, respectively. Colonies expressing typical morphology and biochemistry were identified as described in table 5. In addition, Gram-staining was used when necessary. The antimicrobial susceptibility of the MRSA isolates was tested according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Study I, II, III, V) [268].

Table 5. Classical methods used in this study.

	Culture	Source	Identification tests	Source
MRSA	Chocolate agar	BBL 211047 / BBL 212257	Tube coagulase test	Labema OY, Kerava, Finland
	ORSAB agar (agar base, Oxacillin 2 µg/ml, polymyxin B 50 IU/ml)	Oxoid™ CN 1008	Staphaurex latex test	Remel Europe, Dartford, UK
	MRSA ID	bioMérieux, Bortel, Netherlands	Accu probe	Gen Probe, San Diego, USA
	Selective MRSA enrichment broth	Study V	API staph/ Staph ID 32	bioMérieux,Marcy l'Etoile, France
ESBL	CLED (cysteine lactose electrolyte deficient) agar / CLED + cefuroxime	BBL, Cat. No. 212218, Becton Dickinson, NJ, USA Cefuroxime 16mg/l, Orion Pharma, Espoo, Finland	API20E	bioMérieux,Marcy l'Etoile, France
	chromID® ESBL	bioMérieux,Marcy l'Etoile, France		
A. baumannii	CLED agar	BBL, Cat. No. 212218, Becton Dickinson, NJ, USA	VITEK 2	bioMérieux,Marcy l'Etoile, France
			16S rRNA sequencing	in-house
C. difficile	CCFA agar	[269]	Premier Toxins A&B-test kit	Meridian Biosence Inc., Cincinnati, OH, USA

3.3 Molecular methods

DNA was extracted from clinical samples (MRSA) or colonies on bacterial plates by methods described in table 6 following the manufacturer´s instructions. Both conventional and real-time PCR was used.

Table 6. Nucleic acid methods used in this study.

	DNA Extraction	Source	PCR	Reference
MRSA	Magna Pure LC DNA isolation kit III	Roche, Basel, Switzerland	Real-time duplex (*mecA-nuc*) MRSA-PCR	Study V
	Easymag	bioMérieux,Marcy l'Etoile, France		
	UltraClean Microbial DNA isolation kit	MoBio Laboratories, Solana Beach, CA, USA		
ESBL	Boiling a bacterial suspension for 10 min at 95°C	[270]	Conventional PCR for ESBL genes (THL)	[270]
	UltraClean Microbial DNA isolation kit	MoBio Laboratories, Solana Beach, CA, USA		
A. baumannii	Boiling in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8,0) buffer		Real-time multiplex carbapenemase gene-PCR (17 carbapenemase genes)	Study I
	UltraClean Microbial DNA isolation kit	MoBio Laboratories, Solana Beach, CA, USA		
C. difficile	Boiling in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8,0) buffer		Conventional multiplex *C. difficile*-PCR	Study VI
	UltraClean Microbial DNA isolation kit	MoBio Laboratories, Solana Beach, CA, USA		

3.3.1 Real-time MRSA-PCR based on probes (Study V)

The primers and probes for MRSA-PCR were designed with Primer Express® software (v2.0; Applied Biosystems, Foster City, CA, USA) and the specificity was characterized by sequencing the amplification products. The designed assay is based on 5' nuclease chemistry utilizing two primers and a hydrolysis probe (Taqman®) and it employs exonuclease activity of Taq DNA polymerase. The PCR reaction mixture included QuantiTect Multiplex PCR master mix (cat No. 204543; Qiagen, Valencia, Spain), optimized primer-probe-mix, Urasil-DNA-glycosylase (UDG, 2U/ml; New England Biolabs, Ipswich MA, USA), and template DNA. The PCR run was performed using the Rotor-Gene 2000 Real-time amplification system (Corbett Research, Sydney, Australia) or using Stratagene Mx3005P (Stratgene, La Jolla CA, USA), and the amplification was performed as follows: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, followed by 40 cycles with a denaturation step at 95 °C for 15 sec, and annealing and an extension step at 60 °C for 1 min.

Table 7. Primers and probes designed for MRSA-PCR.

Gene	Primer	Sequence 5´- 3´	Probe	Sequence 5´- 3´
nuc	*nuc*-F	GCTTAGCGTATAT TTATGCTGATGGA	***nuc***	5´-FAM-AAATGGTAAACGAAGCTT-BHQ1-3´
	nuc-R	TTTAGCCAAGCCT TGACGAACT	***mecA***	5´-VIC-CCCTCAAACAGGTGAATT-BHQ1-3´
mecA	*mecA*-F	GATTATGGCTCAG GTACTGCTATCC		
	mecA-R	ATGAAGGTGTGCT TACAAGTGCTAA		

3.3.2 Real-time carbapenemase gene-PCR based on an intercalating dye (Study I)

The design for carbapenemase gene-PCR was performed using AlleleID software [271], taking into account all the globally known sub-variants in the National Center for Biotechnology Information (NCBI) data base. The assay was designed to utilize an intercalating dye (SYBR® Green) which is a non-sequence-specific fluorescent dye that exhibits a large increase in fluorescence emission when introduced into double stranded DNA. The assay was divided into two multiplex reactions consisting of nine and eight gene families, respectively (Table 8) and validated using 43 control strains confirmed at the National Institute for Health and Welfare [145]. In addition, synthetic gene constructs for SFC, CMY-1/10, SIM, SME, OXA-25 and OXA-58 genes were used. All gene products were confirmed by sequencing with reference primers or the gene specific primer alone. The OXA-51 gene with or without IS*Aba*1 was detected separately using primers: F_OXA51_ AATTTATTTAACGAAGCACACACTACGG and R_OXA51_001 (Table 9). The PCR reaction mixture included Maxima SYBR green qPCR master mix (2X) (Scientific Fermentas, Schwerte, Germany), optimized oligomix 1 or 2 (IDT, Integrated DNA Technologies, Inc), and template DNA. The PCR run was performed using Stratagene Mx3005P. The amplification was performed as follows: 95 °C 10 min initial denaturation, 30 cycles 95 °C 20 sec denaturation, 58 °C 30 sec annealing and extension, final extension at 58 °C 1 min and final denaturation 95 °C 30 sec. The melting curve was determined between temperatures 58-95 °C.

Table 8. Primers used for the amplification of carbapenemase genes by PCR.

OLIGOMIX 1:		**OLIGOMIX 2:**	
Primer	**Sequence 5´- 3´**	**Primer**	**Sequence 5' - 3'**
F_gim	CGAATGGGTTGGTAGTTCTGGATAATAATC	F_oxa24	ACTTTAGGTGAGGCAATG
R_gim	ATGTGTATGTAGGAATTGACTTTGAATTTAGC	R_oxa24	TAACTTCTTGTACTGGTGTAA
F_vim	GTGTTTGGTCGCATATCGCAAC	F_oxa27	ATATTTTACTTGCTATGTGGTTGCTTCTC
R_vim	GCTGTATCAATCAAAAGCAACTCATC	R_oxa27	TCTCCAATCCGATCAGGGCATTC
F_spm	CCTACAATCTAACGGCGACCAAG	F_IS51	GTCATAGTATTCGTCGTTAGA
R_spm	AACGGCGAAGAGACAATGACAAC	R_IS51	GTAAGAGTGCTTTAATGTTCATA
F_ges	ACACCTGGCGACCTCAGAGATAC	F_oxa58	GACAATTACACCTATACAAGAAG
R_ges	ACTTGACCGACAGAGGCAACTAATTC	R_oxa58	CGCTCTACATACAACATCTC
F_kpc	CAGCGGCAGCAGTTTGTTGATTG	F_cmy	CAGGTGCTCTTCAACAAG
R_kpc	CCAGACGACGGCATAGTCATTTG	R_cmy	CGCCCTCTTTCTTTCAAC
F_oxa48	TTACTGAACATAAATCACAGGGCGTAG	F_sfc	CCTGGTGATGATAGAGATAC
R_oxa48	ATTATTCGTAAATCCTTGCTGCTTATTCTC	R_sfc	ATAATCGTTGGCTGTACC
F_imi1	AAACAAGGGAATGGGTGGAGACTG	F_ndm	CGATCAAACCGTTGGAAG
R_imi1	AAGGTATGCTTTGAATTTGCGTTG	R_ndm	AAGGAAAACTTGATGGAATTG
F_imp	AATAATGACGCCTATCTAATTGACACTCC	F_sim	CTGCTGGGATAGAGTGGCTTAATAC
R_imp	ATTCCACCCGTACTGTCGCTATG	R_sim	TCAATAGTGATGCGTCTCCGATTTC
F_imp	TGACGCCTATCTGATTGACACTCC		
R_imp	GCTGTCGCTATGGAAATGTGAGG		
F_sme	CAGATGAGCGGTTCCCTTTATGC		
R_sme	CAGAAGCCATATCACCTAATGTCATACC		

All positive isolates with an expected melting curve were confirmed by further analysis using conventional PCR and by sequencing the carbapenemase gene (Table 9). The reaction included HotStar Taq polymerase (Qiagen, Helsinki, Finland), polymerase buffer 10 X, dNTP-mix (2,5 mM), forward and reverse primers for genes predicted from the melting curve, and template DNA. Amplification was performed as follows: initial denaturation 95°C 15 min, 35 cycles with denaturation 94°C 30 sec, variable annealing temperature 55/60/62°C 30 sec depending on the carbapenemase gene to be amplified, extension 72°C 1 min, and final extension 72°C 10 min, using the DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA).

Table 9. Primers used for sequencing carbapenemase genes.

Gene	Primer	Sequence (5'- 3')	Size (bp)	T (m)	Reference
IMP-1	F_IMP-1 R_IMP-1	TGAGCAAGTTATCTGTATTC TTAGTTGCTTGGTTTTGATG	740	55	[134]
IMP-15	F_IMP-2 R_IMP-2	GGCAGTCGCCCTAAAACAAA TAGTTACTTGGCTGTGATGG	737	55	[134]
IMI	F_IMI R_IMI	ATAGCCATCCTTGTTTAGCTC TCTGCGATTACTTTATCCTC	818	55	[134]
KPC	F_KPC R_KPC	ATGTCACTGTATCGCCGTCT TTTTCAGAGCCTTACTGCCC	893	55	[134]
GES	F_GES R_GES	GTTTTGCAATGTGCTCAACG TGCCATAGCAATAGGCGTAG	371	60	[134]
VIM-1	F_VIM-1 R_VIM-1	TTATGGAGCAGCAACCGATGT CAAAAGTCCCGCTCCAACGA	920	60	[134]
VIM-2	F_VIM-2 R_VIM-2	AAAGTTATGCCGCACTCACC TGCAACTTCATGTTATGCCG	865	60	[134]
OXA-48	F_OXA-48 R_OXA-48	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	744	62	[134]
GIM	F_GIM-1 R_GIM-1	AGAACCTTGACCGAACGCAG ACTCATGACTCCTCACGAGG	748	60	[134]
SPM	F_SPM-1 R_SPM-1	CCTACAATCTAACGGCGACC TCGCCGTGTCCAGGTATAAC	650	55	[134]
SME	F_SME R_SME	AAGGCTCAGGTATGACATT GGCATAATCATTCGCAGTA	410	60	Study I
SIM	F_SIM-1 R_SIM-1	TACAAGGGATTCGGCATCG TAATGGCCTGTTCCCATGTG	571	60	[134]
OXA-24 family	F_OXA24 R_OXA24	GAATATGTCCCTGCATCAAC ACCAGTCAACCAACCTAC	477	60	Study I
OXA-23 family	F_OXA23 R_OXA23	GTGTCATAGTATTCGTCGTTAG TATCAACCTGCTGTCCAAT	592	60	Study I
ISaba1/ OXA-51	F_IS51 R_OXA51	GTCATAGTATTCGTCGTTAGA GCACGAGCAAGATCATTACCATAGC	301	60	Study I
CMY-10	F_CMY R_CMY	TAAGATACTTCGGATGAGGAG GCATCTTCTCGGATGAATC	695	60	Study I
SFC	F_SFC R_SFC	CTCATTCTCCTGTGACTGA TTGCTCCTCCTGTTGTATT	351	60	Study I
NDM	F_NDM R_NDM	GACAACGCATTGGCATAAG AAAGGAAAACTTGATGGAATTG	447	60	Study I

3.3.3 Conventional C. difficile-PCR (Study VI)

For the detection of toxin-producing *C. difficile* a conventional multiplex PCR was designed. This PCR detects the genes *tcdA*, *tcdB*, *cdtA*, *cdtB*, *tcdC* and the size of the possible deletion in the *tcdC* gene (Table 10). Primers for amplifying the 139 bp fragment of the *tcdC* gene were designed using PRIMER EXPRESS-software (version 1.5; Applied Biosystems, Foster City, CA, USA). The specificity of amplification products was characterized by sequencing. The PCR reaction included 0,2 mM dNTP-mix (Finnzymes, Espoo, Finland), 0,5 mM $MgCl_2$ (Applied biosystems), 1 U Phusion-polymerase (Finnzymes), 1 X enzyme buffer, optimized primer mix and template DNA. The PCR run was performed using the DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad) and the amplification was performed as follows: initial denaturation step at 98 °C , 36 cycles at 98 °C for 10 sec, 60 °C for 20 sec and 72 °C for 20 sec, followed by final extension at 72 °C for 10 min. The PCR products were run in 2,5 % Nusieve GTG agarose (Lonza, Basel, Switzerland) in TBE buffer, 120 V for 2,5 h.

Table 10. Primers used for the amplification of *C. difficile* toxin producing genes.

Gene	Primer	Sequence (5´-3´)	Reference
cdtA	cdtApos cdtArev	TGAACCTGGAAAAGGTGATG AGGATTATTTACTGGACCATTTG	[272]
cdtB	cdtBpos cdtBrev	CTTATTGCAAGTAAATACTGAGAGTACTATATC ACCGGATCTCTTGCTTCAGTC	Modified from Stubbs [272]
tcdA	TA1 TA2	ATGATAAGGCAACTTCAGTGG TAAGTTCCTCCTGCTCCATCAA	[273]
tcdB1	TB1 TB2	GAGCTGCTTCAATTGGAGAGA GTAACCTACTTTCATAACACCAG	[273]
tcdB2	NK104 NK105	GTGTAGCAATGAAAGTCCAAGTTTACGC CACTTAGCTCTTTGATTGCTGCACCT	[274]
tcdC	tcdC52/121-F tcdC121-R	AAGCTATTGAAGCTGAAAATC GCTAATTGGTCATAAGTAATACC	Study VI

3.3.4 DiversiLab (Study I, II, III and IV)

The automated repetitive extragenic palindromic sequence-based PCR (rep-PCR), DiversiLab, was validated in this study for analyzing outbreaks caused by hospital-acquired bacteria. The DiversiLab analysis was performed on all bacteria examined (Table 11). DNA was amplified using the bacterium specific DiversiLab kits (Bacterial Barcodes, Athens, GA, USA), and DNA fingerprinting was performed following the manufacturer´s instructions. The PCR run was performed using the preheated DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA). The rep-PCR products were detected and the amplicons were separated using the microfluidics lab-on-chip technology (Agilent 2100 Bioanalyzer, Agilent Technologies, Inc. Paloalto, CA, USA), and analyzed using the Diversilab system. Further analyses were performed using the web-based DiversiLab software (version 3.4) applying the band-based modified Kullback-Leibler distance for the calculation of percent similarities. For MRSA, pattern overlay analysis was done, as well. This method was compared with other typing methods, such as

PFGE, PCR ribotyping and *spa* typing (SLST). Two different gene sequencing based methods, the CTX-M gene sequencing in ESBL strains and the *tcdC* gene sequencing in *C. difficile* strains, were also used.

Table 11. Typing methods used in this study.

	Typing method	**Reference**
MRSA	DiversiLab (Study III) PFGE (THL) *spa* typing (THL)	bioMérieux,Marcy l'Etoile, France [275, 276] [66]
ESBL	DiversiLab (Study II) PFGE (THL) CTX-M gene pyrosequencing (THL)	bioMérieux,Marcy l'Etoile, France [277] [270]
A. baumannii	DiversiLab (Study I)	bioMérieux,Marcy l'Etoile, France
C. difficile	DiversiLab (Study IV) PCR ribotyping (THL) PFGE (THL) Conventional PCR and *tcdC* gene sequencing (Study VI)	bioMérieux,Marcy l'Etoile, France [227] [232] [273]

All of the molecular methods used in this study are concluded in table 12.

Table 12. Conclusion of the molecular methods used in this study.

PCR METHODS		
Conventional PCR	**Real-time PCR**	
• Detection of ESBL genes • Verification of positive carbapenemase genes • Multiplex PCR for the detection of toxigenic *C. difficile*	• Multiplex PCR for the detection of *mecA* and *nuc* genes of MRSA • Multiplex PCR for the detection of carbapenemase genes	
TYPING METHODS		
Typing methods based on sequencing	**Typing methods based on PCR**	**Typing methods based on the analysis of chromosomal DNA**
• *spa* typing (SLST) • ESBL gene sequencing • Carbapenemase gene sequencing • *TcdC* gene sequencing	• PCR ribotyping	• PFGE • rep-PCR

4. RESULTS AND DISCUSSION

4.1 Identification of bacteria causing outbreaks by new multiplex PCRs (Study I, V, VI)

In this study three different in-house multiplex PCR methods were established for our clinical laboratory. The goal was to detect the possible HAI strains faster and more easily. In addition, a MRSA enrichment broth was developed, and this broth is still used in the routine diagnostics in our laboratory. The multiplex PCR methods were 1) detection of MRSA by duplex PCR detecting *nuc* and *mecA* genes, 2) detection of 17 different carbapenemase gene groups of *Enterobacteriaceae* by real-time multiplex PCR, and 3) detection of toxigenic *C. difficile* by multiplex conventional PCR, including detection of toxin hyperproducer strains. All of these three in-house methods are still in our daily routine use, as well.

4.1.1 MRSA-PCR (Study V)

1250 clinical samples were isolated for the validation of duplex PCR detecting *nuc* and *mecA* genes. Among these isolates 31 (2.5%) MRSA strains from 21 patients were found. FIN-21 (*spa* type t041, MLST ST type 228) was the most common type (46%, 15 strains from 14 patients), and was one of the epidemic types in Finland at that time [275]. The sensitivity of the test was 93.5%, specificity 88.6%, positive predictive value 17.3% and negative predictive value 99.8% as compared to culture (Study V). Using the MRSA-PCR combined with enrichment broth, 89% of the truly negative samples can be reported correctly. This was in agreement with other methods using a *S. aureus* specific marker combined to *mecA* and a cefoxitin-based enrichment medium [81, 278, 279]. Cefoxitin enhances the growth of heteroresistant MRSA-strains more efficiently and the use of a cefoxitin-based enrichment medium increased the MRSA detection rate as compared to plate culture only. This increased sensitivity brought about by broth enrichment is commonly acknowledged [78].

A clear correlation between the Ct values of *nuc* and *mecA* was seen in the samples containing MRSA, whereas in the samples negative for MRSA such correlation did not exist when the cut-off value was set to 30 cycles. A heavy load of methicillin-sensitive *S. aureus* and methicillin-resistant coagulase negative *Staphylococcae* (*nuc* negative) is often found in MRSA screening samples potentially increasing the number of false positives. Therefore *nuc* (*S. aureus* specific gene) was combined with the methicillin-resistance gene, *mecA*, for the screening method. This MRSA-PCR screening method enables us to provide clinicians rapid reliable negative MRSA results. Broth enrichment combined to the duplex PCR-method allows the reporting of negative samples on the following day after receiving the sample. Using the culture technique a negative report can be given on day three, thus the PCR method speeds up the process by two days which may be considered beneficial. The sooner the result of the MRSA screening test is available in the clinic, the sooner the control measures, namely isolation, cohorting and surveillance of cultures of contacts, can be undertaken [280].

The designed MRSA-PCR is a high throughput method and applicable to all machinery compatible with a 5' nuclease chemistry utilizing a hydrolysis probe. It uses standard probes and laboratory personnel time is taken up only moderately. The costs are relatively low and it has a very high throughput. The method also optimizes the capacity of a laboratory having opening hours from 8 a.m. to 8 p.m. It is suitable for the work flow to combine MRSA culture and MRSA-PCR for verifying the positive result if needed. Options for these kinds of solutions are needed when cost-effectiveness is demanded of clinical laboratories. Many commercial solutions for screening have recently come up [85, 281, 282]. Thus, the molecular diagnostics of MRSA remains challenging even in this era of several commercial, validated MRSA-PCR-tests. At the time of the study one option, the BD GeneOhm™ MRSA assay, was studied in our laboratory. This assay was not able to reliably detect strains belonging to PFGE types FIN-7, FIN-31, FIN-35. The strains harbored SCC*mec* types IV, V, VI, or a novel type. In addition, single false-negative or weak-positive reactions were seen in a few other PFGE types. It was concluded that we could not rely on the BD GeneOhm™ MRSA assay in our epidemiologic situation and it was not tested with any actual patient swabs [205]. To conclude, the combining of the enrichment broth to the MRSA-PCR suggested by us was considered to be more reliable and applicable.

This reasonably rapid, robust, enrichment broth based on the *nuc-mecA*-PCR-application is suitable for diagnostic microbiological laboratories for the screening of MRSA. The method is not able to detect the *mecC* gene. For cases where the *mecA* result is negative and the susceptibility to oxacillin or cefoxitin is reduced our laboratory has set up a new real-time PCR for detecting the *mecC* gene (unpublished). Up to now only one *mecC* positive strain has been found in the HUSLAB material.

4.1.2 Carbapenemase gene-PCR (Study I)

The carbapenemase detection assay was developed to detect carbapenemase producing *Enterobacteriaceae*, but was found to be a useful tool for *Pseudomonas aeruginosa* and *A. baumannii* as well. Earlier, a multiplex PCR for carbapenemase genes had been developed by Poirel et al., but this multiplex did not include OXA-23, OXA-58 and OXA-24/40, the genes commonly found in *A. baumannii* [283]. The assay developed here detects 17 different carbapenemase genes (Study I), and the multiplex PCR proved to be sensitive and highly specific. The assay was tested using 710 isolates of *A. baumannii*, *P. aeruginosa* and *Enterobacteriaceae* with reduced carbapenem susceptibility. CPE strains are increasingly found in Finland, but still at a very low prevalence. In the HUSLAB material from 2008-2014 a total of 53 enterobacterial strains (including IMI, KPC, OXA-48, VIM, NDM, GES, OXA-181, SME and NCM-A enzymes) were found, the most prevalent genes being KPC (38 %) and NDM (30 %) (Figure 7) [148]. The spread of KPC, NDM and OXA-48 is already known worldwide [35, 284].

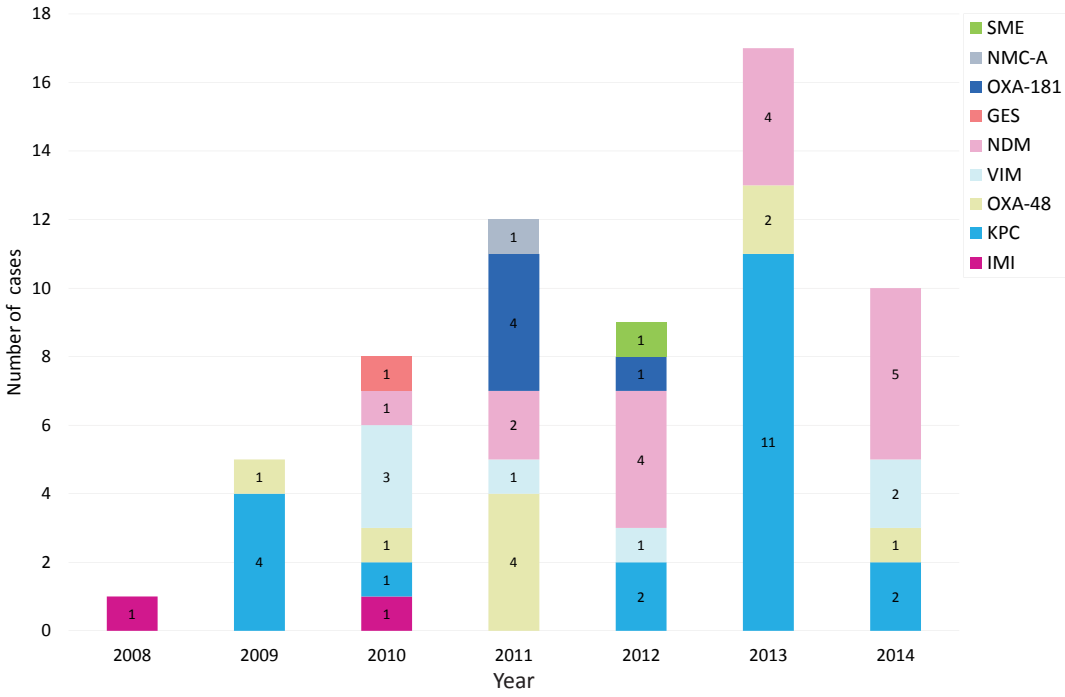

Figure 7. Carbapenemase producing *Enterobacteriaceae* in HUSLAB between 2008 and 2014.

This carbapenemase gene-PCR was used to characterize the carbapenemase genes of *A. baumannii* isolates with reduced susceptibity to carbapenem. In *A. baumannii*, only oxacillinase genes were found, of which the most prevalent were OXA-23-like gene (33/55, 60 %) and the the OXA-58 gene (8/55, 15 %). Only one OXA-24/40-like gene was found. The emergence and spread of several outbreaks of sporadic *A. baumannii* strains producing an OXA-23-like enzyme have been reported around the world [285]. The IS*Aba1*-OXA-51 junction PCR was negative in all strains. IS*Aba1* is providing a strong promoter activity for the *bla*OXA-51-like gene, and probably for the OXA-23-like gene [286]. The IS*Aba1* associated OXA-23 gene in *A. baumannii* was found to increase imipenem resistance and dissemination in an intensive care unit in China [287].

One major problem related to molecular detection is the appearance of new genomic variants. This problem is affecting the detection of many antibiotic resistance genes. For example six OXA-48 variants (OXA-48, OXA-162, OXA-163, OXA-181, OXA-204 and OXA-232) have been identified, with OXA-48 being the most widespread. The variable regions of the OXA-181 enzyme differ from OXA-48 by only four amino acid substitutions and have been found to be associated with other carbapenemase genes [142]. Up to now the GES family includes 27 variants, KPC includes 24 variants and NDM includes 16 variants [107]. The phenotypes of the carbapenemase producing bacteria are highly variable and many overlapping resistance mechanisms can appear. Finding a simple screening method for detecting them all is very challenging. At the time of the study no simple method suitable for routine clinical screening was available. Primers for this assay were designed for conserved gene regions to achieve optimal amplification of all the current and hopefully forthcoming sub-variants. This multiplex assay is very comprehensive compared to the commercial multiplex PCRs, such as Check-Direct CPE, easyplex® SuperBug complete A or Xpert® Carba-R, which will usually detect only five main carbapenemase families, KPC, OXA-

48, NDM, VIM and IMP (usually including only IMP-1 variant) associated with outbreaks. These tests can be used to detect genes directly from a clinical screening sample or they may be used to search for the genes from isolates with reduced susceptibility [288]. The multiplex PCR developed in this study has also been tested directly from clinical faecal samples (spiked samples) using a reduced gene set and the results were promising (data not shown).

Today there are many commercial rapid screening tests to identify carbapenemase producers in *Enterobacteriaceae* [150, 289]. The carba NP test is based on a technique that identifies the hydrolysis of the betalactam ring of carbapenem in carbapenemase-producing *Enterobacteriaceae* (290). Carba NP and Rapid CARB screen Kit (Rosco Diagnostica A/S, Taastrup, Denmark) both base on in vitro hydrolysis of imipenem. The New NucliSENS EasyQ KPC (BioMérieux) has been shown to be highly sensitive and specific for the detection of KPC in *K. pneumoniae* [202]. The recently introduced rapid screening test CarbAcineto (modified version of Carba NP test) efficiently detects OXA-type carbapenemases and offers a cost effective solution for detecting carbapenemase producers in *Acinetobacter* spp [291].

The very early identification of the carbapenemase producers, especially in hospital settings, may contribute to limiting their spread. Today it may be reasonable to use some rapid diagnostics based on phenotypic techniques and combine it with PCR methods. Rapid screening tests can efficiently indicate the strains to be further tested and verified by PCR and submitted to sequencing.

4.1.3 C. difficile-PCR (Study VI)

The *C. difficile* multiplex PCR for the detection of virulence genes of *C. difficile* proved to be sensitive and specific (both 100 %) in identifying and screening for the hypervirulent PCR ribotype 027-like strain. Today it is known that the same toxin profile (A+B+, binary toxin+, tcdC 18 bp del) includes several PCR ribotypes [292, 293]. In this study (Study VI) 730 samples were tested using multiplex *C. difficile* PCR, and 18 % of the strains had a similar profile with PCR ribotype 027. Most prevalent (80 %) was a profile similar to PCR ribotype 001. With this multiplex PCR it is possible to detect strains with large deletions (39 bp and 54 bp) in the *tcdC* gene as well. Such strains are also toxin hyperproducers and are associated with severe disease and mortality [186, 294, 295].

Clostridium difficile infection (CDI) diagnosis is usually based on a combination of clinical symptoms and laboratory tests. The optimal diagnostic algorithm for CDI is yet to be adequately defined and may vary with the underlying clinical circumstances. Laboratory tests can fall into three groups: 1) methods that detect free toxins A and B from stools (enzyme immunoassay (EIA) for toxins, stool cytotoxicity assay), 2) methods that detect the presence of a toxigenic strain (nucleic acid amplification tests, NAATs, and toxigenic culture, TC) or 3) methods that detect the presence of *C. difficile* (culture, EIA for glutamate dehydrogenase, GDH). A two-step protocol is recommended with the initial detection of GDH (*gluD* gene) by EIA or PCR followed by toxin EIA [194, 296]. Today it is known that the sensitivity of EIA is low compared to PCR, and NAATs are recommended [297, 298]. NAATs are promising due to their high sensitivity and rapidity, despite their cost. Nevertheless, there are limits to these new methods, in particular the lack of specificity. These methods can also detect asymptomatic patients who carry a toxigenic strain,

but who have diarrhea for some other reason [299]. It is shown that the detection of toxin is an essential step in the diagnosis of *C. difficile* infection [300]. After the publication of the multiplex PCR assay described here, the detection of GDH has been introduced into the process. More recently, several commercial real-time PCR solutions have been developed, such as Prodesse ProGastro CD, the BD GeneOhm Cdiff, and GeneExpert assays, for detecting toxigenic *C. difficile* [301]. The multiplex assay in this study is designed to be performed from colonies on CCFA plate and is not validated for clinical samples. This robust, low-cost, conventional multiplex PCR correlated excellently with the PCR ribotypes, especially with PCR ribotype 027-like, and also excluded strains with large deletions. More rapid screening using commercial solutions may be desirable. A possible further study is to establish a multiplex real-time PCR performed straight from a clinical faecal sample.

In HUSLAB the multiplex PCR has been used to screen every culture-positive *C. difficile* sample since the end of 2007, and it was a current trend in diagnostics *of C. difficile* at the time [302]. Interestingly, the proportion of hypervirulent PCR ribotype 027 strains is dramatically decreased in the HUSLAB material. In 2008 the proportion of hypervirulent strains was approx. 20 % of all toxigenic *C. difficile* isolates and in 2014 it was less than 1 % of the isolates (Figure 8.) It is known that the molecular epidemiology of *C. difficile* is varied; a certain ribotype can predominate in a particular area during certain periods and at the same time be extremely rare elsewhere [293, 303]. More controlled prescription of antibiotics may also have an effect on the disappearance of CDI. In addition, rates of CDI, most notably PCR ribotype 027 in the UK and other parts of Europe, appear to have declined in the last 4 years and between the years 2007/2008 and 2010/2011, there was a 42.9% decrease in the number of PCR ribotype 027 strains isolated from *C. difficile* [304]. This has occurred simultaneously with an increase in a variety of other PCR ribotypes, especially 014/020, 015, 078, 005, 023 and 016. This phenomenon may reflect the success of control measures to reduce cross-infection in hospitals. The annual incidence rate of CDIs decreased by 24 % from 2008 (119/100 000) to 2010 (90/100 000) in Finland [305]. A total of 5725 cases of *C. difficile* was found in Finland in 2014 and 90 % of them were toxin producers. The incidence varied between 37–206/100 000 depending on the regions [306]. The reason for this might be the differences in the diagnostic methods or differences in the prevention and controlling of *C. difficile*. Increased sample submission may also be expected to lead to an amplification of the relative contribution of other *C difficile* ribotypes.

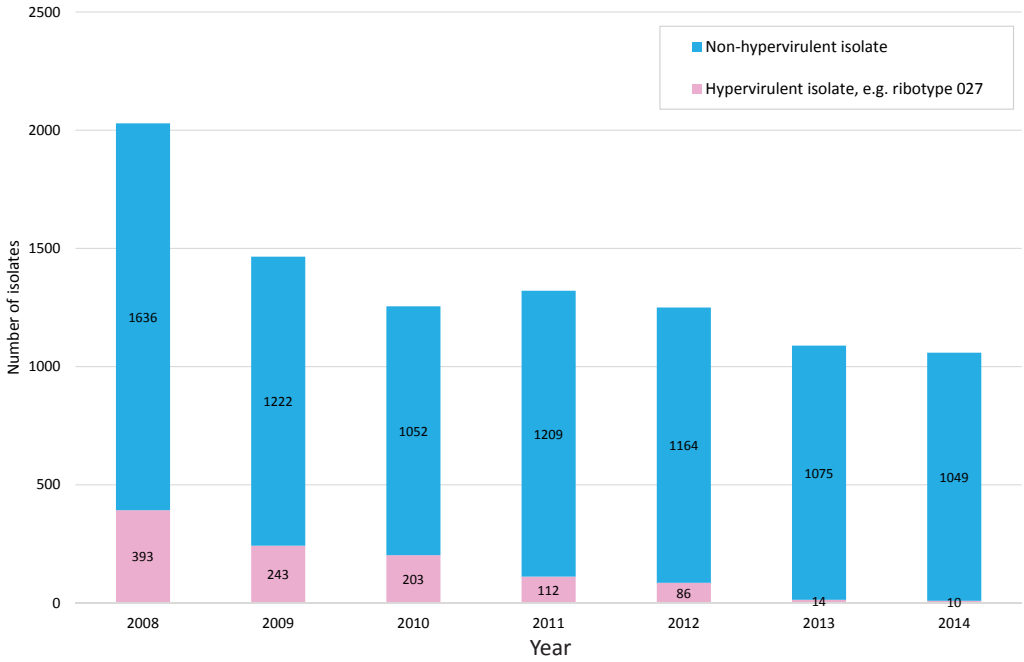

Figure 8. Toxin producing *C. difficile* in HUSLAB between 2008 and 2014. (One isolate per patient per year, including screening samples)

4.2 DiversiLab results compared with other typing results (Study I, II, III, IV)

The repetitive PCR based typing method, Diversilab, was evaluated for detecting high-risk clones of bacteria causing HAIs, and on the other hand for typing the bacteria by comparing this method to other typing methods, so called gold standards. In this study, comparison of the typing methods has been made without any epidemiological tools, and subjective results are presented in table 13.

Table 13. Typing results for repetitive PCR, DiverisiLab.

Bacterium	Result in this study
MRSA	Suitable only for initial screening in an outbreak investigation. Overlay analysis and a more discriminative method (PFGE) is needed.
ESBL *Enterobacteriaceae*	Suitable. Harbored the CTX-M-15 clone.
A. baumannii	Suitable. Harbored the OXA-23-like and OXA-58 clones.
C. difficile	Suitable for monitoring the spread of *C. difficile* locally. Harbored the NAP1/027 clone. Multible methods for typing are needed.

Generally, this study showed the DiversiLab results to be reproducible. The effect of DNA isolation, the performing technician, and PCR conditions, and the electropherogram of each isolate type was studied with a set of isolates (data not shown). If the process was carried out

carefully the profiles were not affected by the performer of the DNA extraction or PCR with a representative set of samples. Even though more reproducible DNA amplification and isolation methods would improve the test, it is reassuring that the same conclusion has been reached by other investigators as well [252]. Some variation in the quality and amounts of the isolated DNA used for amplification was observed and also the thermocycler used may have been a source of variation. The DNA isolation stage must be maximally successful in order for reproducible results to be obtained. In addition, with some isolates the freezing-melting cycle of the template DNA between separate PCRs seemed to result in somewhat distinct rep-PCR profiles. With Gram-positive bacteria (especially *C. difficile*) there were more difficulties in the DNA isolation step than with the Gram-negatives. This may be due to the remarkable differences in the structure of the bacterial cell walls. Furthermore, *C. difficile* has a notably unstable genome [307].

The rep-PCR-system provided results that were easy to analyze, were reproducible and were rapid to perform, thus making this system a suitable option for clinical use. A set of 13 samples could be performed within a single day. In practice, the rep-PCR profiles were easily stored in the web-based library and conveniently analyzed by the software of the system. The profiles of isolates analyzed at separate time points can be archived in the library and compared at any time. The system also allows joint libraries for separate laboratories when necessary, but in this study the library generated by us was more useful than the one provided by the manufacturer.

4.2.1 MRSA (Study III)

Numerous molecular typing methods, such as PFGE [308] *spa* typing [309] SCC*mec* typing [310], MLST [311] and whole genome sequencing [312] have been developed to identify the clonality of MRSA strains and monitor their spread. The usefulness of DiversiLab and the comparability to other methods e.g. PFGE, MLST and *spa* typing is well known today [238, 313-316]. In this study 69 MRSA strains belonging to five distinct outbreaks with precise epidemiological data from the district of Vaasa hospital were examined. DiversiLab was able to assign the isolates into only three different clusters, but when the pattern overlay function was used, two additional sub-clusters were identified. In addition, the DiversiLab system was evaluated using 132 MRSA strains from the HUSLAB culture collection. 13 different DiversiLab clusters were identified when the combined 201 isolates were examined. Only in four out of the 13 clusters all the methods (*spa* typing, PFGE and DiversiLab) compared yielded identical results. As a result, the DiversLab system proved to be a useful tool for a first-line outbreak analysis, but overlay analysis is needed. Unfortunately DiversiLab lacks resolution to differentiate genetically and epidemiologically unique MRSA strains and DiversiLab has been shown to be less discriminatory than PFGE when comparing outbreak strains [313]. On the other hand, the results obtained with *spa* typing had a weaker discriminatory power than the overlay analyses of DiversiLab, causing limited feasibility in an outbreak situation. Even though the discriminatory power of PFGE is higher than that of *spa* typing, *spa* typing is useful to complement the detailed epidemiological investigations [317]. *Spa* typing has been claimed to have strong discriminatory power [318] and even though the region X of the *spa* gene was reported to be stable [319], the variability in *spa* types was seen in this study. Several PFGE strain types collected from different part of Finland were typed as *spa* t008, which was the most prevalent *spa* type in this study. The PVL positive MRSA *spa* type t008 is identified as a variant of USA300 [317], which is a predominant community associated MRSA in the USA [73, 320].

Combined with epidemiological data, PFGE and DiversiLab with overlay analysis were the typing methods converging best. The same result has been obtained by other researchers as well [314, 315]. PFGE is often considered the gold standard for typing MRSA isolates in epidemiological studies. This method is known to be highly discriminatory, but technically demanding and time-consuming [223, 321]. Therefore, the determination of sequence polymorphism in the variable X region of the *spa* gene has become one of the primary typing methods for regional and national MRSA surveillance programs.

Controlling MRSA epidemics is of outstanding importance and therefore rapid detection of MRSA clusters and outbreaks is needed. Predominant focus on control efforts take place in health-care facilities. HA-MRSA lineages are usually poorly adapted for persistence in the community [71]. Lineages of community-associated MRSA, such as USA300, have undergone a fundamental shift and these strains can be carried for long periods by healthy people. Today whole genome sequencing holds great promise for rapid and accurate identification of bacterial transmission pathways and characterization of outbreaks in hospitals and community settings [312, 322].

4.2.2 ESBL producing Enterobacteriaceae (Study II)

DiversiLab has been evaluated as a useful tool for screening *E. coli* outbreaks [249, 323]. In the outbreak characterizated in this study, 39 ESBL producing *Enterobacteriaceae* (16 *E. coli*, 6 *K. pneumoniae*, 5 *K. oxytoca* and 12 *E. cloacae*) isolates from the pediatric surgery ward were examined. The main β-lactamase enzymes found were CTX-M-15 (23 isolates) and CTX-M-9 (four isolates). In addition OXY enzymes in *K. oxytoca* K1 strains were detected. CTX-M-9 emerged first and was then replaced with the CTX-M-15 enzyme (Table 14). It is speculated by Peirano et al. that the successful spread of *E. coli* producing CTX-M-15 is due to the following mechanisms: the spread of an epidemic clone (such as ST131) with selective advantages (such as multiple antibiotic resistance and enhanced virulence factors) between different hospitals, long-term care facilities or wards and the community, and the horizontal transfer of plasmids or genes that carry CTX-M-15 alleles [112].

Table 14. The time continuum of the ESBL isolates found in the pediatric surgery ward. Due to the clonality of *E. cloacae* isolates they were all considered as CTX-M-15-positive.

Year	2004	2005	2006	2007							2008				
				Apr	May-Jun	Jul	Aug-Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
***E. coli* -main clone**						XX		X			X		XXXXX	X	X
E. coli	X	XXX											X		
***K. pneumoniae* -main clone**				X					X				X		
K. pneumoniae	X			X									X		
***K. oxytoca* K1 strains**		XXX	X												
K. oxytoca							X								
***E. cloacae* -main clone**							X		XXX		X	X	XXXXX		X

red: CTX-M-15, blue: CTX-M-9, black: no CTX-M found

Clearly one epidemic clone among the *E. coli*, *E. cloacae* and *K. pnemoniae* isolates was identified by rep-PCR (DiversiLab, DL, study II) in the outbreak. All isolates of the main clones were shown to harbor CTX-M-15 (due to the clonality of *E. cloacae* isolates they were all considered as CTX-M-15-positive). In addition, the epidemic *E. cloacae* strain was isolated from the shower of one patient room. These organisms can spread from one patient to another and may also colonize the environment in the hospital [324]. It seems that *E. cloacae* ST114 might be the most prevalent clone in the dissemination of CTX-M-15, but its dissemination has not been evidenced in one clone [325].

In comparison of the isolates typed with PFGE the main clones were PFGE type A (two subtypes being A1 in *E. coli* and *K. pneominiae*). The main DiversiLab clones embodied eleven of the sixteen *E. coli* isolates and all twelve detected *E. cloacae* isolates. In addition three of the four *K. pneumoniae* isolates with CTX-M-15 formed one clone.

With *Enterobacteriaceae* the discriminatory capabilities of DiversiLab and PFGE were comparable. The DiversiLab results were supported by PFGE, even though it was more discriminative. It is reported that DiversiLab may overestimate the genetic relatedness [249]. In this study the MLST types were not determined. It is also known that DiversiLab may be slightly more discriminative than MLST with *K. pneumoniae* and *E. coli* [251, 326]. The deficiency of this study was that plasmid analyses were not performed.

The majority of the ESBL isolates of this study represented two distinct populations of *E. coli* and *E. cloacae*. The main clones found with rep-PCR harbored the CTX-M-15 enzyme. The clonal dissemination of a distinct strain of either species has occurred in the ward. CTX-M-15 is the most universal type of ESBL among *E. coli* isolates and has been associated with the presence of clone ST131 [140, 327, 328]. The worldwide dissemination of *E. coli* harboring CTX-M enzymes has been very efficient and has involved health care settings, community, livestock, companion animals, wildlife and the environment [329]. Also rapid dissemination of CTX-M-genes in commensal *E. coli* isolates in healthy children [330] and within households [331] has been described. The DiversiLab method could be a rapid option in outbreak investigations and it has the potential to indicate the occurrence of a clonal spread [252].

In conlusion, a clonal outbreak of *E. coli* and *E. cloacae* was detected by DiversiLab in one ward, and the patients with ESBL were cohorted in the ward at the end of March 2008. The ward underwent thorough cleaning and no new patients were admitted, until all the cohort patients were discharged or placed in an isolation ward. The control measures to inhibit the spread of ESBL-producing *Enterobacteriaceae* present a major challenge, because the epidemiology of ESBL-producing bacteria has become more complex with increasingly blurred boundaries between hospitals and the community [93]. In our case the DiversiLab method was found to be suitable for the rapid investigation of a local outbreak. In addition, the DiversiLab system has been proved to successfully identify the CTX-M-15 producing *E. coli* clone ST131 [248]. In the future the next generation sequencing has the potential to provide typing results and detect resistance genes in a single assay [332].

4.2.3 A. baumannii (Study I)

For *Acinetobacter* the DiversiLab system has been proved to be a suitable system for screening clonal spread [155, 247, 323, 333]. Among 55 *A. baumannii* isolates two main clonal groups were detected, one harboring OXA-23-like genes and the other harboring OXA-58 genes. A time-dependent clonal variation was clearly observed. The OXA-58 clone predominated and was later substituted by OXA-23 clone. This replacement has been reported since 2009 in many countries and has been explained by the selective advantage associated with the higher carbapenemase activity of OXA-23 and/or acquisition of carbapenemase resistance through horizontal gene transfer [160]. In this study OXA-58 isolates having lower MIC-values to meropenem and imipenem than OXA-23 isolates were found, as well. The insertion of the IS*Aba1* element located upstream of OXA-51 gene was not found. This junction provides promoter sequences that trigger the overproduction of carbapenemase, resulting in a high level of carbapenem resistance [334].

It has been approx. 20 years since the first description of European clonal lineages I and II [335] and 30 years since the first acquired OXA was found (161). The spread of acquired OXAs is well documented. In 2010 eight clonal lineages in *A. baumannii* (WW1-WW8) distributed worldwide were typed by Higgins et al. using DiversiLab [155]. The majority of the carbapenem-resistant *A. baumannii* from European countries belonged to EUI and EUII and harbored OXA-23, OXA-24/40 or OXA-58 genes. They found identical rep-PCR patterns associated with isolates from different continents harboring OXA-23 or OXA-58 genes, suggesting clonal spread of a resistant organism. Resistance determinants were not associated with a particular cluster; rather each cluster contained more than one resistance gene type. This suggests that carbapenem resistance has developed after or during the spread of the clonal lineages [155]. Variation within isolates harboring the OXA-23 gene identifying seven different clones among OXA-23 isolates has been detected by Cielinski et al. [247]. In our study only four isolates with the OXA-23 gene did not belong to the main clone, but instead six isolates without an acquired OXA gene were clustered in the main clone. Higgins et al. have also used DiversiLab in studing the clonality of other *A. baumannii* complex members (*A. pittii* and *A. nosocomialis*) and noticed that these do not apper to be as widespread and are less clonal in nature than *A. baumannii*. With *A. baumannii* they also found good correlation between MLST and rep-PCR clusters [333].

The current worldwide dissemination of the OXA-23 gene is driven by more than seven MLST types (most common STs were ST22/ST2) associated with different genetic structures and plasmids. A single clone can have a different genetic structure at the origin of the *bla*OXA-23 acquisition. The OXA-23 gene associated with composite transposon Tn2006 can be located in the chromosome or in a plasmid [285]. Complex and dynamic spreading of OXA-23 will be difficult to control because this spread is not associated with a single entity.

Controlling an endemic *A. baumannii* infection in hospitals is very difficult. *Acinetobacter* is widely present in different ecological niches. The ubiquitous presence of *A. baumannii* in nature and consequent isolation from water, animal, and soil samples may have led to its significance being underestimated [336]. The existing approaches consist of environmental disinfection involving the use of potent products together with an intensive educational program that promotes hand hygiene and contact isolation into cohorts and even selective decontamination [337]. The clonal spread of OXA-23-producing *A. baumannii* has led to an increased need for appropriate

infection control measures that can prevent further spread of these multidrug-resistant organisms [338, 339]. The suggestion is that the DiversiLab method might be a useful tool for rapid epidemiological screening of *A. baumannii* and help to prevent the clonal spread. In the future large-scale sequencing, more closely NGS, and genotyping of *A. baumannii* strains provides a comprehensive genetic data that is useful in molecular epidemiology [340].

4.2.4 C. difficile (Study IV)

C. difficile rep-PCR fingerprints were performed for 181 clinical isolates by DiversiLab. In addition, isolates with large *tcdC*-deletions (n=24) were studied. The results of the clinical isolates were compared to PCR ribotyping, PFGE, virulence genes and *tcdC* gene sequencing (unpublished data) (Table 15). The most prevalent PCR ribotypes were 027 (64/181, 31%) and 001 (54/181, 26%). The rest of the isolates (63/181) represented a total of 29 different PCR ribotypes. PCR ribotypes 002, 014 and 020 were the most prevalent among them (25/63, 40 %) and over half of the PCR ribotypes 002, 014 and 020 isolates were grouped to a single clone with DiversiLab. It is known that ribotypes 014 and 020 are closely related, and in PCR ribotyping differ only by one band [341]. PCR ribotypes 014 and 002 were common among the clinical isolates in Europe at the time of the sudy by Barbut [342]. These types of isolates have not been reported to cause a severe disease. They are common in humans and are also present in water and various animals [343].

At the time of the study by Kotila et al [344], the PCR ribotype 027 was found to be the most prevalent PCR ribotype in Finland, followed by ribotypes 001, 002 and 014. Type 027 was usually the dominant PCR ribotype among the isolates from severe cases and outbreaks [344].

Using rep-PCR analysis, a total of 28 different profile groups were identified, but only six major rep-PCR groups could be detected (DL1-DL6). In 75% (135/181) of the isolates the classification obtained by rep-PCR and PCR ribotyping (including toxin hyperproducers PCR ribotypes 027, 001, 023) was comparable (Table 15). The correlation of rep-PCR, PCR ribotyping and PFGE was excellent with the two major groups (DL1 and DL2) of isolates, PCR ribotypes 027 and 001. Within other isolates, the grouping obtained by these three methods was more heterogeneous and there were inconsistencies in results between PCR ribotyping and rep-PCR. However, in some cases PFGE supported the rep-PCR clustering.

Table 15. Comparison of methods in studying the clonality of *Clostridium difficile*.

PCR ribotybe	No. of isolates	Rep-PCR[a] (DL[b])	PFGE	Virulence genes					Deletion	*tcdC*-type
				ToxA	ToxB1	ToxB2	BinA	BinB		
027	64	DL 1	NAP1, sub. 1 ja 2	+	-	+	+	+	18 bp	sc 1
001	54	DL 2 sub.1,2,3,4	Unique 3 and unique 3 sub. 1	+	+	+	-	-	-	sc 3
023	4	DL 5	Unique 14 sub. 1 ja 2	+	+	+	+	+	54 bp	A_{var}
078	2	DL 9	Unique 11 sub. 1 and unique 13 sub. 2	+	+	+	+	+	39 bp	A
005	2	DL 18, DL 28	Unique 5 sub. 2	+	+	+	-	-	-	sc 3
unknown 3	1	DL 15	Unique 22 sub. 1	+	+	+	-	-	-	wt[e]
020	8	DL 3, DL 6, DL 11	Unique 1, unique 1 sub. 4 and 5	+	+	+	+/-	+/-	-	wt[e]
002	6	DL 3, DL 4, DL 6, DL 25	Unique 21, unique 21 sub.1, 2 and 4	+	+	+	-	-	-	wt[e]
012	2	7	Unique 1 sub. 3 and nd[d]	+	+	+	-	-	-	wt[e]
017	1	DL 17	nd[d]	+	+	+	-	-	-	sc 7
unknown 10	1	DL 3	nd[d]	+	+	+	-	-	-	wt[e]
unknown 11	1	DL 3	nd[d]	+	+	+	-	-	-	wt[e]
014	11	DL 3, DL 6, DL 11, DL 12, DL 21, DL 22	Unique 1, unique 1 sub. 2, unique 15, unique 15 sub. 3 and nd[d]	+	+	+	-	-	-	wt[e] and one sc 3
056	2	DL 4, DL 8	Unique 15 sub. 4 and nd[d]	+	+	+	-	-	-	wt[e] and sc5
003	1	DL 4	Unique 7	+	+	+	-	-	-	wt[e]
018	3	DL 3, DL 8	Unique 6 and unique 6 sub.1	+	+	+	-	-	-	sc 15
unknown 19	1	DL 19	nd[d]	+	+	+	-	-	-	wt[e]
010	1	DL 4	Unique 3	+	+	+	-	-	-	nd[d]
unknown 22	2	DL 10	Unique 5 sub. 1 and 3	+	+	+	-	-	-	wt[e]
029	1	DL 26	Unique 5 sub. 3	+	+	+	-	-	-	wt[e]
unknown 30	1	DL 20	Unique 29	+	-	+	+	+	18 bp	sc 2
070	2	DL 4, DL 13	Unique 7	+	+	+	-	-	-	wt[e]
011	1	DL 23	Unique 1 sub. 1	+	+	+	-	-	-	sc 15
unknown 44	1	DL 1	NAP1 sub. 1	+	-	+	+	+	18 bp	sc 1
unknown 52	2	DL 4, DL 7	Unique 28 and unique1 sub. 4	+	+	+	-	-	18 bp / -	sc 15 + del18 and Sc3
unknown 53	1	DL 27	Unique 3	+	+	+	-	-	-	sc 3
unknown 55	1	DL 5	Unique 14 sub. 1	+	+	+	-	-	-	A_{var}
unknown 63	1	DL 3	Unique 13 sub. 3	+	+	+	-	-	-	sc 15
unknown 64	1	DL 14	Unique 1	+	+	+	-	-	-	sc 15
unknown 66	1	DL 24	Unique 1 sub. 4	+	+	+	-	-	-	wt[e]
unknown 68	1	DL 7	Unique 18 sub. 1	+	+	+	-	-	-	sc 15
Total	**181**									

[a]Repetetive PCR, [b]DiversiLab, [c]Pulsed-field electrophoresis, [d]Not detected, [e]Wild type

One clone was clearly detected within PCR ribotype 027 by rep-PCR. Contrary to the results of this study, subgroups among the PCR ribotype 027 have been found (Healy et al. 19th European Congress of Clinical Microbiology and Infectious Diseases, abstract P1731) [264, 292, 345]. In these studies four different subgroups have been identified among PCR ribotype 027 when comparing rep-PCR to PCR ribotyping and PFGE. Heterogeneity within PCR ribotype 027 has been reported by MLVA as well [345]. MLVA has been used to discriminate between isolates with identical PCR ribotypes belonging to types 001, 017 and 027 [346]. This heterogeneity of PCR ribotype 027 may be due to actual differences between the strains circulating in the different geographic areas [303]. PCR ribotype 027 had recently emerged in Finland at the time, the first PCR ribotype 027 having been identified in 2007 [193]. The isolates in Finland were most likely clonal because the variation over time had not yet evolved. Today it is known that PCR ribotype 027 is genetically variable and it appears that the recently identified PCR ribotypes 019, 176, 244 and 198 [293], and in addition PCR ribotypes 016, v046, 080 [347], have evolved from the 027 lineage. These are closely related outliers to hypervirulent 027 clones that can be misclassified as 027. It is important to develop more accurate methods to distinguish between these highly virulent strains. Understanding the evolution of clones such as the PCR ribotype 027 will be important in predicting the early emergence (or disappearance) of highly virulent *C. difficile* strains [292].

When all of the 181 clinical isolates were compared to each other, the most prevalent PCR ribotypes (027 and 001) clustered in their own rep-PCR groups and both had a typical well identifiable electropherogram. Interestingly, when comparing only PCR ribotypes 001 to each other, four rep-PCR subgroups and one outlier could be detected. It proved that sometimes DiversiLab is more discriminatory than PCR ribotyping. This phenomenon within PCR ribotype 001 has also been reported by other reseachers [348, 349].

In this study the two other potentially hypervirulent toxin hyperproducers (PCR ribotypes 023 and 078) were also found to cluster in their own rep-PCR groups when all the consecutive 181 clinical isolates were compared to each other. Within the large deletions more specific investigation was performed. Among *tcdC-A* (39 bp deletion, classification as in Curry *et al.* [179] three different PCR ribotypes, 045, 078 and 126 were detected and results with DiversiLab were highly heterogenic. Genetic diversity among PCR ribotype 078 has been shown, but PCR ribotypes 078, 126, 033 and 045 have proved to be highly related in MLST [350]. The variation is suggested to be due to multiple sources of this ribotype [187, 264]. With these isolates, PFGE supported neither PCR ribotyping nor rep-PCR but led to a third delineation of isolates. Among *tcdC-A* variant (54 bp deletion, classification in this study) all but one of the isolates were of PCR ribotype 023 and with DiversiLab one main clone harboring 11 out of 14 isolates was detected. In PFGE these *tcdC-A* variants represented one PFGE type and its three subtypes.

TcdC gene sc-classification for all the 181 clinical isolates was determinated according to Curry et al. [179]. Most prevalent *tcdC* genotypes were *tcdC-sc1* (65/181) and *tcdC-sc3* (59/181) (Table 11). *TcdC-sc1* genotypes contain 18 bp deletion at nucleotides 330 to 347 and were all but one typed as PCR ribotype 027. This one exception was an unknown 44 which was typed as NAP1 with PFGE and had the same DiversiLab type, DL1 as PCR ribotype 027. In addition, one genotype of *tcdC-sc2*, which is identical to genotype *tcdC-sc1* except for the insertion of a single nucleotide at position 212 was found. Two genotypes of the *tcdC-A* (39 bp deletion) and five genotypes of the *tcdC-A* variant (54 bp deletion) were found. In addition to wildtypes, one

genotype of *tcdC-sc5*, one genotype of *tcdC-sc7* and six genotypes of *tcdC-sc15* t were found. It is known today that *tcdC* deletions may not be the sole factor responsible for the increased toxin production observed. It could be predicted that toxin production could be amplified as the result of a missense or nonsense *tcdC* mutation. Recent studies, however, have failed to confirm such effects in the settings of deletions of larger gene sequences [182]. The presence of a binary toxin gene and *tcdC* SNPs at position 184 and 117 has proved to strongly predict the recurrence of CDI [351].

Several typing methods have been used for *C. difficile*. It has been shown that MLVA, REA, *slp*AST (surface layer protein A gene sequence typing) and PFGE are more discriminative than PCR ribotyping, MLST and AFLP [197]. The rapid clonal spread of the hypervirulent PCR ribotype 027 strain has brought along the need for rapid detection. At the time of this study PCR ribotype 027 strains were clearly separated to one cluster using rep-PCR. Thus, the rep-PCR method may be adequate for the investigation of a local outbreak by rapid screening of the hypervirulent PCR ribotype 027 strains. It is a useful tool for local clinical laboratories in monitoring the spread of *C. difficile* for instance in hospital wards, but all the analyses should be performed in one laboratory. *C. difficile* has a highly mobile, mosaic genome, which may have an effect on the results and DNA degradation, typical for *C. difficile*, interfering with the analysis [307]. None of these three methods (DiversiLab, PCR ribotyping and PFGE) were unanimously more discriminative than the other two, and the results revealed discrepancies.

PCR ribotyping as a current European reference typing technique relies more on the comparative ease of the technique and repeatability than on the discriminatory ability. PCR ribotyping can be performed in two days for a set of 17 samples [352]. More recent studies have begun to recognize the comparative lack of discrimination of this technique and are favoring more discriminatory techniques, such as PFGE and MLVA, for differentiating isolates having identical PCR ribotype profiles [197, 226]. Even if PFGE is discriminatory, it has a very low throughput due to the length of the procedure (taking four to eight days to complete) in comparison with PCR-based genotyping methods [232]. PFGE is claimed to be labor-intensive and it delivers optimal results when performed by a technician with extensive experience using this method. In conclusion, it is advisable to use multiple methods for typing *C. difficile*. The whole-genome sequencing (WGS) has significantly furthered the knowledge of the genetic diversity, evolution, epidemiology, and pathogenicity of this once enigmatic pathogen. The numbers of sequenced and analyzed partial genomes of *C. difficile* have increased rapidly [353].

5. CONCLUDING REMARKS AND FUTURE PROSPECTS

5.1 Summary

Rapid detection in clinical laboratories is essential for the prompt recognition of antimicrobial-resistant organisms. Infection-control practitioners and clinicians need the clinical laboratory to rapidly identify and characterize different types of resistant bacteria to efficiently control the spread of these bacteria and to help select more appropriate antibiotics.

There are several problems for a clinical laboratory wishing to adopt a rapid, reliable and sensitive PCR-method to solve, such as opening hours of the laboratory including logistics of the samples, costs, and equipments needed. The aim of the current study was to develop new multiplex PCR methods for the precise characterization of hospital-acquired bacteria and to compare automated rep-PCR to other typing methods in a compact set of consecutive samples or retrospectively collected strains without the associated epidemiological data. Epidemics cause additional costs in health care. Microbiological screening plays a major role in these costs. When molecular biological techniques are applied, the analytical costs decrease.

A rapid negative or positive screening result of hospital-acquired bacteria can be valuable when the colonization status of a patient is needed. For example, on admission to an intensive care unit possible carriers should be detected as soon as possible. Also rapid reporting that the patient is not a carrier is of great importance. The responsibility of the laboratory is to avoid unnecessary and costly screening methods and to aim at achieving reliable and sensitive methods of detection of notorious hospital-acquired bacteria.

Many of the molecular techniques currently used for typing still rely on electrophoretic separation of DNA fragments of different molecular lengths. The electrophoretic result is represented by a pattern of bands on a gel. Since these patterns may be extremely complex, the ease with which the patterns are interpreted and related is an important factor in evaluating the utility of a particular typing method. As ease of use is important, the technical difficulty, cost, and time to obtain a result must be evaluated in assessing the utility of a particular typing method.

In this study the samples were collected mainly from the district of Helsinki and Uusimaa, during a limited time frame. At the time of the study the first fatal hypervirulent *C. difficile* strain (PCR ribotype 027) and the first CPE strains emerged in Finland. Regarding HAIs in general, however, the situation in Finland is quite good, except for *C. difficile* where the success in controlling the disease has varied between regions. Three different usable multiplex PCRs for rapid identification of bacteria causing outbreaks were established and each of them is still in daily use in routine diagnostics. We succeeded in performing a reliable outbreak analysis at hospital level with methods used in this study. The semi-automated repetitive PCR based typing method was found to be beneficial in the first-line outbreak analysis at local level. The utility of DiversiLab was more obvious with ESBLs and *A. baumannii* than with MRSA and *C. difficile*. The usefulness of the rep-PCR with isolates collected from a larger geographical area, or during a longer period of time remains yet to be studied. Differences in specific bacterial properties and the levels of discrimination required by a particular bacterial species must be taken into account

when selecting the most applicable typing technique. In conclusion, the gold standard methods are still needed in global epidemiology.

The automated rep-PCR system proved to be an easy-to-use, reproducible, fast and discriminative tool. In outbreak situations it is of vital importance that the screening method does not fail to identify related epidemic isolates. The automated rep-PCR system was found to be reliable in this respect. We thus suggest that it may be useful in the rapid analysis of outbreaks caused by ESBL producing *Enterobacteriaceae* and *A. baumannii*.

5.2 Future prospects

Fast and accurate identification and typing of pathogens is essential for effective surveillance and detection of outbreak. The current routine procedure is based on a variety of techniques, making it laborious, time-consuming and expensive. In this study we concluded that the costs of the DiversiLab method are quite high, thus limiting its use. The future will show whether high throughput sequencing methods (next generation sequencing or massive parallel sequencing) will replace the classic DNA-based typing methods. Whole genome sequencing might be the solution which has huge potential in both diagnostic and routine surveillance.

NGS has many advantages over other existing molecular approaches, including throughput, higher resolution, better efficacy, cost efficiency, flexibility, scalability and less hands-on-time. Thus it may potentially replace a multitude of assays currently used simultaneously in a diagnostic microbiology laboratory. It has been demonstrated that SNPs mined from whole genome sequence (WGS) data as well as gene-by-gene (MLST) comparisons provided greater resolution for the detection of an outbreak and for the tracking down of microbial strains for a wide range of bacterial pathogens than current gold standard methods. Additionally, the growth of public databases harboring reference genomes continues to enhance the utility of NGS in public health and in clinical practice [354]. There are still some methods not easily replaceable, such as PFGE, methods based on repetitive sequence, serotyping within certain species, and expressed microbial drug resistance.

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) has become a widely used technique in routine clinical laboratories for bacterial identification. It has replaced previous techniques, but a lot of work remains to be done before it can be used for the detection of, for example, antibiotic resistance in the clinic.

A challenge for future research is to sequence the strain settings studied comprehensively in this thesis using NGS techniques. We aim to start with ESBL producing *Enterobacteriaceae* and compare the sequence results with typing results received in this study. This study, however, suggests the need for new gold standard approaches for the typing of molecular epidemiological strains.

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