Use of core genome MLST for infection prevention and control of *Enterococcus faecium* at a Danish university hospital

PhD thesis

Sanne Grønvall Kjær Hansen, MD

Research Unit of Clinical Microbiology
Department of Clinical Research
Faculty of Health Sciences
University of Southern Denmark

January 2024

PhD thesis

Use of core genome MLST for infection prevention and control of *Enterococcus faecium* at a Danish university hospital

Author

Sanne Grønvall Kjær Hansen, MD
Department and Research Unit of Clinical Microbiology, Odense University Hospital
University of Southern Denmark, Odense, Denmark; and
Department of Bacteria, Parasites and Fungi,
Statens Serum Institut, Copenhagen, Denmark

Supervisors

Marianne Nielsine Skov, DVM, PhD, MPQM
Head of the division for Molecular Biology in the Department of Clinical Microbiology
Department and Research Unit of Clinical Microbiology, Odense University Hospital
University of Southern Denmark, Odense, Denmark

Anette Marie Kühle Hammerum, MSc, PhD
Senior Scientist
National Reference Laboratory for Antimicrobial Resistance
Department of Bacteria, Parasites and Fungi
Statens Serum Institut, Copenhagen, Denmark

Anette Holm, MD, PhD
Consultant and head of the Department of Clinical Microbiology
Department and Research Unit of Clinical Microbiology, Odense University Hospital
University of Southern Denmark, Odense, Denmark

Assessment committee

Chairman
John Coia, MD, Professor Emeritus in Clinical Microbiology
Institute of Regional Health Research (IRS)
University of Southern Denmark

Kristin Hegstad, Professor, PhD
Senior-researcher and part-time Professor
DCM and IPC Hospital of Northern Norway

Henrik Pierre Calum, MD, PhD
Consultant and External Clinical Associate Professor
Department and of Clinical Microbiology
Hvidovre Hospital, Denmark
University of Copenhagen (CU)

Table of contents

Preface

This PhD Thesis is structured as a review, based on the results and publications of three sub-projects.
The scientific work was mainly conducted at the Department and Research Unit of Clinical Microbiology at Odense University Hospital (OUH) and the Department of Bacteria, Parasites, and Fungi at Statens Serum Institut (SSI), Denmark.
The three other Departments of Clinical Microbiology (DCMs) in the Region of Southern Denmark have assisted in providing data.

The work was conducted over a four-year period from February 2020 until January 2024, instead of the scheduled three years. This was due to the COVID-19 pandemic, which required my assistance in the Infection Prevention and Control (IPC) Unit at OUH in 2020 and 2021.

The idea for this thesis took form in 2017, when a rise in vancomycin-resistant *E. faecium* in clinical samples was seen at OUH. At that time, sequencing data in the DCMs was mainly used for research purposes and was almost absent in the daily work with IPC. When whole-genome sequencing (WGS) and single nucleotide polymorphism (SNP)-analysis were used in outbreak control, data were provided with a delay and were difficult to interpret for unskilled users. However, in 2017, an easy-to-use interpretation method of sequencing results, core genome multilocus sequence typing (cgMLST) had recently been invented and used in research, although only sparsely if at all in a real-time setting. It was therefore of interest to investigate if this typing system could be a solution for enhancing IPC in daily work. Furthermore, attempts were made to develop the identification system matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for rapid and low-price bacterial typing. It was therefore an original goal of this PhD to investigate the usability of MALDI-TOF MS along with cgMLST in IPC.
However, in 2020, the COVID-pandemic became a game changer for my project. Due to COVID-19, the staff in the laboratory were allocated to other functions, time was limited, and I paused working on the PhD to help in the hospital.

When I returned to the PhD study, many of my colleagues were still busy controlling COVID-19 and without sufficient resources to be involved in new projects, including my PhD study. The Danish hospitals were still in daily need of isolation rooms for COVID-19 infected patients, and at the same time, our hospital used many resources on screening and isolation to prevent the transmission of vancomycin-resistant *E. faecium* (VREfm). In this scenario, and since the clinical staff observed very few patients with VREfm infections, the IPC unit began to question the purpose of the practice of isolating patients with VREfm. In addition, a growing number of studies revealed risks associated with such isolation. Consequently, the IPC unit decided that an investigation of the VREfm practice was needed.

To facilitate this need with a scientific approach, I decided to modify the aim of my PhD and investigate the role of *E. faecium* in IPC. I wanted to investigate vancomycin-susceptible-, vancomycin-resistant-, and vancomycin-variable *E. faecium* (VSEfm/VREfm/VVEfm) to gain insights into the epidemiology and transmission, by using the experience with cgMLST obtained in my first study in this PhD. Furthermore, I wanted to investigate the impact of ending screening and isolation regimes regarding VREfm and VVEfm.

I hope you will enjoy reading this thesis, and that the results will lead to discussions of your procedures in your hospitals.

Financial support

This PhD thesis was financially supported by the following institutions and funds:
The University of Southern Denmark, SDU
The Region of Southern Denmark, RSD
The Department and Research Unit of Clinical Microbiology, Odense University Hospital, Denmark
Statens Serum Institut, Denmark

Acknowledgements

I am truly grateful for the support from all my colleagues, friends, and family during the whole process in this thesis - it would not have been possible without them. In particular, I would like to thank:

Marianne N. Skov, for guiding and giving me freedom through the academic process, but also challenging the path I wanted to walk.

Anette M. Hammerum, for sharing her huge knowledge about enterococci, her sustained guiding in manuscript writing and the PhD processes, and for never giving up on me and the project, even though she had to struggle with illness and periods of absent herself.

Anette Holm, for her support, sparring, and never-ending calm in any situation of the academic process.

Henrik Hasman, for sharing his knowledge about sequencing data interpretation and EpiLinx, and especially for stepping in when Anette M. Hammerum was absent.

Louise Roer and Kasper Thystrup Karstensen, for their priceless help in constructing and evaluating sequencing data and their patience in teaching me these skills.

Flemming Rosenvinge, for sharing his knowledge on microbiologic data interpretation, and giving advice in any clinical matter.

Elisa Knudsen, the essential Laboratory technician, a second mother to all the persons in the research laboratory at the DCM, OUH and always available with a smile.

Michael Kemp, for introducing me to the sequencing world of clinical microbiology and the possible use of the methods in IPC.

My colleagues at the DCM, OUH for sharing their PhD experiences with me and being cheerful.

My colleagues at the other DCMs in the Region of Southern Denmark for always being ready to cooperate and sparing time for me and the project.

And last but not at least my very dear and beloved family for their endless support and encouragement. My parents, sister, and husband always ready to challenge and discuss my academic questions and findings, giving advice in use of the British language, listening to my complaints, and keep me going.

My dear children, Signe, and Nanna,
Thank you so much for your little messages and drawings on my whiteboard, they have pleased and encouraged me every single day I have been sitting in the home office.
Thank you, Signe, for all your care, which you are a master at sensing the need for despite your young age and thank you Nanna for letting me use your beautiful drawings and photos for presentations of my results out in the world.

List of papers/manuscripts and posters

Paper I, II and III have been accepted by the Journals for parallel publication in this thesis.

I. **Paper I**
Sanne Kjær Hansen, Lise Andersen, Mette Detlefsen, Anette Holm, Louise Roer, Panagiotis Antoniadis, Marianne Nielsine Skov, Anette M. Hammerum, Michael Kemp; 'Using core genome multilocus sequence typing (cgMLST) for vancomycin-resistant *Enterococcus faecium* isolates to guide infection control interventions and end an outbreak'. J Glob Antimicrob Resist. 2021 [1].

II. **Paper II**
Sanne Groenvall Kjaer Hansen, Louise Roer, Kasper Thystrup Karstensen, Silje Vermedal Hoegh, Frank Hansen, Kasper Klein, Flemming S. Rosenvinge, Anette Holm, Marianne N. Skov, Anette M. Hammerum, and Henrik Hasman; 'Vancomycin-sensitive *Enterococcus faecium* bacteraemia – hospital transmission and mortality in a Danish University Hospital'. Journal of Medical Microbiology, 2023 [2].

III. **Manuscript III (referred to as Paper III)**
Sanne Groenvall Kjaer Hansen, Kasper Klein, Anita Nymark, Lise Andersen, Kim Oren Gradel, Joanna Lis-Toender, Claus Oestergaard, Ming Chen, Raluca Datcu, Marianne N. Skov, Anette Holm, Flemming S. Rosenvinge; 'Vancomycin-resistant *Enterococcus faecium* – impact of ending screening and isolation in a Danish University hospital'. Submitted for publishing, 2023 November [3].

Posters

I. "Core genome MLST reveals a more differentiated transmission than MLST in a rise of vancomycin resistant *Enterococcus faecium* in a University Hospital"
Healthcare infection society conference (HIS) 2018, Liverpool, United Kingdom, 26/11/2018, 2018-11-26 | Conference poster | *Author*
Contributors: **Sanne Kjær Hansen**; Lise Andersen; Mette Detlefsen; Anette Holm; Marianne Nielsine Skov; Panagiotis Antoniadis; Michael Kemp
URL: https://portal.findresearcher.sdu.dk/en/publications/f0a66e5d-7bea-4dd1-80bd-3df4d1519357

II. "To die or not to die from *Enterococcus faecium* bacteraemia"
33rd ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, København, Denmark, 15/04/2023, 2023-04 | Conference abstract | *Author*
SOURCE-WORK-ID: f64f9286-7362-404a-a9bf-f67977fc5423
CONTRIBUTORS: **Sanne Kjær Hansen**; Kasper Klein; Flemming Rosenvinge; Marianne Nielsine Skov; Anette Holm

Abbreviations

AFLP	Amplified fragment length polymorphism
AICC	Antibiotics and Infection Control Committee
APACHE	Acute Physiology and Chronic Health Evaluation
AREfm	Ampicillin-resistant *Enterococcus faecium*
ASEfm	Ampicillin-susceptible *Enterococcus faecium*
BAP	Bacterial Analysis Pipeline
BAPS	Bayesian Analysis of Population Structure
Bp	Base pairs
CAS	the National Center for Hospital Hygiene (Den Centrale Afdeling for Sygehushygiejne)
CC	Clonal complex
CDC	Communicable Disease Center/Centers for Disease Control and Prevention
CEI	the National Center for Infection Control (Central Enhed for Infektionshygiejne, CEI)
CEO	Chief Executive Officers
CGE	Center for Genomic Epidemiology
cgMLST	core genome MLST
CPO	Carbapenemase-producing organisms
CPE	Carbapenemase-producing Enterobacteriaceae
CT	Complex type/clonal type
DANMAP	the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
DCM	Department of Clinical Microbiology
DTU	the Technical University of Denmark
eBURST	Electronic version of the clustering algorithm 'based upon related sequence types'
ECDC	European Centre for Disease Prevention and Control
EAS	Electronically Assisted Surveillance
ESGEM	European Society for Clinical Microbiology and Infectious Diseases
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FES	Fully automated Electronic Surveillance
GIT	Gastrointestinal tract
HAI	Hospital-acquired infection/healthcare-associated infection
HAB	Hospital-adapted bacteria
HAIBA	Healthcare Associated Infections Database
ICU	Intensive Care Unit
IPC	Infection prevention and control
MADS	Mikrobiologisk Afdelings DataSystem
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MDRO	Multidrug resistant organisms
MGE	Mobile genetic elements
MiBa	The Danish Microbiology DataBase/Den danske mikrobiologidatabase
MLE	Maximum likelihood estimation
MLST	Multilocus sequence typing
mRNA	Messenger RNA

MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MSTree	Minimum spanning tree
NDTree	Nucleotide difference tree
NGS	Next-generation sequencing
NIR	Nationale Infektionshygiejniske Retningslinjer/National IPC Guidelines
NJTree	Neighbor-joining tree
NytOUH	The future OUH which is under construction
OLC	Overlap Layout Consensus
ONT	Oxford Nanopore Technologies (ONT)
OUH	Odense Universitetshospital/Odense University Hospital
PacBio	Pacific Bioscience SMRT technology
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PPE	Personal protective equipment
qPCR	Quantitative PCR
rMLST	Ribosomal MLST
RSD	Region of Southern Denmark
SDU	University of Southern Denmark (Syddansk Universitet)
SEI	Sundhedssektorerhvervede infektioner
SKA	Split k-mer analysis
SLC	Single-linkage clustering
SMRT	Single-molecule real-time
SNP	Single nucleotide polymorphism
SSI	Statens Serum Institut
ST	Sequence type
TBP	Transmission-based precautions
TM	Translational Medicine
UPGMA	Unweighted pair group method with arithmetic mean
UTI	Urinary tract infection
VRE	Vancomycin-resistant enterococci
VREfm	Vancomycin-resistant *Enterococcus faecium*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
VSEfm	Vancomycin-susceptible *Enterococcus faecium*
VVEfm	Vancomycin-variable *Enterococcus faecium*
wgMLST	Whole-genome MLST
WGS	Whole-genome sequencing
WHO	World Health Organization

Summary in English

During the past decade, the number of multidrug-resistant organisms (MDRO) have increased worldwide, and especially the vancomycin-resistant *Enterococcus faecium* (VREfm) has entered the stage.

E. faecium is a part of the microbiota in the gut, causing a variety of infections, and associated with a high 30-day mortality – even higher than methicillin-resistant *Staphylococcus aureus* (MRSA). The microorganism is intrinsically resistant to many antimicrobials and acquires easily new resistance. Due to few treatment options, high mortality, and widespread transmission, many resources including the use of screening and isolation regimes in hospitals are used to prevent VREfm from spreading.

VREfm was detected in Denmark from the beginning of the 2000s. From 2012-13, a spread on a large scale in the Danish hospitals began, with most of the VREfm containing the resistance gene complex *vanA*. In 2015, vancomycin-variable *E. faecium* (VVEfm) was introduced in Denmark. The VVEfm also contains the *vanA* gene complex, but has a deletion in the *van*X gene, making it phenotypical vancomycin susceptible and difficult to detect by traditional antimicrobial susceptibility tests. The VVE clone ST1421-CT1134 *vanA E. faecium* spread in the entire country during the following years and almost outnumbered VREfm. From 2019 onwards, there has been an incipient shift in *van*-gene occurrence with a steady increase in VREfm containing the *vanB* complex.

In the same period as the VREfm incidence increased, surveillance and outbreak investigation requested typing methods with a higher discriminatory power, reduced turnaround time, and an internationally standardized nomenclature allowing for comparison of results within and between laboratories. Furthermore, the typing methods had to be applicable on different bacterial species. Several bacterial whole-genome sequencing (WGS) based typing systems fulfilling most of these requests were developed. Due to a simultaneous reduction in costs, the use of WGS and the WGS-based typing systems became an option for clinical laboratories. Some of the bacterial WGS-based typing systems available were pulsed-field gel electrophoresis (PFGE), single nucleotide polymorphism (SNP), multilocus sequence typing (MLST), and core genome MLST (cgMLST). Until 2017, cgMLST in Denmark had mostly been used for outbreak investigations and as a retrospective research tool at the national reference laboratory, Statens Serum Institut. However, cgMLST was marketed as an easy-to-use system that could be used with a small degree of molecular biology training. Furthermore, cgMLST was introduced to increase the specificity of the phylogenetic relationship between isolates compared to MLST combined with PFGE, and with an easier interpretation of data than when using SNP or PFGE.

Until mid-2016, VREfm was detected sporadically at Odense University Hospital (OUH), Denmark. From 2017, an increasing number of VREfm cases were detected at OUH, and MLST and cgMLST were introduced to help track transmission. In 2018, VVEfm emerged and increased rapidly.

The introduction of VREfm and VVEfm left the vancomycin-susceptible *E. faecium* (VSEfm) strains almost unnoticed in this period. However, VSEfm is still interesting to investigate as it is the origin

of the resistant counterpart, and as the transmission mechanisms are the same regardless of resistance. In the same period, there was a widespread use of screening and isolation against VREfm and VVEfm at OUH without the efforts being evaluated.

The specific aims of this PhD were:

- to investigate if cgMLST could be used in real time for IPC of VREfm transmission (Paper I)
- to investigate for unrecognised transmission of VSEfm by use of cgMLST (Paper II)
- to investigate if cgMLST data of VSEfm could be used to predict VREfm occurrence (Paper II)
- to investigate the clinical relevance of VSEfm and VREfm/VVEfm (Paper II and Paper III)
- to investigate the impact of ending screening and isolation of VREfm/VVEfm patients at a Danish university hospital (Paper III)

In Study I, 38 patients with a clinical sample containing VREfm and suspected to belong to an on-going outbreak at OUH, in the period January 2014-June 2017, were included. WGS with subsequent MLST and cgMLST was performed at the Department of Clinical Microbiology at OUH. Data revealed that 13 of the VREfm isolates were related and belonged to a cluster of ST80-CT993 *vanA E. faecium*. Due to the detailed interpretation of the cgMLST data together with use of epidemiology data, departments with need for infection prevention and control (IPC) interventions were reduced from several to one. IPC precautions were initiated at that one department and ended the outbreak (Paper I).

In Study II, MLST and cgMLST analysis were performed on *E. faecium* isolates from blood cultures from patients at OUH. In total, 630 VSEfm and 27 VREfm isolates from the period 2015 to 2019 were included.
The medical records of VSEfm patients were investigated regarding different clinical parameters including mortality.
The sequence types (STs) and complex/clonal types (CTs) of VSEfm were changing and diverse, belonging to 42 different STs and 131 CTs in several clusters. A widespread transmission of VSEfm belonging to multiple CTs in the hospital were detected, indicating the presence of transmission risk factors.
No connection between the VSEfm and VREfm clones in number or types (ST-CT) were detected.
By investigating the mortality, we identified a discrepancy between the 30-day mortality and the cause of death. In most of the cases, the patients died from their severe underlying diseases and not from the VSEfm bacteraemia in itself (Paper II).

Based on a preliminary quality survey of patients at OUH with VREfm infections and the related mortality, the VREfm/VVEfm screening and isolation practice were ended at the hospital by the end of 2021.
Study III was conducted to investigate the impact of this cessation. It was conducted as a retrospective cohort study of all patients with a first time VREfm/VVEfm clinical isolate (index

isolate) detected at OUH in the period 2015-2022. A total of 436 patients were included: 285 in the intervention period (2015-2021) and 151 in the post-intervention period (2022).
From the intervention to the post-intervention period there was a significant change in the *van*-gene distribution, but no differences in the investigated patient characteristics in relation to each of the *van*-genes. After ending screening and isolation, an increased number of index VREfm/VVEfm isolates was found, but nothing else to support a reintroduction of screening and isolation. As for VSEfm, we found that 30-day mortality did not reflect whether death was attributable to VREfm/VVEfm (Paper III).

This PhD has made us aware of the possibilities of use of WGS and cgMLST in real time in our daily work with IPC. The actual process of extracting sequencing data and ensuring good quality requires insight into molecular biology. The cgMLST data are easy to convey, but to avoid misinterpretation when comparing isolates, it is important to be aware of the choices and possible limitations in the underlying algorithms. Furthermore, sequencing data without a simultaneous use of epidemiological data have a limited application in IPC.
This PhD has shown that there is a need to resume surveillance of transmission of the less resistant bacteria. Perhaps this can pave the way for better prevention of transmission of the more resistant bacteria rather than extinguishing fires once the problem has arisen. Furthermore, the present data suggest that screening and isolation for VREfm/VVEfm can be ended in a low incidence setting. However, the development in VREfm/VVEfm incidence at OUH will be closely followed in the coming years, and hopefully data from other low-incidence environments will help shed light on the area.

Dansk resumé (summary in Danish)

I løbet af det seneste årti er antallet af multiresistente mikroorganismer (MDRO) steget på verdensplan – her i blandt vancomycin-resistente *Enterococcus faecium* (VREfm).
E. faecium er en del af den humane mikrobiota i tarmen. Den kan forårsage en række forskellige infektioner, hvoraf bakteriæmi er forbundet med en høj 30-dages dødelighed – endda højere end ved bakteriæmi med methicillin-resistente *Staphylococcus aureus* (MRSA).
Mikroorganismen er naturlig resistent over for mange antimikrobielle stoffer og erhverver sig let ny resistens. På grund af få behandlingsmuligheder, en potentiel høj dødelighed og udbredt spredning, er der brugt mange ressourcer, herunder brug af screenings- og isolationsregimer på hospitalerne, til at forhindre VREfm spredning.
VREfm blev påvist i Danmark i begyndelsen af 00'erne. Fra 2012-13 begyndte en spredning i større skala på de danske sygehuse, hvor de fleste isolater af VREfm indeholdt *van*A-genkomplekset.
I 2015 blev vancomycin-variabel *E. faecium* (VVEfm) introduceret i Danmark. VVEfm indeholder også *vanA*-genkomplekset, men har en deletion i det såkaldte *vanX*-gen. Dette gør, at VVEfm er fænotypisk følsom men resistent ved behandling, hvilket er vanskeligt at påvise ved brug af de traditionelle resistensbestemmelsesmetoder.
I løbet af de efterfølgende år spredte VVE-klonen ST1421-CT1134 *vanA E. faecium* sig i hele landet og udkonkurrerede næsten VREfm. Fra 2019 og frem er der sket et begyndende skifte i *van*-gen forekomsten med en støt stigning i VREfm indeholdende *vanB*-komplekset.
I samme periode blev typningsmetoder med en højere diskriminationsevne, en reduceret ekspeditionstid, en internationalt standardiseret nomenklatur med en anvendelighed inden for og mellem laboratorier, samt en mulig anvendelighed på forskellige bakteriearter, efterspurgt til overvågnings- og udbrudsundersøgelser.
Der blev udviklet flere helgenomsekventerings- (WGS) baserede typningssystemer, opfyldende de fleste af disse ønsker. På grund af en samtidig reduktion i omkostningerne blev brugen af WGS og de WGS-baserede typningssystemer en mulighed i flere af de lokale danske mikrobiologiske afdelinger.
Nogle af de bakterielle WGS-baserede typningssystemer der blev til rådighed var pulsed-field gel electrophoresis (PFGE), single nucleotide polymorphism (SNP), multilocus sequence typing (MLST) og core genome MLST (cgMLST).
I 2017 havde cgMLST næsten udelukkende været brugt i Danmark til udbrudsudredning og retrospektiv forskning på det nationale referencelaboratorium på Statens Serum Institut. cgMLST blev dog markedsført som et let anvendeligt system, der kunne bruges af personer med en lille grad af molekylærbiologisk uddannelse. Endvidere blev cgMLST introduceret som en metode, der havde en mere specifik angivelse af slægtskab mellem isolater end MLST kombineret med PFGE, og med resultater der var lettere at kommunikere ud end data var for SNP og PFGE.
Indtil midt-2016 blev VREfm kun sporadisk påvist på Odense Universitetshospital (OUH), Danmark. Fra 2017 blev der påvist et stigende antal VREfm tilfælde på OUH, og på det tidspunkt blev MLST

og cgMLST indført på OUH for at hjælpe med at spore mulig transmission. I 2018 dukkede VVEfm op og steg hurtigt i forekomst.
Introduktionen af VREfm og VVEfm efterlod de vancomycin-følsomme *E. faecium* (VSEfm) stammer næsten ubemærket i samme periode. VSEfm er dog fortsat interessant at undersøge, da den er ophav til den resistente modpart, og da spredningsmekanismerne menes at være de samme uanset tilstedeværelse af antibiotika-resistens. Samtidig var der i perioden en udbredt brug af screenings- og isolationsinterventionsregimer mod VREfm og VVEfm, men uden at indsatsen blev evalueret.

De specifikke formål med denne ph.d. var:

- at undersøge om cgMLST i realtid kan anvendes til infektionsforebyggelse og kontrol af VREfm spredning (Paper I)
- at undersøge for ikke-erkendt transmission af VSEfm ved at bruge cgMLST (Paper II)
- at undersøge om cgMLST af VSEfm kan benyttes til at forudsige forekomst af VREfm/VVEfm (Paper II)
- at undersøge den kliniske betydning af VSEfm og VREfm/VVEfm (Paper III)
- at undersøge betydningen af at ophøre med screening og isolation af VREfm/VVEfm patienter på et dansk universitetshospital (Paper III)

I studie I blev 38 patienter med en klinisk prøve indeholdende VREfm og mistænkt for at tilhøre et igangværende udbrud på OUH i perioden januar 2014-juni 2017, inkluderet. WGS med efterfølgende MLST og cgMLST blev udført på Klinisk Mikrobiologisk Afdeling, OUH.
I alt blev 13 af VREfm-isolaterne fundet beslægtede og tilhørende ST80-CT993 *vanA E. faecium*. På grund af den høje detaljeringsgrad i cgMLST-data i samspil med brug af epidemiologiske data kunne antallet af afdelinger med behov for infektionshygiejniske indsatser reduceres fra adskillige til én. Der blev iværksat infektionshygiejniske interventioner på den pågældende afdeling, hvorefter udbruddet ophørte (Paper I).

I studie II blev der udført MLST og cgMLST analyse på *E. faecium* isolater fundet i bloddyrkninger fra patienter på OUH. Seks hundrede og tredive VSEfm- og 27 VREfm-isolater blev inkluderet. Journalerne fra patienterne med VSEfm blev undersøgt med hensyn til forskellige kliniske parametre, herunder dødelighed.
Data viste, at VSEfm sekvenstyperne (STs) for MLST og kompleks/klonal typerne (CTs) for cgMLST var skiftende og multiple - isolaterne tilhørte 42 forskellige ST og 131 CT i flere klynger (clusters). Der blev fundet udbredt transmission af VSEfm med forskellige CTs, hvilket indikerer tilstedeværelsen af sprednings-risikofaktorer på hospitalet.
Vi fandt ingen sammenhæng mellem VSEfm- og VREfm-klonerne i antal eller i typer (ST-CT).

Ved at undersøge dødeligheden identificerede vi en uoverensstemmelse mellem 30-dages dødelighed og dødsårsagen. I de fleste tilfælde døde patienterne af alvorlige underliggende sygdomme og ikke af VSEfm-bakteriæmi i sig selv (Paper II).

Baseret på en overordnet kvalitetsundersøgelse af patienter på OUH med VREfm-infektioner og den relaterede dødelighed, ophørte hospitalet med VREfm/VVEfm-screening og isolation af disse patienter i slutningen af 2021.
Det tredje studie blev udført for at undersøge virkningen af dette ophør.
Studiet blev udført som et retrospektivt kohortestudie af alle patienter med et første gangs VREfm/VVEfm klinisk isolat (indeks isolat) påvist på OUH i perioden 2015-2022.
I alt blev 436 patienter inkluderet, hvoraf 285 patienter var fundet i interventionsperioden (2015-2021) og 151 i post-interventionsperioden (2022).
Fra interventions- til post-interventions- perioden var der en signifikant ændring i *van*-gen-fordelingen, men ingen forskelle i de undersøgte karakteristika i forhold til hver enkelt *van*-gen.
Der blev fundet et øget antal indeks VREfm/VVEfm isolater efter ophør med screening og isolation, men intet andet til at støtte en genindførelse. Ligesom for VSEfm blev det fundet, at 30-dages dødeligheden ikke afspejlede, om døden kunne tilskrives VREfm/VVEfm (Paper III).

Denne ph.d. har gjort os opmærksomme på mulighederne for brugen af WGS og cgMLST i vores daglige arbejde med infektionshygiejne. Selve processen med at udtrække sekventeringsdata og sikre en god kvalitet af data kræver dog indsigt i molekylærbiologien. cgMLST-data er nemme at formidle, men det er vigtigt at være opmærksom på valgene og mulige begrænsninger i de underliggende algoritmer, for at undgå fejlfortolkning. Endvidere har sekventeringsdata uden samtidig brug af epidemiologiske data en begrænset anvendelse i infektionsforebyggelse og -kontrol.
Dette ph.d.-studium har også vist, at der er behov for at genoptage overvågningen af spredningen af de mindre resistente bakterier. Måske kan en sådan overvågning bane vejen for en bedre forebyggelse af spredning af de mere resistente bakterier frem for at vi slukker ildebrande, når først problemet er opstået. Ydermere tyder de foreliggende data på, at screening og isolation af VREfm/VVEfm kan ophøre i et lav-incident miljø. Udviklingen i antallet af VREfm/VVEfm tilfælde på OUH vil dog blive nøje fulgt i de kommende år, og forhåbentlig kommer der data fra andre lav-incident miljøer, der kan hjælpe med at belyse området.

1. Background

1.1 Infection prevention and control – challenges of today

In the 21st century, the number of healthcare-associated infections (HAIs) are a very important factor in healthcare service and social economy, because HAIs account for billions of extra costs, losses of earnings, and lives [4, 5]. The terms 'Nosocomial infection', 'hospital-acquired infection', and 'healthcare-associated infection' are used alternately, and covers the World Health Organization (WHO)-definition 'an infection occurring in a patient during the process of care in a hospital or other healthcare facility, which was not present or incubating at the time of admission' [5].

In 2011, WHO estimated the average number of HAIs for patients divided by income. In high-income countries 7% of the patients suffered from a HAI at any given time, compared to 15% in low-and middle-income countries. At the Intensive Care Units (ICUs) in high-income countries, the rate of HAI was estimated as high as 30-40% [4, 5]. The numbers of HAI has been estimated by the European Centre for Disease Prevention and Control (ECDC) to 4.5 million HAIs occurring each year in the period of 2016-17 in the European acute care hospitals, affecting 3.5 million patients [6].
In Denmark, the number of HAI has been estimated to 8-10% or 60,000 patients each year, but the overall level of HAI has not been estimated since 2012 [7]. Despite the introduction of a national reduction target in 2016, the level of hospital-acquired bacteraemia has changed only slightly in Denmark during the last five years [7–9].

Infections contribute heavily to the economic burden in healthcare service, because they can lead to serious complications for the affected patients, need of broad-spectrum antibiotics, and increase the mortality [10]. The Communicable Disease Center (CDC) in Atlanta estimated the costs of HAIs in the United States hospitals in 2009 to US$ 35-45 billion [11]. In a study from 2013, one of the most expensive HAIs were the central-line-associated bacteraemia with an estimated cost of US$ 45,814 each [12].
Outbreaks in hospitals with several patients involved also contribute significantly to costs, especially due to lost revenue because of closed beds. European studies have estimated outbreak costs to range from € 10,778 to € 356,754 for a single outbreak, and with increasing costs if the microorganism was multidrug-resistant [13, 14].

The population in high-income countries are getting older and have more comorbidities. Furthermore, the continuing improvements of medical skills lead to more patients being treated for severe illness and kept alive. In the process of surviving severe illness the patients are fragile and can be infected by their own otherwise harmless flora as well as hospital-adapted bacteria (HAB) [15–17]. They get several infections which are often treated with broad-spectrum antibiotics. The antibiotic treatment alters their microbiota and enhance the development of

resistance. In 2017, the increasing occurrence of antibiotic-resistant bacteria, promoted WHO to publish a list of bacteria against which there are an urgent need for development of new antibiotics [6, 18].

Studies from the 1980s to the 2010s have revealed that 30-75% of HAIs can be prevented through use of multimodal and multidisciplinary prevention and control programs, in an interaction with hospital organisation, bed occupancy, and staffing [19–23]. Such programs are not necessarily cheap, and this raises the dilemma of whether to serve the patient in the best possible way or to be most cost-effective [24]. It may be difficult to implement all the recommended guidelines in a busy everyday life, and it is therefore important to evaluate what we do and do not do to find the right balance.

1.2 The history of Infection prevention and control

Cholera, Plague, and Diphtheria have all caused epidemics, which have devastated the World. Records of epidemics can be found way back in time, and outbreaks of Diphtheria and Plague have been described together with the use of hygiene by Hippocrates as far back as before 400 BCE [25–27]. The necessity of hygiene for the welfare of people has also been described by the Roman Emperor Hadrian in his memoires around 120 CE [28]. The name hygiene derives from the Greek Goddess Hygeia, protector of health, cleanliness, and hygiene. Her origin is turbulent, but she is today described as the daughter of the Greek God Asclepius, the protector of Medicine. Both Hygeia and Asclepius were popular among the people, and were worshipped in each their cult from 500 BCE to 500 CE [29–31].

Specific places where fragile and sick people have gathered are known from the same period. Best known are the ancient Greek healing temples Asclepieia and the military roman hospitals - and for as long as there have been hospitals there have been hospital infections.

Until the late 18th century, it was a general opinion that infections could appear out of the blue, but in 1767, the Italian physiologist L. Spallazani published his results on growth of microorganisms and heat sterilisation thus rejecting the idea of spontaneous generation of life [32]. During the 19th century an increasing knowledge of microorganisms and transmission of infections were achieved. The findings of Spallazani were supported by the work of the French chemist L. Pasteur, and the British physicist J. Tyndall in the 1850s and 1860s [33].

In 1847, the Hungarian physician I.P. Semmelweis described how death due to puerperal fever could be reduced by using chlorine as a disinfectant, but it was not until the 1870s that the terms sterilising, disinfection, and pasteurisation were introduced [34]. The Scottish surgeon J. Lister was inspired by the work with microorganisms of L. Pasteur, and in 1865, J. Lister described his own discovery of microorganisms in wound infections, and how these could be avoided by disinfection with carbolic acid of instruments, skin, hands, and linen [35].

With J. Lister and I.P. Semmelweis, the use of aseptic and antiseptics were introduced in medical work. Aseptic techniques meaning practices and procedures that prevents contamination with

microorganisms, and antiseptics being the use of chemical agents that prevent the growth of microorganisms.

Other infection prevention and control (IPC) precautions were the various kinds of public- or self-isolation regimes that had been used for persons with infections like leprosy and plague since ancient times, and the use of quarantine introduced in Italy in the 14th century [36, 37]. However, this ancient knowledge seemed to have been forgotten over time, until it was re-introduced as an approach in nursing by Florence Nightingale in 1856. Florence Nightingale had witnessed the poor conditions in the infantries during the Crimean war, and she took the practice with her, when she later on opened the 'Nightingale Training School' at St. Thomas Hospital in London in 1860 [38]. After that, the hospitals began to isolate contagious people in epidemic wards, and the hospital staff was introduced to the use of gloves, facemasks, and gowns for surgical use. Hand wash and hand disinfection were also introduced [38].

A third approach used in the fight against infections is vaccination. Vaccination was introduced by E. Jenner during the smallpox epidemic in Europe in 1796, but vaccination had at that time been known for more than 1000 years in China. In 1890, the Diphtheria epidemic in Denmark resulted in the production of diphtheria-antitoxin in serum, and in the first clinical controlled trial of serum-treatment in the World. The trials were performed in 1896-1897 [39]. In some of the epidemics, before the time of antibiotics and vaccines, the mortality rate increased to above 50%. With the introduction of vaccines and later the antibiotics sulphonamides and penicillin in the 1930s, the mortality decreased drastically. This led to a kind of loss of respect for infections, and nearly a hundred years after I.P. Semmelweis and J. Lister had introduced the aseptic and antiseptic principles, the use of most IPC precautions deteriorated.
In 1946, the United States opened the CDC in Atlanta, with the aim to prevent malaria from spreading in the country [40]. A year after the opening of CDC, a huge outbreak of staphylococci among patients admitted to Australian hospitals was registered. The rest of the World soon followed with similar outbreaks, and it has been estimated that more than 50 million people were infected. This prompted the CDC to begin surveillance of infectious diseases.
Today, most countries in the world have a national surveillance system, and CDC has been joined by a European counterpart – ECDC in 2005 [41].

1.3 Infection prevention and control in Denmark

Statens Serum Institut (SSI) in Denmark opened in 1902, with the mission to produce anti-diphtheria serum. At the same time, the manager of SSI was made advisor to the Danish Health Authority (Sundhedsstyrelsen) in questions concerning epidemic diseases [26].
Due to the staphylococcal pandemic in the 1940s, the IPC in Denmark was further organized and recommendations were published by the Danish Health Authority and SSI in cooperation to bring awareness to IPC [26].

To combat infections in Danish hospitals, a three-faceted approach was established including the following [26, 42]:

- a sustained improvement and modification of infection control
- an enhanced education for all kinds of hospital staff to achieve good infection control and rational use of antibiotics, and
- a restriction of antibiotic consumption in humans and animals, and surveillance of development of resistance

In the 1970s, a Study on the Efficacy of Nosocomial Infection Control (SENIC) was provided by CDC. The SENIC-study found a 32% reduction of HAIs in hospitals where infection control programs had been established compared to hospitals without a program [19].
In Denmark, the IPC approach was modified and built on the SENIC-study, which it still is today, and contains the following elements [42]:

- Organisation
- Surveillance
- Recommendations
- Education
- Antibiotic policies

The National Center for Hospital Hygiene (Den Centrale Afdeling for Sygehushygiejne, CAS) was established in 1978, and in 2010 CAS changed its name to the National Center for Infection Control (CEI) (Central Enhed for Infektionshygiejne, CEI) [26].

In Denmark, the current definition of IPC is 'the professional work that has the intention to prevent infections obtained by care and treatment in the primary sector as well as the hospitals – and the in-betweens'. These infections are in Danish known as 'Sundhedssektorerhvervede infektioner' (SEI), and correspond to HAIs [43].

1.3.1 Organisation

Today, IPC is organized under the Ministry of Health as the supreme authority. The Danish Health Authority is also established under this ministry.

Some of the Danish Health Authority's primary tasks are to promote the health of the citizens, plan the structure of the healthcare system, establish guidelines for the training of healthcare staff, and be responsible for health emergency services and preparedness in Denmark. The Danish Health Authority also publish lists of notifiable diseases as well as specific guidelines regarding precautions concerning multi-drug-resistant microorganisms [44].
The antimicrobial agent consumption and resistance are monitored by the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP). DANMAP was

established by the Danish Ministry of Food, Agriculture and Fisheries and the Danish Ministry of Health in 1995 [45].
Both the Ministry of Health and the Danish Health Authority are advised by CEI.
Among other things, CEI publishes the National Infection Prevention and Control Guidelines, known as the 'Nationale Infektionshygiejniske Retningslinjer' (NIR), which are used by all healthcare institutions in the country, and offer advice on local work on IPC [46].

In Danish hospitals, IPC is organized in IPC units mainly located in the Department of Clinical Microbiology (DCM) (Fig. 1).

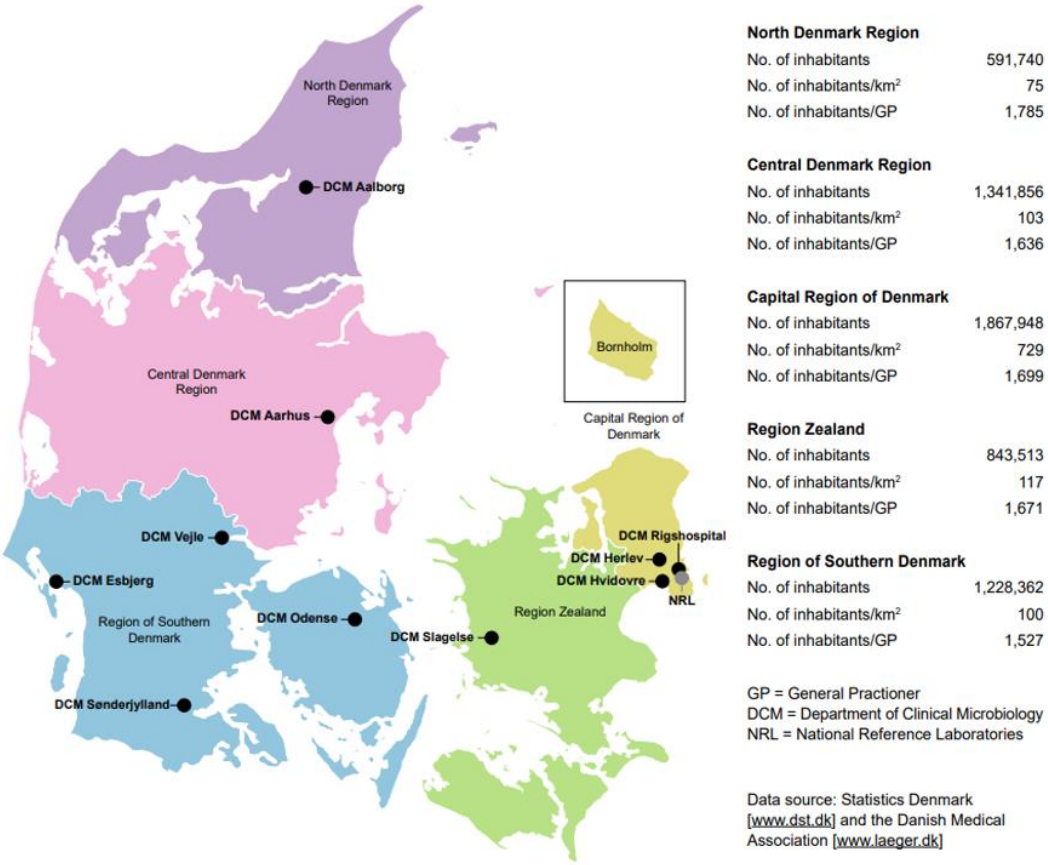


Figure 1: The five Danish healthcare regions and their associated DCMs and population distribution. The grey dot indicates the national reference laboratories (NRL) at Statens Serum Institut. Adapted and modified picture from DANMAP 2022, Statens Serum Institut [47].

In the IPC unit, IPC-nurses work in close collaboration with doctors specialised in clinical microbiology. The majority of Danish IPC-nurses are nurses with extensive experience in daily clinical nursing work, who have subsequently participated in a Nordic master program in IPC or passed similar courses offered by other countries. The doctors specialised in clinical microbiology have as part of their education received training and participated in courses in IPC. They can also participate in the same courses as the IPC-nurses.

In the Danish hospitals, the organisation of infection control varies slightly.
At OUH, the organisation is as follows:
One of the Chief Executive Officers (CEO) at the hospital has the main responsibility and is chairman of the Antibiotics and Infection Control Committee (AICC).
The AICC consists of professionals with management responsibilities and competencies within the various areas in the hospital – e.g. clinical departments, facility services, cleaning, and IPC.

In each department at the hospital one or more IPC coordinators have been appointed, who, in collaboration with a doctor responsible for antibiotics and IPC, form part of the department´s antibiotic and IPC team - the AIK-team. This AIK-team, in collaboration with the IPC unit, must help implement and monitor compliance with guidelines, as well as assist in outbreak investigations in their own department.

1.3.2 Surveillance

Surveillance of infections has several purposes.
The surveillance can in itself be preventive due to the attention on the infections and the elements that are used for prevention. Besides that, surveillance in the Danish hospitals are used for [48]:

- detecting outbreaks
- identifying the need for an intervention
- measuring the effect of an intervention
- estimating the economic consequences of the infections
- as a part of surveying the quality in a hospital

In 1978-79, prevalence surveillances were initiated every sixth month in the Danish hospitals.
The prevalence investigation was carried out in the wards and registered by hand. Since then, digital systems for this purpose were introduced.
The electronic surveillance systems can be divided in two: the Electronically Assisted Surveillance (EAS) and the Fully automated Electronic Surveillance (FES). Both kinds of systems have their challenges. EAS requires a person to confirm if the surveillance definition of a HAI is met, and sensitivity may therefore be favoured above specificity in these systems. FES systems may have a high proportion of false positives, if the rate of HAI is low [49].
In Denmark, all DCMs register all samples analysed in a laboratory information system. In these systems the bacteriological, serological, and molecular results such as identification and antimicrobial susceptibility are registered together with data of the sample and the patient. However, differences in the laboratory processes between the individual laboratories can lead to differences in which data are recorded and the quality of the data. All data are used together with information from other databases in a FES system named HAIBA (Healthcare-Associated Infections Database). HAIBA is a system using continuous surveillance. It was introduced in 2015 and

replaced the former six-monthly prevalence surveillances [26, 42]. HAIBA monitors the following HAIs: bacteraemia, urinary tract infections (UTI), deep infection after planned hip or knee alloplastic surgery, and *Clostridium difficile* gastrointestinal infections. The HAIs are detected by combining information on microbiological results with data on hospitalisation. HAIBA does not include clinical information and therefore to be precise, HAIBA monitors the presence of microorganisms and not infections, i.e. bacteriuria and not UTI.

1.3.3 Infection prevention and control recommendations in Denmark

In IPC various precautions are used to prevent infections, since infections can occur in different ways as described in the section 'Transmission'.
The National Infection Prevention and Control Guidelines (NIR) consists of several very different sets of guidelines. The most frequently used guidelines deal with the standard precautions and the transmission-based precautions (TBP). Other NIR deal with highly specialised areas, e.g. renovation and construction of new buildings in the healthcare sector [46].
The Danish Health Authority has published specific guidelines regarding the multidrug-resistant microorganisms´ methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenemase-producing organisms (CPO) (www.sst.dk).

Standard precautions

Standard precautions apply to all patients in all departments and in all care, treatment, and examination situations [43].
They are basic principles based on aseptic and antiseptics. They are used for the prevention and interruption of possible routes of infection in all tasks at the hospitals. Standard precautions must prevent infections with microorganisms, regardless of being detected in the microbiology laboratory or not, and therefore regardless of the knowledge of whether the patient or staff in fact carry them.
The standard precautions covers everything from hand hygiene, healthcare uniforms, and cleaning, to reprocessing and ventilation [43].

Transmission-based precautions (TBP)

Transmission-based precautions are precautions used in addition to the standard precautions [50]. They are used in special situations and depends on how the transmission takes place, e.g. by droplets or by contact. One or more of the TBP may be used at the same time. The use depends on an evaluation of different parameters including treatment options, transmission rate, mortality, new or unknown resistance mechanism, or if particularly susceptible patients have to be protected. TBP may consist, among other things, of screening, isolation or single-room placement, use of special protective equipment, and intensified cleaning and disinfection [51].

Screening

Screening programs to identify carriers of resistant bacteria are widely used in attempts to reduce infection rates in hospitals. However, use of screening tests increases costs and are time-consuming in laboratories [52, 53].

Several studies have investigated how to obtain the most optimal screening procedures. Studies on VREfm have revealed that for each patient, two to five rectal swabs collected on three consecutive days, and at the earliest three to four days after exposition, should be used for screening [54–56].

Isolation

When using isolation against resistant bacteria, the hospital staff are required to use gloves, gowns, and sometimes a facemask in patient-contact situations. The door to the patient room is often closed, and visitors are not allowed without special instructions. This behaviour leads to less contact with the patient, fewer measurements of vital parameters, and delays in various procedures, which all contributes to a higher mortality rate and poorer patient experience [57–60].

After the patient is discharged from hospital, the patient´s room is often cleaned and disinfected with chemicals such as alcohol or chlorine-based products to ensure that the risk of transmission through the environment is reduced [51, 61]. This increases the costs further and can have side effects on the environment such as a degradation of materials and replacement of the harmless environmental flora with flora containing increased bacterial resistance against the disinfectant used [62, 63].

1.4 HAI and risk factors

The acquisition of HAI can be divided into three main categories [64]:

- self-infection
- cross-infection
- environmental infection

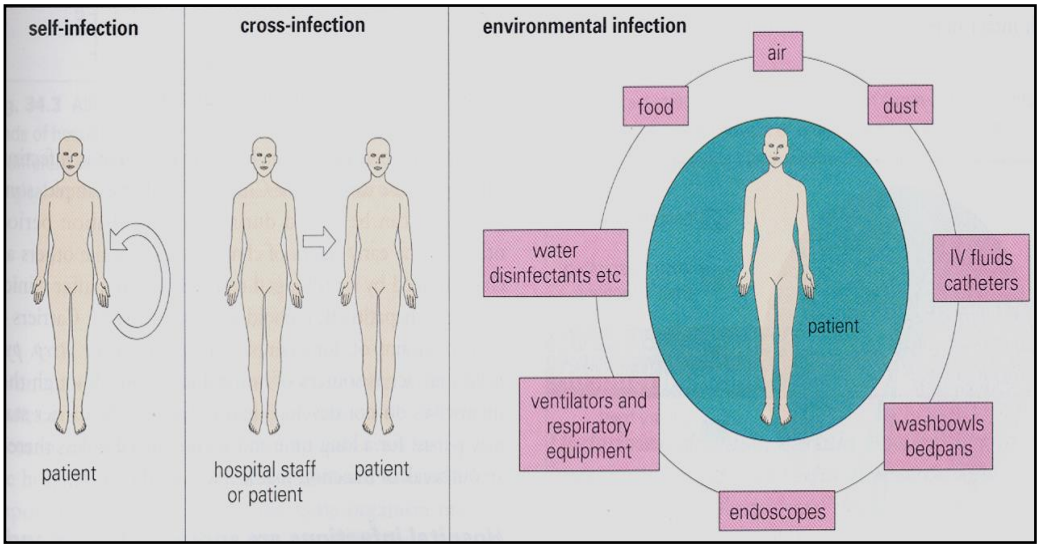


Figure 2: Acquisition types of infection. Adapted from Mims 2ed [64].

Self-infection

Most often, HAI occurs due to self-infection, which means that the infection is caused by the patient's own microbiota. This type of infection occurs because we break the natural barrier (skin and mucosa) due to the examinations and treatments we carry out in the hospitals. This type of infections are also known as endogenous infections [43, 64].

Cross-infection

Cross-infection happens by transfer of microorganisms between patients, or patients and the hospital staff. The cross-infection most often happens by contact – directly or in-directly, as described in the section 'Transmission'. This type of infections are also reported as exogenous infections [43, 64].

Environmental infection

The third type of HAI caused by transfer of microorganisms from the environment.
As shown in Figure 2, several factors in the environment can harbour microorganisms that can cause infection, e.g. legionellosis from tap water, tuberculosis from dust, or shigellosis from food. This type of infections are like cross-infections also reported as exogenous infections [43, 64].

Risk factors

Several studies find that the same risk factors are related to both susceptible and resistant bacteria of the same species causing HAI. The risk factors are factors that have significance beyond the obvious risks involved in interventions such as the use of catheters, prostheses, and operations. The risk factors apply to, e.g. *E. faecium*, *S. aureus,* and *K. pneumonia* infections, and includes the following [65–70]:

- Host susceptibility
    - Reduced resistibility due to damaged tissue or mucosa, reduced immune response, cancer or other underlying illness like renal failure and organ transplantation
- Antibiotic treatment
    - Previous antibiotic treatment, long duration of the treatment, and specific antibiotics
- Hospital settings
    - Transfer between wards, extended hospitalisation, Intensive Care Unit (ICU) stay, and contaminated environment and equipment
    - Patient exposure to hospital staff colonised with specific infectious agents
    - Absence of infection control guidelines and lack of compliance with these, especially insufficient hand hygiene compliance among hospital staff

Furthermore, microbiological factors have an impact on transmission.

1.5 Transmission

Humans harbour bacteria on all non-sterile parts, and each of us live in symbiosis with our specific bacterial mix and refer to this collection of microorganisms as our microbiota [71]. The microbiota consists of more than a thousand different species, and the DNA of the microbiota is named microbiome. The microbiota can be considered the individual person´s second genome.
Besides having our own microbiota, humans exchange microorganisms all the time, e.g. when shaking hands, giving a kiss, or indirectly by touching the same door handle [43]. Mostly this exchange is harmless. However, some of the bacteria in the microbiota can be potential pathogenic microorganisms.
A person colonised for shorter or longer periods with a specific bacterium without getting an infection, is a so-called ‘carrier’ [72]. If the pathogenic bacterium is transmitted to another person, that person may become a new carrier or develop infection. Healthy people can be carriers for a shorter or longer period, but for the fragile patients, and on special occasions where the natural skin and mucous membranes barriers are broken, the exchange can have fatal consequences.

Transmission of bacteria also takes place in the hospitals and along some of the same routes as outside the hospitals. The differences between the hospitals and the homes are the state of health of the people, and what we do to them [43].
The transmission of bacteria can occur through:

- contact
- air
- dust
- vehicles
- vectors

Transmission occurs most often through the direct contact between people or indirectly through contact points – that is, the bacteria can spread directly from patient to patient or from staff at the hospital to patients (e.g. by handshaking), and indirectly from a person to the environment and from there on to other patients (e.g. by touching the same hand rail) [73–77].

One of the microbiological factors that facilitate transmission through the environment is the microorganism's ability to survive in the environment for an extended period while maintaining the virulence. The microorganism also need the ability to colonise the patients, the hands of the hospital staff, and hospital equipment such as thermometers, gloves, and gowns to have a high transmission rate [76, 77]. The six conditions needed for transmission of microorganisms between persons, have been described as follows:

- a microorganism
- a reservoir/a carrier
- an exit-site of the microorganism
- a transmission route
- an entrance for the microorganism
- a recipient

These conditions have been linked and described as 'The transmission chain' (Fig. 3) [43].
If one of the links in the chain is broken, the transmission will end.

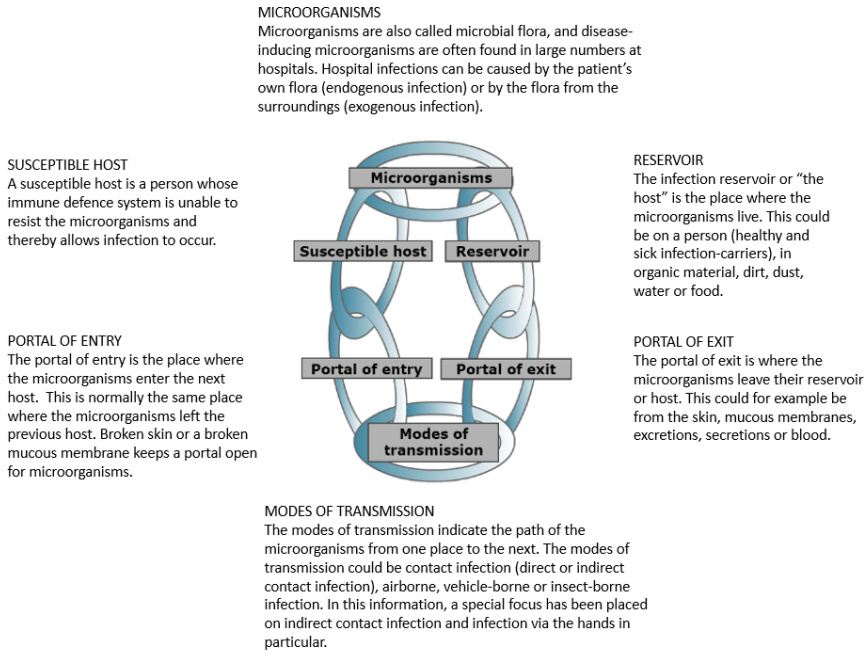


Figure 3: The transmission chain. Modified with acceptance from Statens Serum Institut [43].

The transmission routes of the microorganisms seem to be similar whether the microorganism carries antibiotic resistance or not [78]. If transmission of susceptible strains is prevented, prevention of the resistant counterpart should also occur.

To interrupt transmission there are three possible methods [43]:

- increase the resilience of the patient
- use of antimicrobial treatment
- use of infection control precautions

Of concern is the carriers that make up a reservoir. Sometimes it is desirable to identify these carriers to break the transmission chain. Finding the connections between patients carrying a bacterial species with a unique resistance profile is not difficult in a country with a low incidence rate, because there are few cases. They are like the top of the iceberg very visible. The problem arises when a spread of more common bacteria species with a 'normal' susceptibility profile is to be discovered.
Due to the limited treatment options and the reports of increased mortality and outbreak costs, national and international guidelines recommend or requires screening and isolation as a prevention of the multidrug-resistant organisms (MDROs) MRSA and CPO. In Denmark, there are no legal requirements regarding VREfm/VVEfm - only recommendations in NIR. Furthermore, there are no specific recommended TBP against the susceptible counterparts [79–81].

Transmission is not only happening inside the hospital, but also between collaborating hospitals and other non-hospital institutions by the transfer of patients [82]. It is a two-way transmission, and if IPC precautions are to be successfully implemented, the greatest effect is achieved by starting at the hospital from which most transfers take place [83, 84].
Furthermore, a spread can also occur to and between many different actors outside the hospitals. This is particularly evident when it comes to antibiotic-resistant microorganisms (Fig. 4) [85]. This spread in society and the environment can sometimes make it difficult to find the original source of transmission.

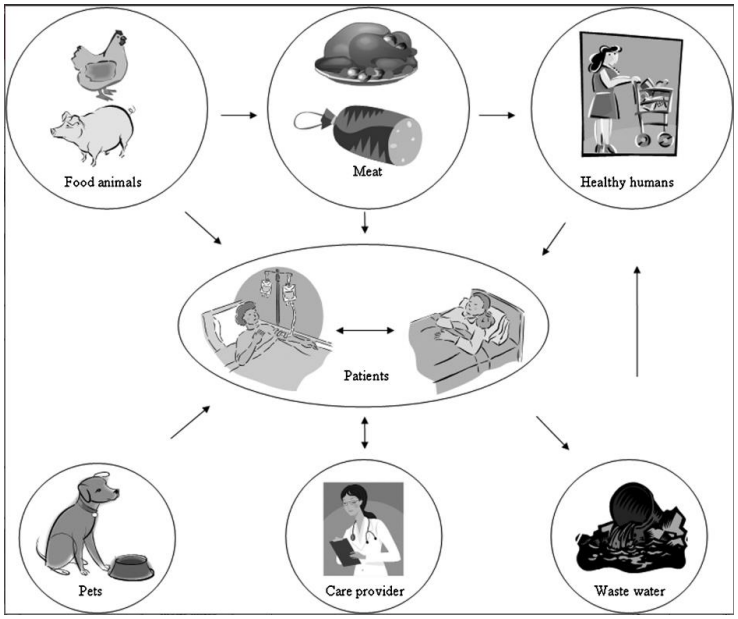


Figure 4: Transmission of microorganisms between hospitals, community, and animals visualised by VREfm. Adapted from Hammerum *et al.* [85].

1.6 Outbreaks

There is no global definition of an outbreak.
According to CDC an outbreak is defined as an epidemic in a limited geographical area. An epidemic is defined as 'the occurrence of more cases of a disease than expected in a given area or among a specific group of people over a particular period of time' [86].
In Denmark, CEI has defined the term as 'accumulation of cases, above what is normally expected' [51]. This definition has been translated at OUH to 'an accumulation of cases in a clearly defined unit or population, where an IPC intervention is necessary' (OUH's intranet, ID-number: 60365).

An outbreak does not necessarily involve only one microorganism.
Outbreaks can be divided into several types involving:

- a specific type of the same species (the classic but narrow definition)
- different types of the same species or different species (e.g. accumulation due to poor IPC)
- the same specific resistant pattern among different species (e.g. overuse/abuse of antibiotics)

At OUH, the TBP depend on the suspected extent of the transmission, e.g.:

- If transmission involve two patients sharing the same room:
  It is assumed that transmission has occurred only in that specific room.
  The room and the associated patients are the only ones involved in an inspection of the IPC precautions used and in the initiation of TBP.
- If transmission involve two or more patients in the same department, but not in the same room:

The entire department is subjected to an outbreak investigation. One or more TBP are used such as patient screenings, decontamination of the entire department, disposal of utensils, use of protective equipment, and closure for intake of new patients in the department – all-together a costly affair.

Outbreaks ought to be stoppable by interrupting the transmission chain, but it is not that easy. The transmission routes and reservoirs are often very complex because they can be multiple and very diverse – from equipment to an invisible carrier.
Some of the essential questions when investigating an outbreak are the date of the start of the outbreak and when it has come to an end. There is no global consensus on how to determine this period, making it difficult to pinpoint the number of patients and staff to be investigated, and to calculate the economic burden of the outbreak [13].

Costs

Estimating the economic burden of nosocomial outbreaks and comparing results are difficult, because there is no common methodological approach – especially with regards to determining which elements to include, the duration of the outbreak, and who the payers of the costs are [52, 87].
Despite the difficulties in estimating the costs, several studies have found that the highest economic burden was due to missed revenues because of closed beds, and that outbreaks with MDROs are more expensive than outbreaks with antibiotic susceptible bacteria [13, 14, 52, 88].
It has been pinpointed by others, that it is important, that costs are adjusted for confounders such as comorbidities, because differences between VREfm/VVEfm and VSEfm may be due to comorbidities rather than the infections in-itself [87, 89]. In a German study from 2018, this adjustment was made and the attributable cost of a VREfm case compared to VSEfm was calculated to be EUR 13,157 per patient [90]. The major costs were for antimicrobial drugs, nursing staff, medical products, and assistant medical technicians. The study did not describe in detail what these headings covered, but it could appear to cover the tasks involved in the use of screening, isolation, and decontamination.
Some studies have found that using the international guidelines for IPC alone was insufficient to detect and control outbreaks with e.g. vancomycin-resistant enterococci (VRE), and that sustained screening of the patients was necessary [56, 91]. In a Danish study of MRSA, in a setting of screening and isolation, the largest independent financial item was the inquiry about risk behaviour. The additional cost of using isolation was estimated to DKK 2,000-4,000 per patient per day, or approx. DKK 8,700 per colonised/infected patient [92].
Although both screening and isolation are expensive to carry out, studies from several countries have found them to be cost-effective compared to the cost of an outbreak. Early identification of carriers of the microorganism involved in the specific outbreak with an implementation of contact precautions as well as search and destroy policies were the most cost saving strategies. Due to

that, it has been concluded that faster, cheaper, and more accurate diagnostics are needed for all MDROs to save costs [13, 52, 53, 79, 88, 93].

1.7 Microorganisms

The microbes can be divided in to four major types: bacteria, yeasts, virus, and parasites. All types of microbes can cause HAI, but the prevalence of the types varies greatly between different parts of the world. To understand which identification and prevention options are available against bacteria, knowledge of the bacteria's structure is required.

1.7.1 The bacterial structure

A bacterium consists of an outer cell wall and a cytoplasmic membrane. Inside is the cytoplasm with the bacterial chromosomal genome as one circular DNA-nucleoid, the ribosomes, proteins, and metabolites. The outer cell wall might be enveloped by a capsule and have fimbriae (pili) and flagella anchored (Fig. 5). The cell wall are structured differently in gram-positive and gram-negative bacteria (Fig. 6) [94].

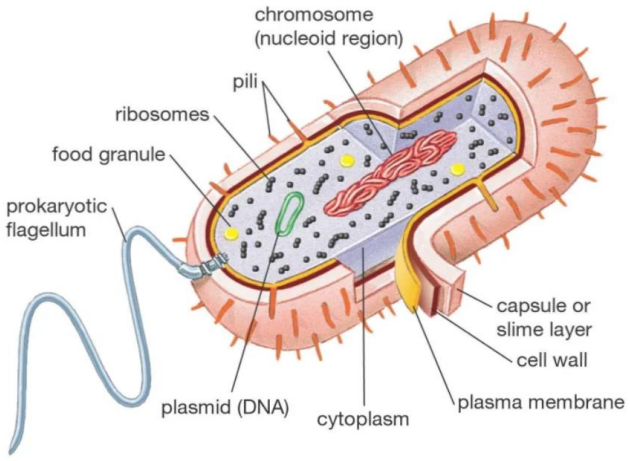


Figure 5: The structure of a typical bacterium [95].

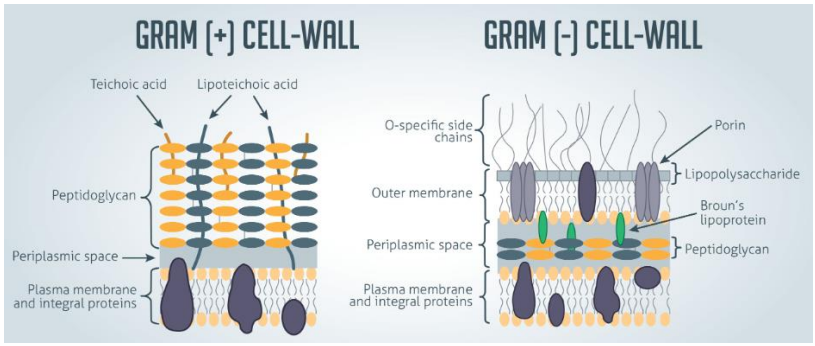


Figure 6: The cell wall structure of a gram-positive and a gram-negative bacteria [96].

The DNA is transcribed to messenger RNA (mRNA), which is transported to the ribosomes and translated to proteins [94].
The proteins in the cell are located in the outer cell wall, the cytoplasmic membrane, the cytoplasm, and in structures like flagella, fimbriae, and capsule. More than 20% of the total cell protein is derived from the ribosomal proteins [97].
The genome can be divided into the chromosomal and extrachromosomal DNA, with the extrachromosomal DNA being physically separated from the chromosomal DNA.
The chromosome is located as a double stranded DNA circle. This DNA consist of millions of bases/nucleotides spread over thousands of genes. Some of the genes are present in all members of the species and are named the core genome, while the rest of the genes are variably present and named the accessory genome [94]. Some of the genes in the core genome are essential for the metabolic functions to keep the bacterium alive. These genes are named housekeeping genes and are always present [98]. The core genome account for approximately 10% of the genome, while the remaining 90% consists of accessory genes [99].
The extrachromosomal DNA in bacteria is also located in the cytoplasm, and are mostly circular double stranded DNA, also known as plasmids.

Reproduction

The reproduction of bacteria occurs by DNA replication followed by cell division, leaving the genome in the offspring identical to the mother-genome. This passing on of genes is known as clonal or vertical gene transfer. Minor changes can appear due to point-mutations, but to secure genetic development, the bacteria can exchange genetic material by recombination, also known as horizontal gene transfer.
Usually, the recombination takes place in the accessory genome and rarely in the stable core genome.
Changes in the core genome can instead be caused by point mutations and are estimated to 1-29 events per six months for *E. faecium.* The recombination rate differs between the bacterial species, and a high recombination rate with up to 44% of the genome involved is seen in *E. faecium* [100, 101].

Recombination happens either by [102]:

- uptake of naked DNA from the environment (transformation),
- transfer of DNA through a bacteriophage (transduction), or
- by passage of plasmids or transposons through a sexual process (conjugation)

By these processes, mobile genetic elements (MGEs) consisting of DNA in the surroundings or in other bacteria are exchanged with the bacterium (Fig. 7) [102].

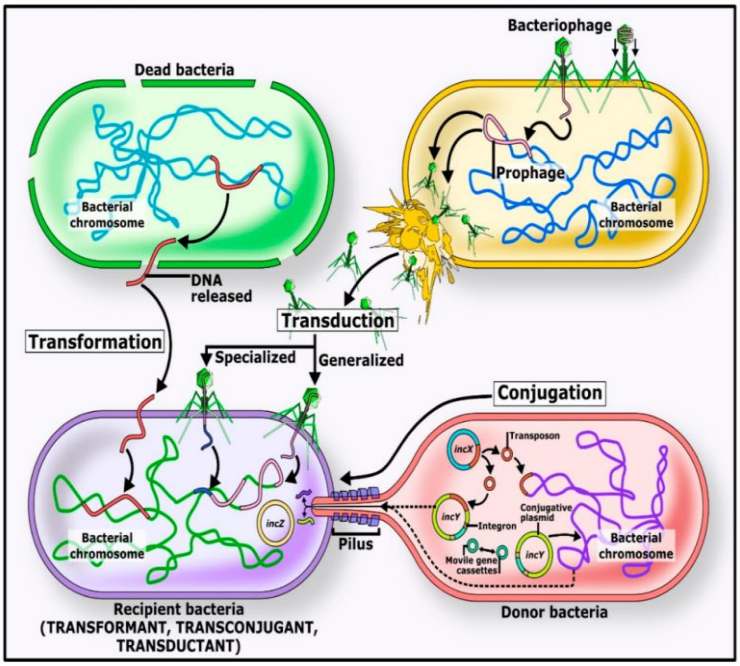


Figure 7: Mechanisms involved in horizontal gene transfer (transduction, conjugation, and transformation). Adapted from Bello-López JM *et al.* [102].

The MGEs can be plasmids, transposons, integrons, insertion sequence (IS) elements, or bacteriophages (Fig. 8). The MGEs can cause either insertions or deletions of DNA [94, 103, 104].
A plasmid is an independent piece of extrachromosomal circular double-stranded DNA. A plasmid can harbour several resistance and virulence genes, and can control its own replication [94, 105].
A transposon is a lager intra-species transposable fragment of DNA that can cause significant changes in the genome. Transposons can carry antibiotic resistance genes [106].
Integrons are transposons consisting of one or more gene clusters called a cassette [107]. The difference to transposons is that integrons incorporate site specifically, whereas transposons integrate randomly in the genome. Both the transposons and integrons can be carried by plasmids.
IS elements only carry genes encoding for enzymes involved in the transposition of the element. IS elements can shift place in the genome and participate in rearranging the chromosome and flanking transposons. In contrast to transposons IS elements does not carry accessory genes [108].

The absorbed DNA can be incorporated in the genome as an insertion in the existing chromosome or placed as a plasmid. After absorption, the resistance and virulence genes can be clonally spread [94]
The virulence of a bacteria may therefore not only be located on genes in the core genome but also on the accessory genes [109].

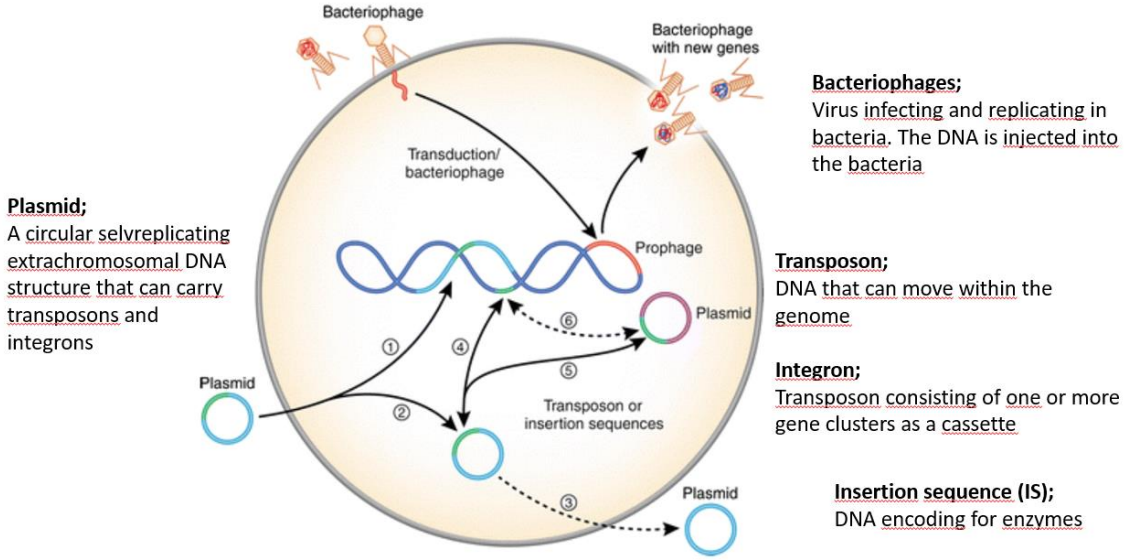


Figure 8: The bacterial core genome and the acquisition of extrachromosomal genome by *Staphylococcus aureus* from mobile genetic elements such as plasmids, transposons, and insertion sequences - 1 Incorporation of plasmid elements into bacterial genomic DNA. 2 Plasmid maintained in the bacterium as free circular DNA. 3 Plasmid excretion from the bacterium. 4 Transfer of a transposon or an insertion sequence between plasmid and genomic DNA. 5 Transfer of a transposon or an insertion sequence between plasmids within the cell. 6 Transfer of a transposon or an insertion sequence from genomic DNA to another plasmid. Picture adapted from Malachowa *et al.* [110].

In the human gastrointestinal tract (GIT) a lot of different bacterial species live together. Many of them contains resistance mechanisms of which some are encoded by genes placed on MGEs. The MGEs can be exchanged between the same kinds of species and sometimes between different species in the conjugation process. Due to the large number of species in the GIT, there is a frequent opportunity to gain resistance genes at this location [111]. Because the spread of a mechanism through MGEs can be vertical and horizontal, and transmission can take place between the same species and different species, this makes MGEs a challenge in IPC.

1.7.2 Pathogenicity

Pathogenicity is the ability of a microorganism to cause disease, whereas the virulence is the degree of pathogenicity of the microorganism [112, 113].
The pathogenicity depends on several factors: the immune system of the host, and bacterial virulence factors such as toxins, capsules, and adherence factors.

Bacteria are divided into three categories:

- Primary pathogens, a probable aetiology of disease
- Opportunistic pathogens, may be the aetiology of disease
- Non-pathogenic bacteria, rarely cause disease

Depending on the host, the bacteria can change category – i.e., non-pathogenic becomes pathogenic.
The virulence is affected by different variables such as the number of infecting bacteria, the virulence factors of the bacterium, the route of entry into the body, and specific and nonspecific host defence mechanisms described in the chapter 'HAI risk factors' [112] .

Worldwide there is an increased focus on MDROs, not only due to the reduced treatment possibilities and difficulties in inventing new antibiotics, but also because of the MDROs increased pathogenicity and mortality compared to their susceptible counterparts [114–117].

1.7.3 Multidrug-resistant bacteria

Emergence of antimicrobial resistance is promoted by the use of antimicrobial agents. The use of antibiotics creates a selection pressure on the microorganisms, which means that only the resistant ones survive (Fig. 9) [118].

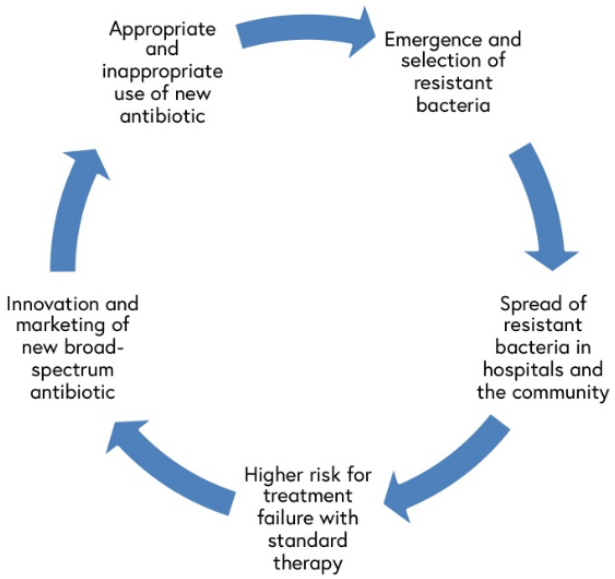


Figure 9: The vicious circle of antibiotic resistance. Adapted from ReAct [118].

Hospital outbreaks with antibiotic susceptible bacteria include methicillin-susceptible *Staphylococcus aureus* (MSSA), vancomycin-susceptible Enterococci (VSE), and cephalosporin susceptible *Klebsiella pneumoniae* [119].
All three bacterial species can convert from a peaceful co-occupant to an invasive enemy. They belong to the group of ESKAPE bacteria, which is an acronym for *E. faecium, S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* species [120]. These bacteria are capable of incorporating resistance mechanisms, thereby turning into a MDRO, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *E. faecium* (VREfm), and carbapenemase-producing Enterobacteriaceae (CPE). Furthermore, they are all capable of

surviving for prolonged periods in the hospital environment - enterococci can survive for several months in the environment without nutrient supply [121–123].

Acquisition of a resistance mechanism in bacteria take place in different ways and results in either a modification of the antibiotic target site, a decreased antibiotic uptake, or an inactivation of the antibiotic.
The bacteria either:

- have the resistance mechanism from the formation/inheritance (intrinsic)
- acquire the mechanism spontaneous through mutations in the genes (intrinsic)
- acquire the mechanism through horizontal transfer of resistance genes from other bacteria (extrinsic)

Horizontal gene transfer consists of genetic elements from species of its own kind (intra-species) or from other bacterial species (interspecies) as described above [103, 104, 124].

In the 1970´s, before widespread antibiotic resistance was introduced, outbreaks in hospitals were caused by non-resistant strains, especially penicillin- and methicillin-susceptible staphylococci [26]. Even though the World´s attention is on the MDROs, there is no reason to believe that outbreaks with susceptible species do not take place, although they are not discovered as easily as outbreaks caused by bacteria with unusual antibiotic resistance patterns [125].
HAI caused by bacteria with unremarkable antibiotic resistance profiles are usually interpreted as originating from the patient´s pre-hospitalisation microbiota, and transmission is therefore rarely detected. HAI caused by susceptible strains do also increase the morbidity, mortality, and the length of hospitalisation, and consequently the financial burden [20, 21, 26, 126]. Transmission of bacteria is therefore important to recognise regardless of bacterial resistance mechanisms. However, neither the frequency nor the costs of these unrecognised ongoing outbreaks are known.

1.8 Identification and typing of bacteria

To reduce the burden of bacterial infections, the most important steps in handling the sample are detection of pathogens, to determine the species, and to perform antimicrobial susceptibility tests.
To uncover the extent of transmission of a specific bacterial species in surveillance and outbreak investigation, the relatedness of the specific species in the involved patients must be determined. The relatedness can be investigated in different ways, as described below.

1.8.1 Classic/conventional clinical microbiology for bacteria

Patient samples are cultured on different substrates to achieve growth of bacteria. The grown bacteria are identified to species level by, e.g. matrix-assisted laser desorption ionization time-of-

flight mass spectrometry (MALDI-TOF MS). For bacteria with a pathogenic potential, antimicrobial susceptibility tests are performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST (www.eucast.org). Altogether this takes at least 36-48 hours [127]. The phenotypical characteristics (growth conditions, susceptibility) of the bacteria are often too non-specific to detect relatedness due to transmission. For this, methods with a high discriminatory power that can distinguish between closely related isolates are required. If the method is to be used in the routine laboratory or during outbreaks, it furthermore has to be rapid, easy to perform, inexpensive, applicable on different bacterial species, and have an internationally standardized nomenclature that makes it applicable within and between laboratories (intra- and inter-laboratory) [128, 129].

1.8.2 Mapping the microbiota

A relevant issue in relation to all the available typing methods is the uncertainty of the extent to which the method has in fact uncovered all the different types of each species present in the sample.

Another relevant issue in relation to transmission investigation is the prevalence of each of the types of each species that may be present in the human microbiota [130–132]. For *E. faecium* it has been shown that persons can be colonised with one or more types simultaneously [133]. Investigations and mapping of the common bacteria in the human microbiota has only been done to a limited extent, but there is a need to be able to distinguish between possible transmission and normal occurrence. Furthermore, mapping the microbiota of hospitalised patients are needed, because their microbiotas differ due to the use of antibiotics and due to the changed composition of bacteria that they encounter in hospitals [134].

1.8.3 Study of the genome

The development has provided new technologies that makes us able to study the genomics, transcriptomics, proteomics, and metabolomics – 'the omics' (Table 1) [135]. The omics creates the opportunity to investigate most of the biological molecules, identify pathogenic drivers, and personalise the medicine [135]. By investigating these technologies, the desired transmission-investigation method can be determined.

Table 1: 'The omics' and their interpretation.

Name	Interpretation
Genome	The total chromosome or sequences of nucleotides
Genomics	The sequence of nuclei acids/a genotype
Transcriptome	The transcription of DNA to coding messenger RNAs (mRNA) and non-coding RNAs (ncRNA)
Transcriptomics	The gene expression profile/intermediate phenotype
Proteome	The process of constructing the polypeptides from mRNA (translation), and the transportation to their cellular destination
Proteomics	The analysis of all the proteins in an organism at a particular time
Metabolome	The complete set of metabolites developed from enzymes catalysing biochemical reactions
Metabolomics	Analysis of quantitative and qualitative variations of the complete set of metabolites

Table created by use of EUSTM - European Society for Translational Medicine. *Translational medicine* [135].

Today, the most widespread method is still the study of the genomics.
For investigation of the genome, the microbiologic methods PCR, PFGE, Sanger sequencing, and WGS are highly specific. The methods can therefore be used for studying the epidemiology of bacteria.

WGS (whole-genome sequencing)

The discovery of the DNA structure as a double helix was made in 1953 [136]. In the 1970s, the first DNA-sequencing was performed, and in particular the first-generation sequencing method developed by F. Sanger, Cambridge UK became widespread and known as capillary electrophoresis sequencing or dideoxy-chain-termination sequencing. By this automated method one single DNA-fragment could be sequenced at a time [137].
During the 1980s, the first fully automatic sequencing machines were marketed, and large-scale sequencing experiments were carried out. The first complete bacterial genome to be sequenced was of a *Haemophilus influenza* and happened in 1995 [138]. Sanger-sequencing was the method of choice until the mid-2000s.
New methods for sequencing were continuously developed, and in the 2000s, next-generation sequencing (NGS) also called next-seq or high-throughput sequencing was introduced. Sequencing was carried out using different techniques such as pyrosequencing and massively parallel sequencing. As a critical difference to Sanger-sequencing the new methods could sequence millions of fragments simultaneously. Furthermore, the speed was increased, the reproducibility high, and at the same time at a reduced cost [129].
Today, third-generation sequencing such as nanopore DNA-sequencing is gaining ground. It continues to increase throughput with longer reads, improve assembly of chromosomal- and extrachromosomal DNA, increase turn-around time, and reduce the costs.

All the sequencing methods can be used for sequencing one or several specific targets and for whole genome sequencing. All the methods consist of a DNA-sequencing followed by different interpretation analysis. By using DNA-sequencing, the precise order of the nucleotides in the entire DNA or in a DNA-fragment is attempted to be determined. The entire DNA-sequence can be achieved by whole-genome sequencing, which means that not only the chromosomal DNA of the bacterium, but also the DNA-sequence of plasmids and other extrachromosomal DNA are extracted.
The sequencing results are used for assembly of genomes, genome characterisation, comparative genomics, phylogeny, and complete outbreak analysis [139].
Some of the interpretation methods of the sequence results are SNP, MLST, and cgMLST. These methods are described in detail in the 'method-section'. Using MLST, the isolate is assigned a sequence type (ST), while using cgMLST, a complex type/clonal type (CT) is assigned [140].
In 2017, use of cgMLST in Denmark had almost exclusively been used at the national reference laboratory, SSI, and descriptions of the use in real time outbreak analysis was almost absent. However, due to a suspected outbreak of VREfm at OUH, and since cgMLST was marketed as an easy-to-use method, it was decided to implement cgMLST and thereby investigate its real-time use for outbreak control at OUH.

Although the cost of performing WGS is decreasing, it is still an expensive method which many countries in the world cannot afford to implement at their hospitals. However, despite the high cost, studies have shown that the purchase and implementation of WGS equipment pays off to a great extent by reducing the duration of the hospital outbreaks and thereby save money [141, 142].

1.8.4 Other typing methods

MALDI-TOF MS

A method that has potential to be used as a part of the routine diagnostics for investigation of relatedness between species, is MALDI-TOF MS. This method is easy to carry out in the laboratories and at a low cost.
It is a method which generates a mass spectrum mainly from the ribosomal proteins [97, 143–145]. The method is used all over the world, including all Danish DCMs, for identification of bacteria and fungi.
The method is based on analysis of the proteome and metabolome products, but mainly the ribosomal proteins, nucleic binding proteins, and cold shock proteins in the mass range of 2,000 – 20,000 Dalton (Da) [97, 127, 146].
Regardless of the subtype, a part of the ribosomal proteins is always present and used in the ID-spectra, by which naming of the species takes place.
The method has been further developed to compare individual isolates of the same species, allowing estimation of relatedness using a reference library. By comparing the spectra's, the

method is thought to be able to detect if isolates are different, and hence indirectly detect if the strains could be identical. The spectra depends on the gene expression and regulation [97, 127]. Variability in the expression of the proteins may occur in the individual strain (intra-strain) and among strains of the same subtype (inter-strain) [145]. In using MALDI-TOF MS for typing, it is therefore very important to use proteins with a stability that allows them to be detected every time, as in the selection of genes for MLST and cgMLST.
The use of MALDI-TOF MS for isolate comparison is disputed. While some studies have published results indicating that the system allows a rapid (minutes) comparison of bacteria with sub-differentiation beneath the species level using actual or/and stored data from isolates of the same species, other studies state the opposite [147–149]. The system can be set to distinguish between different clones, allowing for immediate comparison of a new isolate with a panel of previously identified isolates. Isolation of bacteria with identical mass-spectra from different patients give rise to suspicion of transmission, and the bacteria can be further analysed by WGS [147, 148].

1.8.5 The future of the omics

The new technologies in omics have the potential to bring IPC at hospitals to a new level. The turn-around time for many of the bioinformatic technologies can be reduced to a couple of days, and since costs and complexity have been reduced significantly, they are suitable for clinical use and may reduce the overall costs through indirect savings [150]. However, due to the constant rising costs in healthcare and the rapid development of costly new laboratory technologies, it is imperative to find out which technologies that should be implemented for everyday use, and which still belong in the research laboratory. For this purpose, Translational Medicine (TM) may be of use in a common approach – with TM being defined as ‘an interdisciplinary branch of the biomedical field supported by three main pillars – bench-side, bed-side, and community’. ‘The goal of translational medicine is to combine disciplines, resources, expertise, and techniques within these pillars to promote enhancements in prevention, diagnosis, and therapies’ [135].

When evaluating typing systems, the European Society for Clinical Microbiology and Infectious Diseases (ESGEM) has a set of criteria for evaluation [151].
The criteria include:

- Type assignment cut-off value
- Typeability - the ability to assign a type to all the tested isolates
- Reproducibility - the ability to assign the same type to a strain independent on the occasion
- Discriminatory power (D index) - the ability to assign a different type to unrelated strains
- Concordance or agreement between typing methods
- Epidemiological concordance - outbreak strains are correctly grouped together

The cgMLST and MLST methods only include conserved genes in the genome, so for mapping the accessory genome – both in the chromosome and the extrachromosomal DNA, whole-genome MLST (wgMLST) or SNP may be used (described in the 'method-section').
Furthermore, there is a discrepancy between genes contained in the chromosome and genes expressed by the bacterium. This makes up some of the differences between a genotype and phenotype. It could be postulated, that the final potential function of the bacterium is the most important, and for this, the expression of the ribosomal proteins is a far better measure than the genes.
The development creates new possibilities which forces the clinical microbiologists to debate what is best to use in different situations – the genome or the proteome. Furthermore, studies of the importance of epigenetic changes are increasing, and studies of the metabolites may be the next step in increasing our knowledge of gene expression.

1.9 *E. faecium*

The bacteria in the genus *Enterococcus* are facultative anaerobic gram-positive cocci, arranged in pairs or short chains.
Enterococci are a part of the human and animal microbiota in the GIT but are also widely distributed in the environment. The genus consists of several species of which *E. faecalis* and *E. faecium* are the most prevalent in causing infections in humans. [121, 122].
E. faecalis can give rise to infections like UTI, abdominal infections, bacteraemia, and endocarditis. *E. faecium* has been described involved in almost the same kind of infections as *E. faecalis*, but is in contrast considered as an opportunistic pathogen more low-pathogenic, and often involved in infections with a foreign body or in patients with severe underlying illness [152–155].
Several risk factors are associated with *E. faecium* bacteraemia and includes high age, severe underlying disease, immune suppression, invasive devices, long duration of hospitalisation, and prolonged use of broad-spectrum antibiotics [66, 126, 152, 156–159].
The 30-day mortality of *E. faecium* bacteraemia has been reported to be from 24% to 66%, with the highest mortality if the isolates are vancomycin-resistant. The difference in mortality regarding presence of vancomycin resistance is disputed, as some studies detect no difference, if data are adjusted using the Acute Physiology and Chronic Health Evaluation (APACHE) II score and age [66, 69]. Regardless of presence of vancomycin resistance, mortality is higher than for bacteraemia with primary pathogens like *E. coli* and MRSA with mortality rates at 18-31% [160–164]. The 30-day mortality of *E. faecium* has been found to increase with a high APACHE II score and with presence of the same risk factors as mentioned above for acquisition of *E. faecium* bacteraemia - risk factors that all imply a critically ill state of health of the patient [158, 159, 164]. The mismatch found between the supposed pathogenicity, the risk factors of the patients detected with *E. faecium* bacteraemia, and the high 30-day mortality, raised the question and need for investigating to what extend death can be attributed to VREfm/VVEfm.

E. faecium is also a microorganism capable of surviving for long periods in extreme environments, e.g. it has been found capable of growth after month on plastic and after four years in a dried growth bottle [122]. Furthermore, it has been found that *E. faecium* may achieve reduced susceptibility to disinfectants such as chlorohexidine if the substance is used in a low concentration [165].
E. faecium has become a well-known hospital adapted microorganism all over the world. This is probably due to the hospitals being an ideal place for *E. faecium* to thrive because of the combination of frail patients, the antibiotic susceptibility profile, and the ability to survive in hospital environments with a high antibiotic pressure [91]. Due to the ability of *E. faecium* to survive in the environment, several outbreak studies have found it necessary to significantly increase cleaning efforts and to eradicate *E. faecium* from the environment to end the outbreaks [77, 166].

1.9.1 Antimicrobial resistance in *E. faecium*

E. faecium is intrinsically resistant to most of the β-lactam-antibiotics (penicillins, cephalosporins, and carbapenems) and has low-level resistance towards aminoglycosides. In addition, it has often acquired resistance to ampicillin, lincosamides, streptogramins, fluoroquinolones, and high-level aminoglycoside resistance.
The treatment possibilities include beta-lactam (ampicillin), glycopeptides (vancomycin and teicoplanin), oxazolidinone (linezolid), lipopeptide (daptomycin), and glycylcycline (tigecycline) [154]. The drug quinupristin-dalfopristin (Synercid) is a combination of macrolide-lincosamide-streptogramin, and is used as last choice of treatment in many countries [167].
Ampicillin was initially the preferred treatment for *E. faecium* infection, but resistance appeared in the 1970s-80s in the USA and approximately 20 years later in Europe [54, 152]. The glycopeptides such as vancomycin was used for treatment of ampicillin-resistant *E. faecium* (AREfm), but 30 years after the introduction of this drug, resistance appeared. With the occurrence of vancomycin resistance, other antibiotics were needed for treatment, and linezolid and daptomycin were used. Unfortunately, resistance mechanisms against all these antibiotics have today been demonstrated in the enterococci (Fig. 10) [168].

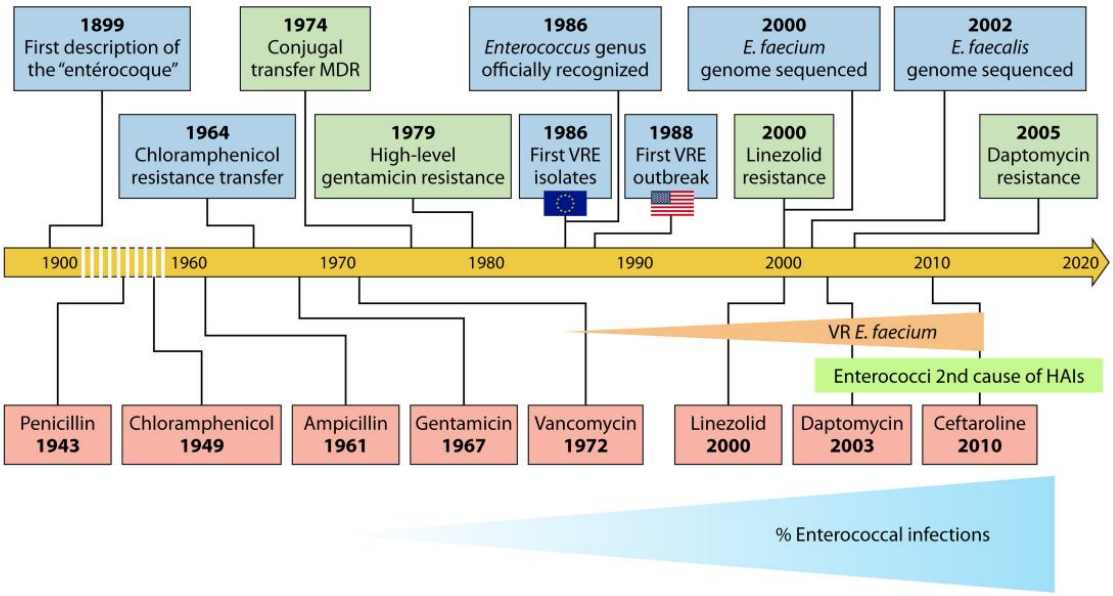


Figure 10: The history of enterococci. Relevant events in the history of enterococci as human pathogens (blue rectangles), appearance of antibiotic resistance (green rectangles), antibiotic clinical debut (red rectangles). Adapted from García-Solache M *et al*. [168].

1.9.1.1 Vancomycin resistance – the mechanism

Vancomycin inhibits the gram-positive cell wall synthesis by binding to a peptidoglycan precursor, and thereby preventing the cross-linking of the peptidoglycan.

The mechanism of vancomycin resistance in enterococci consists of a complex or operon comprising seven different *van*-genes with each its own function, but of which the expression of the *vanHAX* or *vanHBX* respectively are essential for the resistance to be functional (Fig. 11) [169]. The complex encodes enzymes that alter the peptidoglycan binding precursor and thereby reduce the affinity for vancomycin [168, 170].

There are nine different acquired operons or genotypes of glycopeptide resistance in enterococci (*vanA, B, D, E, G, L, M, N, P*), of which the *vanA* and *vanB* genotypes are the most prevalent in *E. faecium* detected in humans [70, 171]. Furthermore, operons with a deletion in the *vanX* gene have spread all over the World since 2017. The genotype is most often *vanA*, and the deletion in the *vanX* gene results in *E. faecium* being phenotypically susceptible to vancomycin, but resistant when treated, hence the name vancomycin-variable enterococci [47].

Based on the content of *van*-genes and the phenotypical antimicrobial susceptibility, *E. faecium* can today be separated into vancomycin-susceptible *E. faecium* (VSEfm), vancomycin-resistant *E. faecium* (VREfm), and vancomycin-variable *E. faecium* (VVEfm) [47].

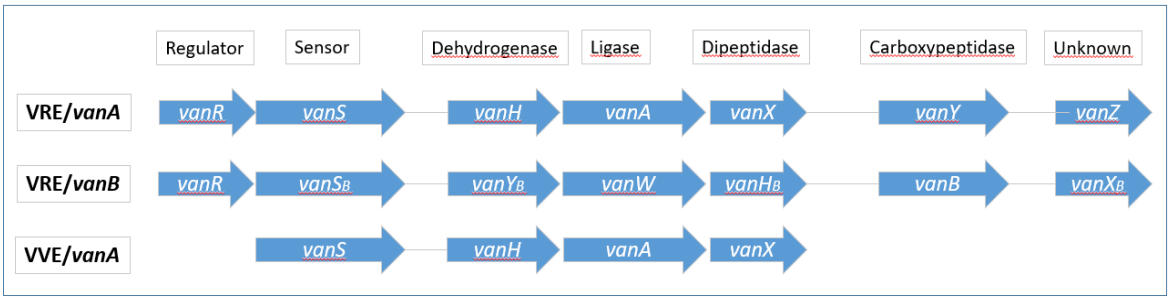


Figure 11: The vancomycin resistance complexes for Tn*1546* and Tn*1547,* and the corresponding genotypes.

The acquired vancomycin resistance in *E. faecium* can be mediated by different mechanisms located on plasmids and/or the chromosome.
The *van*-genes are often placed on mobile genetic elements which makes them horizontally transferable [70, 133]. The *vanA* gene is located on a transposon (Tn*1546*) most often residing on a plasmid and can be spread both horizontally and vertically. The *vanB* gene can be divided in to two subgroups $vanB_1$ and $vanB_2$ carried by different transposons. The $vanB_1$ is the oldest occurring type carried by Tn*1547* and generally without the ability to transfer the mechanism. The $vanB_2$ is carried by Tn*1549/*Tn*5382* and is most often incorporated in to the chromosome and subsequently vertically transferred [172].

1.9.1.2 Vancomycin resistance in other bacterial species

Some environmental microorganisms are known as natural producers of glycopeptides, e.g. *Streptomyces toyocaensis* and *Amycolatopsis orientalis*. For self-protection these microorganisms also contain the antidote - the *van*-genes. It is assumed that these microorganisms are the origin of vancomycin resistance in the human species [173]. Several non-enterococcal gram-positive human species belonging to the intestinal microbiota harbour the $vanB_2$-gene, thereby increasing the risk of transfer of this resistance mechanism between several different gram-positive species in the GIT [173, 174]. Concerns have therefore been raised about vancomycin resistance being transferred to primary pathogens such as *S. aureus*. Complete vancomycin-resistant *S. aureus* (VRSA) was detected for the first time in 2002. It has afterwards been observed in 14 cases in the USA and in very few cases in other countries. The American VRSA contained the *vanA* gene on transposon Tn*1546* located on a plasmid. They all belonged to clonal complex (CC) 5, and there was no suspicion of person-to-person transmission [175, 176]. VRSA in Denmark has not been reported by The Danish national reference laboratory for antimicrobial resistance at SSI [177]. Another concern is vancomycin resistance in *E. faecalis*. However, a spread in *E. faecalis* strains has rarely occurred. In the few cases where resistance has been seen, the isolates all remained susceptible to ampicillin [178, 179].

1.9.1.3 Occurrence of vancomycin-resistant *E. faecium* worldwide

The first reports of vancomycin-resistant *E. faecium* came from hospitals in France and the UK in 1987, and from the USA the following year [180, 181].

In the beginning of the 1990s, only smaller hospital outbreaks of VREfm had been reported from the USA. However, from then on, a rapid transmission occurred, leaving VREfm endemic in all hospitals in the USA just a few years later. At the same time, the incidence of VREfm in the community in the USA kept low [69, 182–184].

In Europe, the situation was somewhat different. In the 1990s, a spread of VSEfm began in European hospitals, resulting in an epidemic situation in the late 1990s. This spread of VSEfm was followed by an increase in VREfm – almost twenty years later than in the USA (Fig. 12) [54].

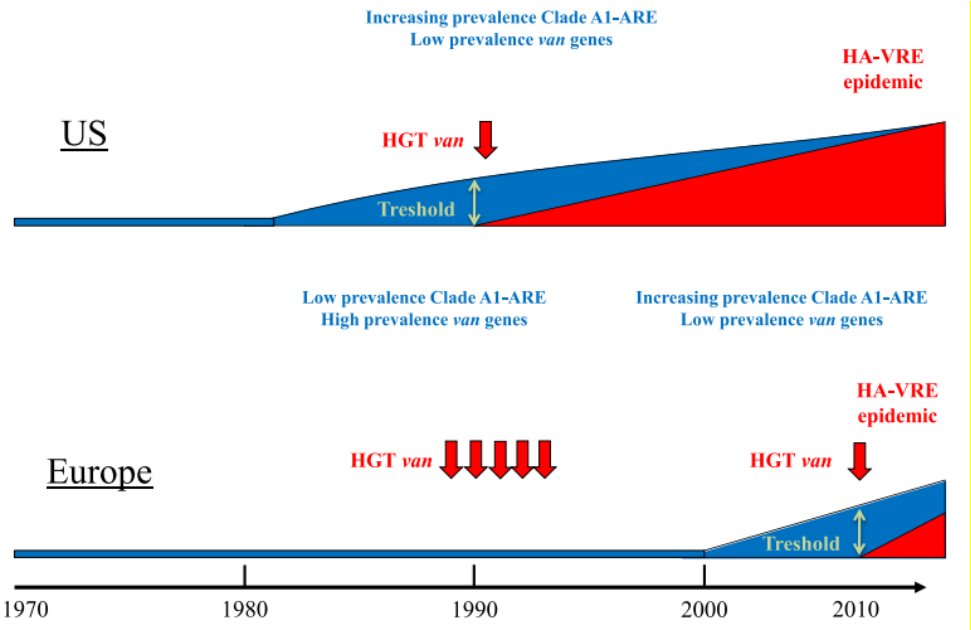


Figure 12: Course of events in the epidemiology of AREfm and VREfm and the differences between the United Stated (US) and Europe from 1970 till 2010. Blue: hospital Clade A1-VSEfm (AREfm). Red: hospital-Clade A1 VREfm. HGT: horizontal gene transfer (of van genes). Threshold: hypothetical critical number of hospital clade A1 AREfm strains needed for the introduction of van genes. Picture adapted from Zhou *et al*. [54].
(Clades are described in the thesis section 'Population structure of *E. faecium*').

Until 2000, VREfm was most frequently detected in isolates from farm animals and in non-hospitalised patients, but with a low prevalence in the European hospitals [70, 185]. Bates *et al*. detected the first non-human reservoir of VREfm in farm animals in the UK in 1993, although vancomycin had not been used for treatment of animals [186]. This study was shortly after followed by other studies describing VREfm in non-human sources like pigs, poultry, horses, dogs, birds, sewage etc. [187]. It was revealed that the glycopeptide avoparcin had been added to animal foodstuff. It had been used as a growth promotor in livestock animals like poultry and pigs in many counties since the 1970s. Danish studies investigated for a connection and detected that avoparcin selected for vancomycin resistance in *E. faecium* [188, 189].

The suspicion of avoparcin leading to a selection of VREfm in animals led to a total prohibition of the use of avoparcin in Denmark and Norway in 1995. Germany followed with a ban in 1996, and thereafter the rest of the European Union (EU) and some countries in Asia and New Zealand joined in [187]. A late ban was implemented in Australia in 2008, and in the USA avoparcin was never licensed [190]. In these two countries the vancomycin resistance in enterococci was not associated with the use of avoparcin as a growth promotor, but due to an extensive use of vancomycin in the treatment of MRSA [133, 191]. The level of vancomycin usage has been estimated to be five to ten fold higher in the USA than in Europe in the 1980s [173].
Despite the ban on avoparcin, VREfm continued to be detected in pigs in Europe. This was found to be associated with the continued use of the macrolide tylosin, co-selecting for the macrolide resistance gene *erm*(B) and for *vanA* [187].
E. faecium strains involved in hospital outbreaks were usually not found related to the non-human strains. However, it has been shown that the *vanA* gene can be transferred from *E. faecium* of animal origin to *E. faecium* in humans via the intestine. In this way, the non-human strains can become donors if the right circumstances are present, e.g. antibiotic treatment which promotes selection [187]. It is assumed that this explains what has happened.
In Europe, the number of VREfm declined rapidly in livestock animals after the ban of avoparcin in farm animal production. However, a decline in the incidence at the hospitals was not seen. In some European countries, the proportion of VREfm has remained low, while the proportion has increased in other [192]. It can be difficult to compare data directly due to differences in how the surveillance data is collected in each country [70].
Due to this increase in incidence and reduced treatment options, VRE has been placed in the high priority group of the WHO priority pathogen list for Research and Development (R&D) need of new antibiotics [193].

1.9.1.4 Vancomycin-resistant *E. faecium* in Denmark

In Denmark, the number of invasive VREfm cases has been surveyed in DANMAP since 2005. Furthermore, data on prevalence of VREfm bacteraemia has been distributed by the European Antimicrobial Resistance Surveillance Network (EARS-Net, ECDC) since 2000 [192]. Until 2010, the VREfm prevalence was less than one percent per year of the invasive *E. faecium* isolates [47, 192]. In the period 2010-2015, this prevalence increased to 5% (Fig. 13) [47, 192]. Furthermore, local hospital outbreaks were detected [170]. In 2019, a Danish study found approx. 0.4% of the patients in emergency departments in RSD colonised with VREfm [194]. In 2021, 9.4% of all invasive *E. faecium* isolates were resistant to vancomycin [47].

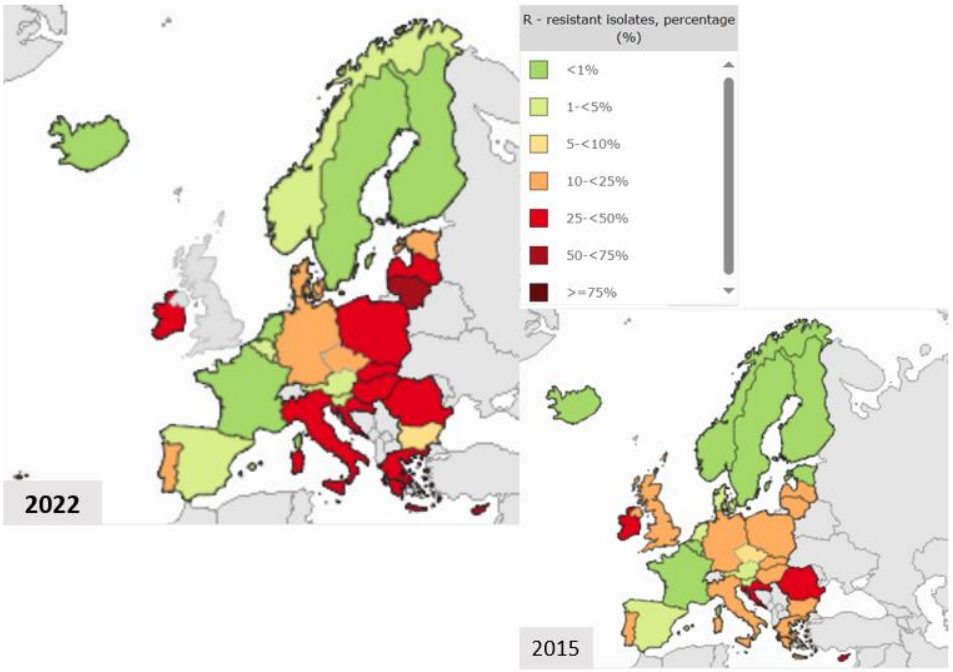


Figure 13: Surveillance data of vancomycin-resistant *E. faecium* in invasive isolates, Europe in the years 2015 and 2022. The pictures are adapted from EARS-Net, ECDC [192].

1.9.2 Population structure of *E. faecium*

In the beginning of the 2000s, ampicillin-susceptible *E. faecium* (ASEfm), ampicillin-resistant *E. faecium* (AREfm subsequently described as VSEfm), and VREfm were divided into host-specific lineages by use of amplified fragment length polymorphism (AFLP) [185, 195, 196]. One lineage contained the isolates from hospitalised patients and other lineages contained the isolates from non-hospitalised patients and animals. The AFLP groups were found to be host-specific, but with isolates from non-hospitalised healthy humans in every group [185, 195, 196]:

- Group A: pigs (and non-hospitalised humans)
- Group B: poultry (and non-hospitalised healthy humans)
- Group C: hospitalised patients (and non-hospitalised healthy humans)
- Group D: different animals but mostly pets (and non-hospitalised healthy humans)
- Group R: human isolates not fitting into one of the other groups

With the development of MLST in 1998 by Maiden *et al*., seven housekeeping loci or alleles were selected for use of typing *E. faecium*: *gdh, purK, pstS, atpA, gyd, adk* and *ddl*. These alleles were chosen due to a low ratio of variance, indicating that environmental selection did not interfere with these sequence variations [195]. It was found that some of the isolates of AREfm/VSEfm and VREfm designated to the C-group had become endemic. These nosocomial isolates mostly had the *purK-1* allele type and contained the *esp* gene – a virulence factor [185, 195]. These isolates were therefore considered a subpopulation of group C and named C1.

In 2004, an electronic version of the clustering algorithm based upon related sequence types (eBURST) was put into use together with MLST to predict the funding genotypes by dividing the isolates into clonal complexes (CCs) [197]. By using MLST and eBURST clustering, the C1 group was re-named CC17 [195].
The isolates of VSEfm and VREfm belonging to CC17 were associated with nosocomial outbreaks all over the world, and ST17, ST18, ST78, and ST192 were the most common types [152, 198–200]. Due to few endemic nosocomial clones of AREfm/VSEfm it was speculated if a few of the AREfm/VSEfm types belonging to CC17 had acquired vancomycin resistance and later on spread this mechanism through horizontal gene transfer of MGEs to many other clones [70, 185, 195, 201]. Only a few years after the introduction of eBURST, studies found that this algorithm was unreliable for phylogenetic use for species with a high recombination rate [202]. Instead, Bayesian Analysis of Population Structure (BAPS) was used to structure the relatedness of *E. faecium* [200]. By using BAPS and SNP trees, *E. faecium* clones were divided into different BAPS groups and clades/branches – clade A and clade B. Later, isolates in the clades were investigated using MLST. Clade A and B were found to have split up 1,000 -10,000 years ago, and clade A was found to have split into two additional branches (clade A1 and A2) around 75 years ago (Fig. 14) [203].

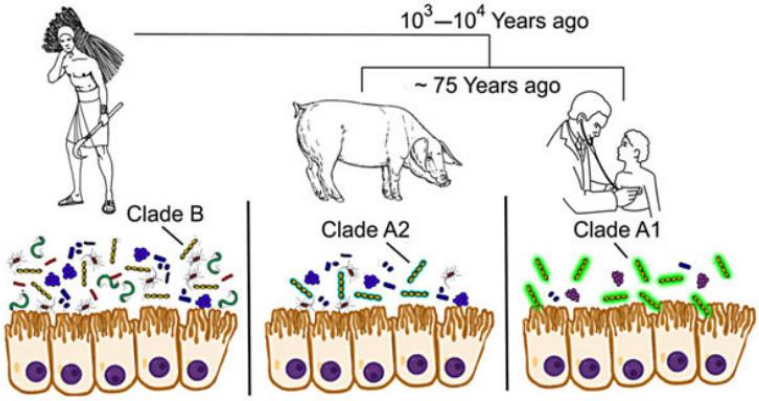


Figure 14: The split of *E. faecium* into two distinct clades in parallel with the urbanization of humans, and the animal lineage split simultaneously with the application of antibiotics in agriculture and human medicine. Picture adapted and modified from Wurster *et al*. [203].

Clade B contained the BAPS 1 group, consisting of all community-acquired isolates but also some hospital acquired isolates. The isolates were mostly ampicillin-susceptible, representing the commensals and having a high clonal diversity with many different *purK*-alleles and STs [152, 153, 185, 204, 205]. Based on molecular investigations the isolates in this branch have be reclassified as *E. lactis* [206].
Clade A contained the rest of the isolates. The isolates were genetically related, and included the isolates originally designated to CC17 [185, 201, 207, 208]. The CC17 isolates belonged to two different BAPS subgroups with BAPS 2-1 containing isolates belonging to ST78, among others, and BAPS 3-3 containing the ST17 and ST18 isolates.

Clade A was split into clade A1 containing strains detected as epidemic in hospitals, while clade A2 contained animal derived strains and strains causing sporadic human infections (Fig. 14) [200, 205].
By investigating *E. faecium* belonging to clade A1 (hospital-associated isolates) from the entire world, it was found that different clusters had appeared. Some were entirely locally distributed, while other clusters were distributed internationally (Fig. 15) [208].

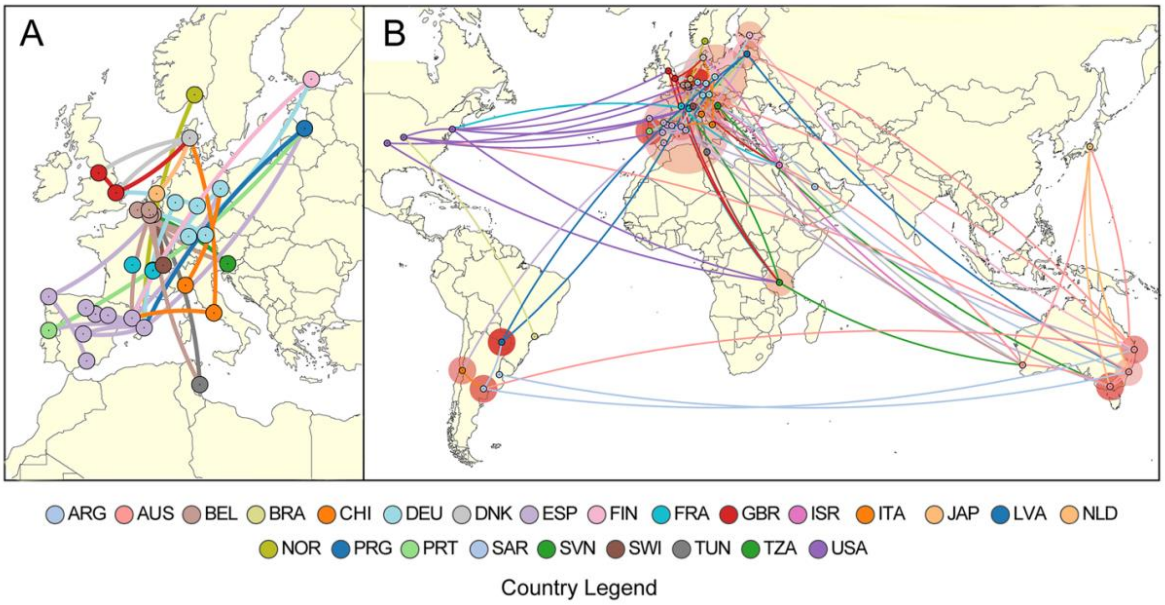


Figure 15: Dissemination routes of hospital-associated *E. faecium* in the World. Coloured nodes represents an isolate´s country of origin. Connecting lines are coloured by destination location. The circle size is proportional to the number of isolates that share the same continuous line of ancestry in that location. A) the spread across Europe, B) the dissemination across the globe. Adapted from van Hal *et al.* [208].

This division into clade A1 and A2 is consistent with the prevalence of MGEs being higher in the *E. faecium* strains adapted to the hospital environment than in the community strains [209].
The hospital-associated *E. faecium* have more virulence factors and higher genome plasticity than other strains, enabling them to succeed in colonisation and to thrive in hospital environments [210]. Furthermore, the dominant VREfm CTs are often equipped with more virulence factors than VSEfm. This also applies to VSEfm and VREfm with identical CTs [211].
Hospital strains compete with commensal strains in the GIT, and when patients are no longer hospitalised and the ecosystem changes, the hospital-acquired strains are replaced by the commensals [210]. It has been observed in a mouse-model, that *E. faecium* strains in healthy human microbiota can outcompete hospital-associated *E. faecium* strains, regarding persistence in the GIT [212]. In contrast, a Danish study from 2022 found that ST1421-CT1134 *vanA E. faecium* (VVEfm clone) had a significantly increased capability to colonise human intestinal cells compared to an *E. faecium* ATCC-strain (ATCC® 51559) [213]. Such a difference in colonisation capacity may

explain differences in transmission of community and hospital strains, but also the successful spread of specific hospital clones in humans.
Certain hospital-associated VSEfm has been found to open the way for dissemination of vancomycin resistance in *E. faecium*, by acquisition of either a *vanA* plasmid or a *vanB* gene, and thereafter establishing nosocomial outbreaks [70, 170, 200, 214]. In some countries, rapid spread of VREfm is thought to be caused by this kind of prior spread of AREfm/VSEfm clones, which upon subsequent introduction of a resistance mechanism, quickly shared the resistance mechanism among themselves [215–217].
In nosocomial outbreaks with VREfm clones where a close relationship to a susceptible clone (VSEfm) was not found, it was interpreted that the VREfm clone had been introduced to the hospital as a VREfm [170, 215]. Over time, the vancomycin resistance-mechanism would spread to many different clones, leaving a picture of multiple VREfm clones, but with a single or a few clones as the dominant ones [133]. This is thought to be the case in Denmark, where most of the detected VREfm clones were also found abroad, especially in Germany [218].

In 2015, a study revealed that MLST was not sufficient for phylogeny analysis on its own [216]. At the same time, a cgMLST scheme for *E. faecium*, developed by de Been *et al*. in 2015 was created. The cgMLST scheme was established by use of 1,423 target genes in the core genome of 40 *E. faecium* isolates, with these target genes being present in more than 95% of the 40 isolates [100].
Since the introduction of the cgMLST scheme the use of CCs and BAPS has almost been replaced by WGS with a combined MLST and cgMLST interpretation (Fig. 16).

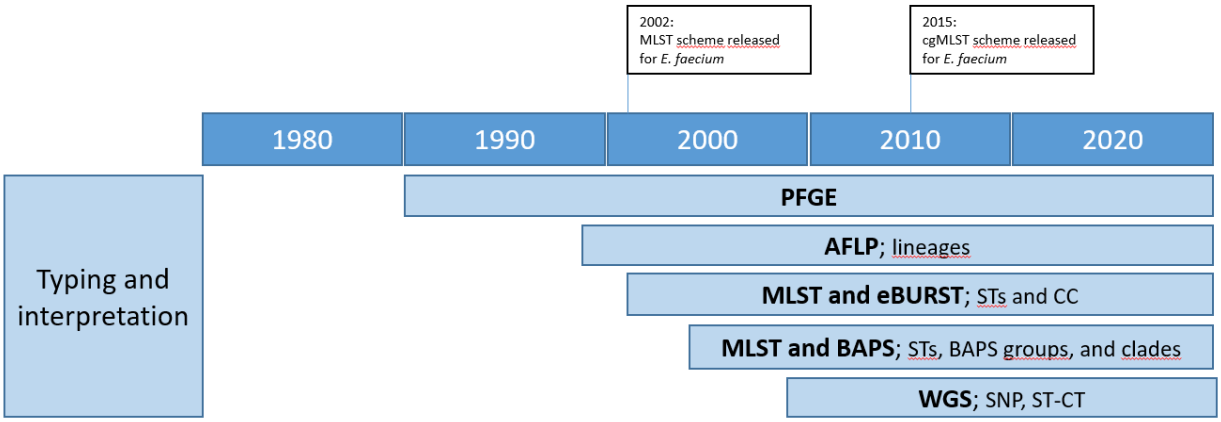


Figure 16: Use of typing and interpretation systems for *E. faecium.* Adapted and modified from Freitas *et al*. [210].

The cgMLST scheme created a possibility not only to investigate VREfm in more details, but also to increase knowledge of types and transmission of VSEfm and the association between type distribution of VSEfm and VREfm in hospitals. However, when this PhD took form, this had not been done yet.

To investigate for transmission across the World, the ST-CT and the *van*-gene content are very useful. However, there is no common database worldwide where this information can be found, which is why data must be found in publications or by contacting the national surveillance centre in each country.

1.9.2.1 Van-genes in Danish *E. faecium* isolates

In Denmark, a voluntary national surveillance of human VREfm isolates began in 2005. The isolates detected in 2005 were found to be imported from other countries and mostly containing the *vanB* gene complex [47].

From 2006 to 2018, the detected isolates were mostly *vanA E. faecium*, but with different sequence types (STs) [101, 198, 219].

In 2015, a *vanA E. faecium* VVE-clone appeared in Denmark, which in the following years came to dominate the picture. This clone, together with other *vanA E. faecium* clones constituted the majority of isolates until 2020/2021, when they were replaced by VREfm clones containing a *vanB*-gene (Fig. 17).

In 2022, the national surveillance laboratory at SSI reported that 92% of the investigated VREfm/VVEfm isolates contained a *vanB*-gene [47].

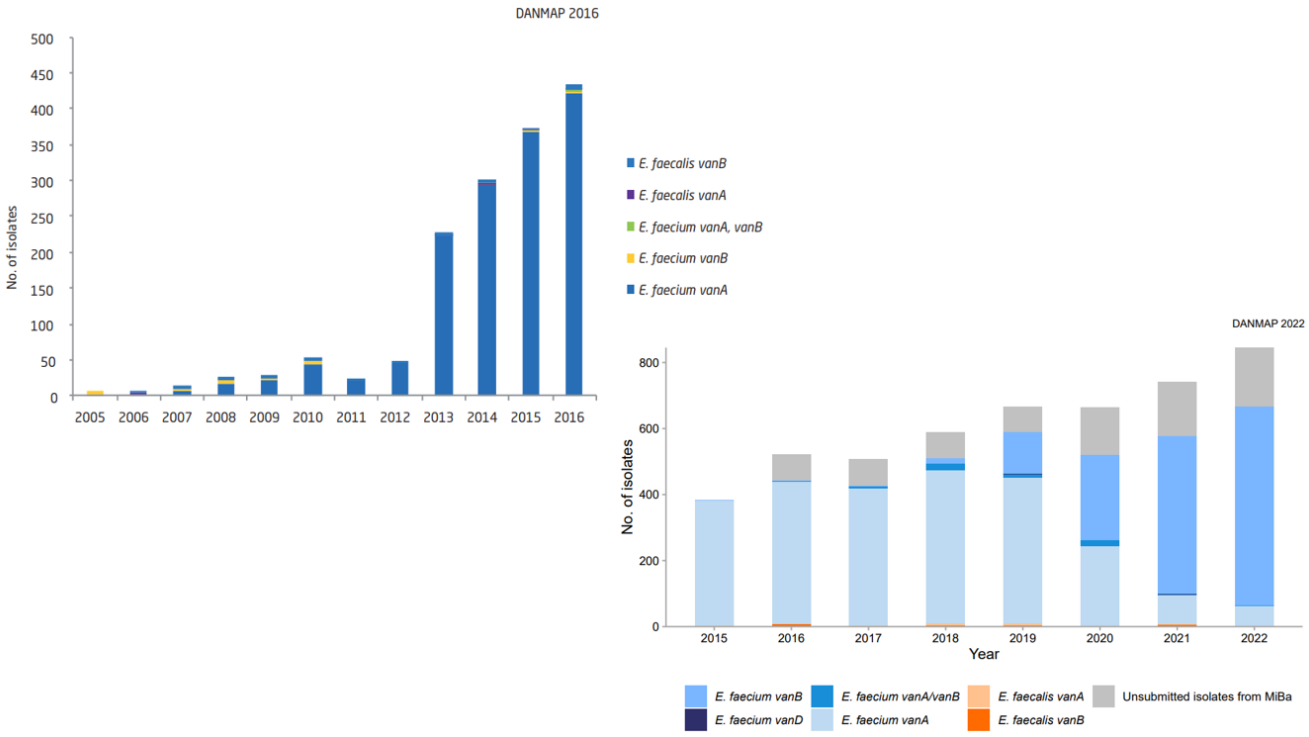


Figure 17: Numbers of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* isolates and *van*-genes from clinical samples in Denmark in the period 2005 to 2022. Pictures are adapted from DANMAP [47].

In the neighbouring countries, the dominant *van*-gene in detected VREfm isolates was slightly different over time (Table 2). Furthermore, a shift from *vanA E. faecium* to *vanB E. faecium* has also been seen in the Netherlands, just as in Denmark [220].

Table 2: Dominating v*an*-genes in *E. faecium* distributed on year and country.

Year	**Denmark** [47]	**Sweden** [221]	**Germany** [172, 222, 223]
2000-2005	*vanB*	*vanB*	-
2006-2010	*vanA*	*vanB*	*vanA*
2011-2015	*vanA*	*vanB*, but *vanA* in 2012	*vanA/vanB*
2015-2020	*vanA*	*vanA*, but *vanB* in 2018	*vanB*
2021-2022	*vanB*	*vanA*	*vanB*

1.9.2.2 ST-CT of Danish *E. faecium* isolates

Until 2005, all of the world, including Denmark, reported the most frequent types of AREfm/VSEfm and VREfm to be ST16, ST17, and ST18 [185, 198, 199, 224]. After 2005, reports on AREfm/VSEfm have been almost absent, which is probably due to focus being directed towards VREfm.
In the beginning of the year 2005, some of the most frequent VREfm types were reported to be ST18 and ST203. Later, ST117 and ST192 were added as the dominating types, and in 2012 ST80 also became high-incident [56, 83, 172, 211, 218, 222, 225–227]. In some countries, ST117, ST80, and ST17 have persisted until today, including in the neighbouring countries Sweden and Germany [172, 221–223].

In Denmark, the dominating VREfm STs shifted from ST203 to ST18/ST117, to ST117/ST80/ST192, and back to ST203 again [47, 101, 218]. Using cgMLST for typing the Danish isolates, the most prevalent VREfm types were ST117-CT24 (year 2010-2016), ST80-CT14 (year 2012—2015), and ST203-CT859 (year 2014-2018) (Fig. 18 and Fig. 19) [47].
ST117-CT24 *vanA E. faecium* has been reported from both Germany in the 1990s and from the Netherlands in 2014 [220]. Reports on ST80-CT14 from other countries have not been found [220]. ST203-CT859 was found to spread from Denmark to Sweden, the Faroe Islands, and Norway, but the country of origin of the introduction to Denmark is unknown [220].
In 2015, the VVE clone ST1421-CT1134 *vanA E. faecium* was detected for the first time in Denmark, and it spread nationwide during the next six years [47, 228]. This clone became the dominant clone in Denmark in 2018-2020. The origin of this clone is unknown, but a VRE ST1421-CT1134 *vanA E. faecium* have been detected in Asia and Australia [220].
During 2019 and 2020, a new increase of clones was observed. Especially ST117-CT36 and ST80-CT2406 were seen, both containing the *vanB* gene complex [47]. In 2021, the ST80-CT2406 *vanB E. faecium* clone superseded ST1421-CT1134 and became the new dominating type (Fig. 18 and Fig. 19) [47]. The first Danish patient detected with ST80-CT2406 *vanB E. faecium* had been transferred

from a German hospital, and the following Danish isolates of this type were detected in patients hospitalised in the same region as this index patient [220].

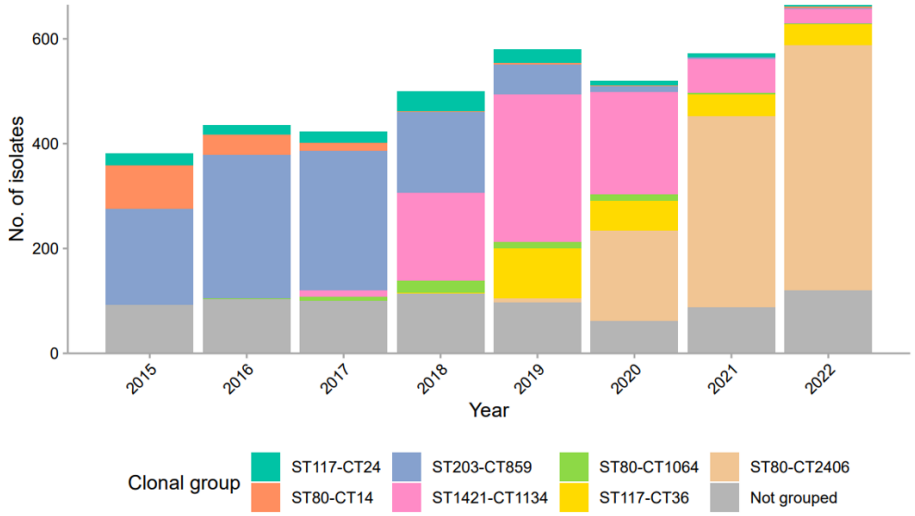


Figure 18: Timeline of the clonal group prevalence in all sequenced VREfm isolates in Denmark in 2015-2022. Adapted from DANMAP 2022 [47].

Types [a]	2016		2017		2018		2019		2020		2021		2022		All years
	(n = 435)		(n = 426)		(n = 518)		(n = 584)		(n = 519)		(n = 565)		(n = 609)		Total
ST117-CT24 group [b]	19	4%	20	5%	38	7%	26	4%	8	2%	7	1%	4	1%	147
ST80-CT14 group [c]	39	9%	16	4%	3	1%	3	1%	1	0%	0	0%	3	0%	147
ST203-CT859 group [d]	273	63%	265	62%	156	30%	57	10%	12	2%	3	1%	2	0%	952
ST1421-CT1134 group [e]	1	0%	12	3%	167	32%	285	49%	197	38%	63	11%	27	4%	752
ST80-CT1064 group [f]	2	0%	7	2%	23	4%	12	2%	13	3%	3	1%	2	0%	62
ST117-CT36 groupG	0	0%	0	0%	3	1%	95	16%	56	11%	43	8%	40	6%	237
ST80-CT2406 group [h]	0	0%	0	0%	0	0%	7	1%	174	34%	356	63%	468	68%	1,005
ST117-CT1686	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	15	2%	15
ST80-CT6438	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	10	1%	10
Other types	101	23%	106	25%	128	25%	99	17%	58	11%	90	16%	120	17%	927

a) ST, sequence type (MLST); CT, cluster type (cgMLST)
b) **CT24**,CT875,CT1180,CT1487,CT1834,CT2456,CT6018
c) **CT14**,CT869,CT1530,CT1797,CT2019
d) **CT859**,CT1051,CT1507,CT1688,CT2257,CT2758,CT5973
e) **CT1134**,CT1749,CT1854,CT2545,CT2911,CT3379,CT5936,CT6048
f) **CT1064**,CT2496,CT6123,CT6520
g) **CT36**,CT991,CT1526,CT2531,CT2659,CT2979
h) **CT2406**,CT2946,CT2949,CT3024,CT3234,CT4189,CT4835,CT5120,CT5143,CT5166,CT5211,CT5215,CT5928,CT5972,CT5974, CT5999,CT6117,CT6132,CT6253,CT6254,CT6417,CT6435,CT6435,CT6436,CT6494,CT6507,CT6531,CT6547,CT6598,CT6610

Figure 19: Description of the most common types according to MLST and cgMLST and including CT-cluster distribution in Denmark from 2016-2022. Adapted from DANMAP 2022 [47].

At OUH, we detected the first cases of VREfm in the year 2014. Apart from a single minor outbreak with ST80-CT993 (n=13), only sporadic findings were made until mid-2017. The dominating types were ST80, ST117, and ST203, all of which harboured the *vanA* gene [47].
In 2018, the VVEfm clone ST1421-CT1134 *vanA E. faecium* was introduced at OUH. It caused an almost endemic transmission in the hospital during the following years [47].

From 2020, an increasing presence of ST80-CT2406 *vanB E. faecium* was seen, and in 2023 it had outcompeted the ST1421-CT1134 *vanA E. faecium* (VVEfm clone) [47].

1.9.3 Treatment and infection prevention and control recommendations for *E. faecium*

1.9.3.1 Treatment of *E. faecium* in Danish hospitals

One of the most important tasks for a clinical microbiologist is to avoid treating bacteria detected in specimens, but instead to treat patients - i.e. to initiate a treatment where the detected microorganisms are in agreement with the clinical status of the patient. The complexity of this evaluation, the presence of catheters, the different patient composition at the hospitals, and differences between the Danish DCMs have probably led to the differences in the approach when detecting *E. faecium* in a blood culture, e.g.: no treatment, removal or change of a present catheter, removal or change of a present catheter including a single dose of relevant antibiotic, removal or change of a present catheter and treatment for seven days, antibiotic treatment until removal or replacement of a present catheter is possible, antibiotic treatment for several days to weeks if a catheter is not present (personal communications from the Danish DCMs).

1.9.3.2 Infection prevention and control recommendations in Danish hospitals

Until 2019, there was no national guideline on IPC to prevent the transmission of VREfm in Denmark, and each hospital used local recommendations. From 2019, NIR regarding TBP recommended that patients detected with VREfm/VVEfm should be isolated, but in making this decision, the sample material, presence of catheters or drains, and whether the patient had diarrhoea should be taken into account. Furthermore, screening was recommended if the patient was transferred from a hospital abroad or if the patient had VREfm/VVEfm detected within the last six month. This reflected and supported a multifaceted approach towards VREfm/VVEfm in the hospitals in Denmark [51].

In 2021, the IPC unit at OUH performed a questionnaire survey among all Danish IPC units to identify the IPC precautions used to control VREfm/VVEfm in the Danish hospitals. The following regimens were identified:

- Search and destroy approach with screening and isolation of multiple patients
- Screening and isolation of patients in high-risk departments
- Screening and isolation of patients with diarrhoea and at the same time receiving antibiotic treatment
- Screening and isolation of patients with diarrhoea
- No screening and isolation at all

Furthermore, in some hospitals, patients known to carry VREfm/VVEfm within the last 6-12 month were screened at re-admission.

Isolation was either in a single-room or as a cohort. For some hospitals, isolation was not used, but the patient was placed in a single-room with a specific allocated toilet in the ward.

Upon discharge, cleaning was either carried out by normal procedures, or normal procedures supplemented with a disinfection of the room, using either an alcohol-, chlorine-, or dihydrogen oxide-based product.

1.9.3.3 IPC measures against VREfm/VVEfm at OUH

At OUH, IPC measures against VREfm/VVEfm were gradually enhanced from 2018 until the end of 2021.
The IPC precautions were:

Screening

Screening was performed as a single rectal swap in case of:

- Hospitalisation of the patient outside the Nordic countries within the last six months
- Detection of VREfm/VVEfm in a fellow patient
- Re-admission within six months of the latest positive VREfm/VVEfm sample (clinical or screening sample)
- Suspicion of an outbreak in the ward (all patients screened)
- Repeated or sustained outbreaks in a ward (periodically screening of all patients on admission and at discharge)

Contact precautions

The contact precautions consisted of standard precautions supplemented with TBP consisting of isolation of colonised or infected patients in six months since the latest detection of VREfm/VVEfm. In isolation rooms, the staff use personal protective equipment (PPE) such as gown and gloves.

Cleaning and disinfection

The cleaning procedures consisting of water and soap were supplemented with use of:

- chlorine-based products in the daily disinfection of VRE/VVEfm isolation rooms
- chlorine-based products at the intervention/operation theatre after procedures on a VREfm/VVEfm positive patient
- dihydrogen-peroxide decontamination of the isolation room after discharge of a VREfm/VVEfm positive patient

The IPC precautions were continuously adjusted during the entire period to deal with local outbreaks. In some wards, frequency of cleaning of toilets was increased, and disinfection of frequently-touched surfaces initiated.
Standard precautions and antibiotic stewardship were emphasised and specific risk factors dealt with; i.e. replacement of rectal thermometers with ear thermometers.

The end of TBP against VREfm/VVEfm at OUH

In 2019-21, OUH used a lot of resources on screening, isolation, and cleaning procedures against VREfm/VVEfm. As the clinical staff observed very few infections with VREfm/VVEfm, and as there was an increased patient risk with the use of isolation, the IPC unit at OUH decided to investigate the need to maintain the VREfm/VVEfm IPC precautions.

A pilot study of the mortality of VREfm/VVEfm was initiated and revealed, that only a few patients received an antibiotic treatment, and that the 30-day mortality did not seem to be in agree with clinical findings of a VREfm/VVEfm infection.

Some hospitals in high-incidence countries, especially the USA, had fully or partially ended their screening and isolation regimes. By doing this, the number of VREfm patients increased, but stabilised within two-three years without increasing the mortality [229–235]. There had been no such investigation in a low prevalence setting such as Denmark, and it was therefore unknown if OUH could expect the same outcome.

Due to this information, the Antibiotics and Infection Control Committee (AICC) at OUH recommended a cessation of all specific infection prevention procedures against VREfm/VVEfm, which was implemented by the end of December 2021. Simultaneously, the committee was informed about the differences in *van*-genes and ST-CTs, and that the hospital had primarily been and was plagued by the ST1421-CT1134 *vanA E. faecium* (VVE clone). Furthermore, the committee was informed that an increasing incidence of ST80-CT2406 *vanB E. faecium* was detected. It was unknown whether a change in type or *van*-gene could lead to a changed clinical significance demanding a re-introduction of the specific IPC precautions against VREfm.

It was decided to follow the development closely in the following years and investigate the impact of ending screening and isolation.

2. Aim of the thesis

The overall aim was to investigate vancomycin-susceptible, vancomycin-resistant, and vancomycin-variable *E. faecium* (VSEfm/VREfm/VVEfm) to gain insights into epidemiology, transmission, and real time outbreak investigation by using cgMLST. Furthermore, the impact of ending screening and isolation of VREfm and VVEfm patients in a low-incident country was to be investigated.

The specific aims were:

- to investigate if cgMLST could be used in real time for IPC of VREfm transmission (Paper I)
- to investigate for unrecognised transmission of VSEfm by use of cgMLST (Paper II)
- to investigate if cgMLST data of VSEfm could be used to predict VREfm occurrence (Paper II)
- to investigate the clinical relevance of VSEfm and VREfm/VVEfm (Paper II and Paper III)
- to investigate the impact of ending screening and isolation of VREfm/VVEfm patients at a Danish university hospital (Paper III)

3. Materials and methods

A schematic overview of the studies and the related papers are found in Figure 20.

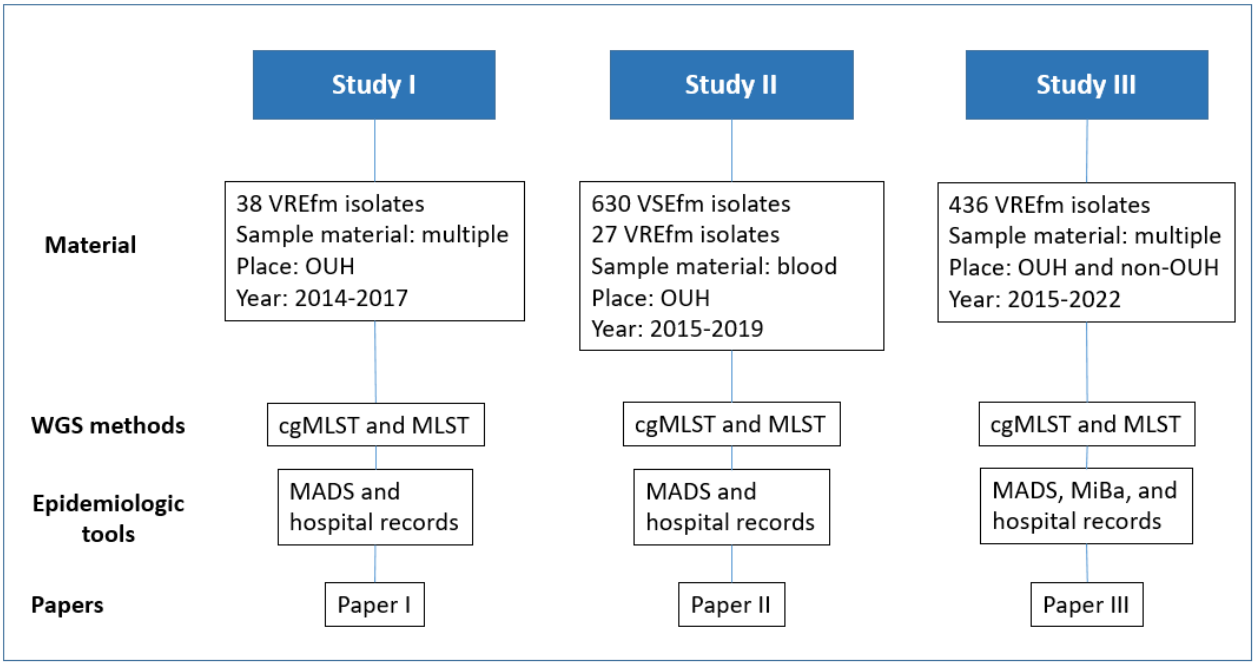


Figure 20: Schematic overview of the materials and WGS interpretation methods used in the three main studies.

A specific description of the materials and methods used in each of the studies can be found in the associated papers.
However, in general, the following sequencing and interpretation methods were used:
DNA purification was carried out using MagNa Pure 96 DNA and Viral NA kit (Roche) and Chemagic 360 CMG-1091 (PerkinElmer) instruments.
Nextera XT kit was used for library preparation and WGS was carried out using the MiSeq or NextSeq Illumina-platform with paired-end reads of at least 2 x 150 bp.
The draft genomes were processed in the pipeline bifrost, SSI (https://github.com/ssi-dk/bifrost). This pipeline performed de novo genome assembly using SKESA, quality control on the raw reads accepting an average coverage more than 30, species identification and detection of resistance genes using the CGE BAP (Center for Genomic Epidemiology).
If there was uncertainty regarding species identification or read-quality parameters, the isolates were submitted to PubMLST-rMLST (https://pubmlst.org/species-id).
The draft genome sequences were analysed by Ridom SeqSphere+ software (Ridom SeqSphere+ - Overview) using pairwise-ignoring missing values. Cluster distance threshold was set to 20 or less allele-differences.
CC, ST, CT, CT cluster groups, MSTrees, SLC, and epicurves were all obtained from Ridom SeqSphere+ software.

3.1 WGS

The methods used for typing of the genome can be divided into amplification and non-amplification based, with amplification being the process of copying the DNA.

3.1.1 Non-amplification based typing

3.1.1.1 PFGE (pulsed-field gel electrophoresis)

This method was developed in 1984 by Schwartz and Cantor, and is a highly discriminative typing method [236]. It can differentiate bacterial isolates on a strain level and is used as the gold standard in the countries that have not yet converted to WGS.

In this method the bacterial DNA is cut into relatively few pieces by using site specific rare cutting restriction enzymes, cleaving the DNA into fragments. Afterwards the fragments are separated according to their size in a gel using a pulsed electric field. This creates a specific bacterial fingerprint of bands. The fingerprint of the strain can be compared to other strains by either investigating the strains at the same time in the same gel, or by taking a visual print of the bands and compare it to other prints or databases, e.g. PulseNet [98, 237].

Pros and cons:

The method is a low-cost method in terms of equipment and reagents, but the use of multiple standardized protocols and variations in the used restriction enzymes makes the results inconsistent and difficult to compare between laboratories. Furthermore, it is a laborious time-consuming method [140].

3.1.2 Amplification-based typing

3.1.2.1 qPCR (quantitative polymerase chain reaction)

The polymerase chain reaction (PCR) is based on an amplification of a specific and known chromosomal or extrachromosomal DNA or RNA sequence (amplicon).

The method consists of a number of identical cycles in which a DNA-replication takes place, doubling the DNA. Normally 30-40 cycles are performed. The PCR can be made quantitative (qPCR) by using fluorophore labelled probes in real time assays. The initial number of amplicons in the sample can be measured by comparing the number of cycles needed to reach a predefined threshold with a dilution series with a known copy content [135].

The traditional PCR analysis is usually designed to detect or amplify a single specific gene, but by using multiplex PCR methods, several genes or targets can be amplified and/or detected in the same run. The analysis can be used for detection of specific genes whatever they are localised in the chromosome or extrachromosomal [135].

The PCR in itself is often not used for comparing isolates, but the replication of DNA is indispensable in the DNA-sequencing methods [140].

Pros and cons:

It is a fast and inexpensive method that can be performed on a daily basis.
As an independent method it can only detect known sequences and is laborious if there is a high number of targets and multiplex PCR is not used.

3.1.2.2 WGS Methods

The determination of the nucleotide sequence takes place in several steps, and especially the amplification and sequencing step have changed from first- to second- generation sequencing. As there is a constant development within the individual sequencing methods, the descriptions must be seen as overall descriptions.

1. Extraction and purification of the bacterial DNA

The extraction of bacterial material often takes place from a pre-cultivated colony.
The bacterial cells are lysed, and the DNA fragments are bound using magnetic beads. Unbound material is washed away, and the pure DNA fragments can be transferred for amplification.

2. Amplification and sequencing

The extracted purified DNA is amplified using PCR technology to have enough DNA material for the sequencing process.

1st generation sequencing (Sanger)

In the first-generations sequencing methods a single DNA fragment is sequenced at a time [137]. During amplification, a single primer, DNA polymerase, deoxy nucleotides, and the four types of nucleotides in a modified form (di-deoxy nucleotides) which can stop replication (stop nucleotides) are added to the purified DNA. These stop-nucleotides are each labelled with a fluorescent substance that can be read by machine.
During the amplification of the DNA fragment, many DNA pieces of different sizes are created, all beginning at the same place on the DNA strand of the fragment but ending in different places and with a different readable stop nucleotide.
Next, electrophoresis is used to separate the DNA pieces according to size. The size corresponds to the number of bases in the sequence. Using the fluorescence emitted from the stop nucleotide in the individual piece, the nucleotide type can be read and the order of the bases in the DNA fragment found.
If the full genome is to be studied all the DNA-fragments from each its Sanger sequencing must be assembled. The assembling process is described later in this section.

Pros and cons of Sanger sequencing:

The method creates reads of up to 1,000 base pairs have a high accuracy at a low cost [238].
The disadvantages in using the method for sequencing the whole genome is the low through-put. This is because only one DNA-fragment is sequenced at a time by this method, which significantly reduce the turn-around time for a full-genome sequencing.

A further disadvantage is that specific primers encoding a gene specific sequence are needed in the process.

2nd generation sequencing

The second-generation sequencing methods are known as short-read sequencing, next-generation sequencing (NGS) or just 'next-seq'.
By these methods several DNA fragments can be sequenced at the same time.
The most used method is probably Illumina - a sequencing-by-synthesis technology. Illumina is used for the further description of second generation sequencing (Fig. 21) [239].

Library preparation:
All the DNA fragments is cut by mechanical or enzymatic shearing to single-stranded DNA fragments each of a length of up to 300 base pairs (bp). Adaptors which is unique DNA fragment barcodes are ligated to all the DNA-fragments – each its own.
The DNA fragments are loaded to a flow-cell, where the attached adaptors are ligated by hybridisation to complementary oligonucleotides already covering the surface of the flow-cell like a carpet.

Cluster generation:
DNA-polymerase are added to the flow-cell and amplification of all the DNA fragments takes place. By this process a complementary strand of each fragment is created and the DNA fragments become double stranded. The DNA double-strand are cleaved, and the primary DNA strand washed away.
Once again DNA-polymerase are added and amplification of the remaining complementary strings takes place, creating double strands of each DNA fragment. The created double strands are separated, and both the forward and the reverse strand are used for the further doubling processes, by which a cluster of each DNA fragment are created.

Sequencing:
All the complementary strands are removed leaving only the forward DNA-strands in the flow-cell. DNA-polymerase, fluorescently labelled nucleotides, and primers specific for each DNA fragment adaptor, are added.
The reward strand of each DNA fragment is built or extended one base at a time. Each time a nucleotide is incorporated, the specific fluorescent dye is cleaved off and a fluorescent signal from the cluster is emitted.
A continuous reading of the fluorescent signals is carried out, which allows reading the sequencing-by-synthesis, and without subsequent electrophoresis.
After the process of building strands by using the forward strands (single-end reads), the forward strands are removed, and the process can be repeated using all the synthesised revers strands.

This is done to obtain longer reads and more DNA. The use of sequencing from both ends of the DNA strand are known as ‘pair-end sequencing’. By using the specific adaptors ligated in the beginning of the process, clusters of forward and revers strands are paired and used in the following alignment.

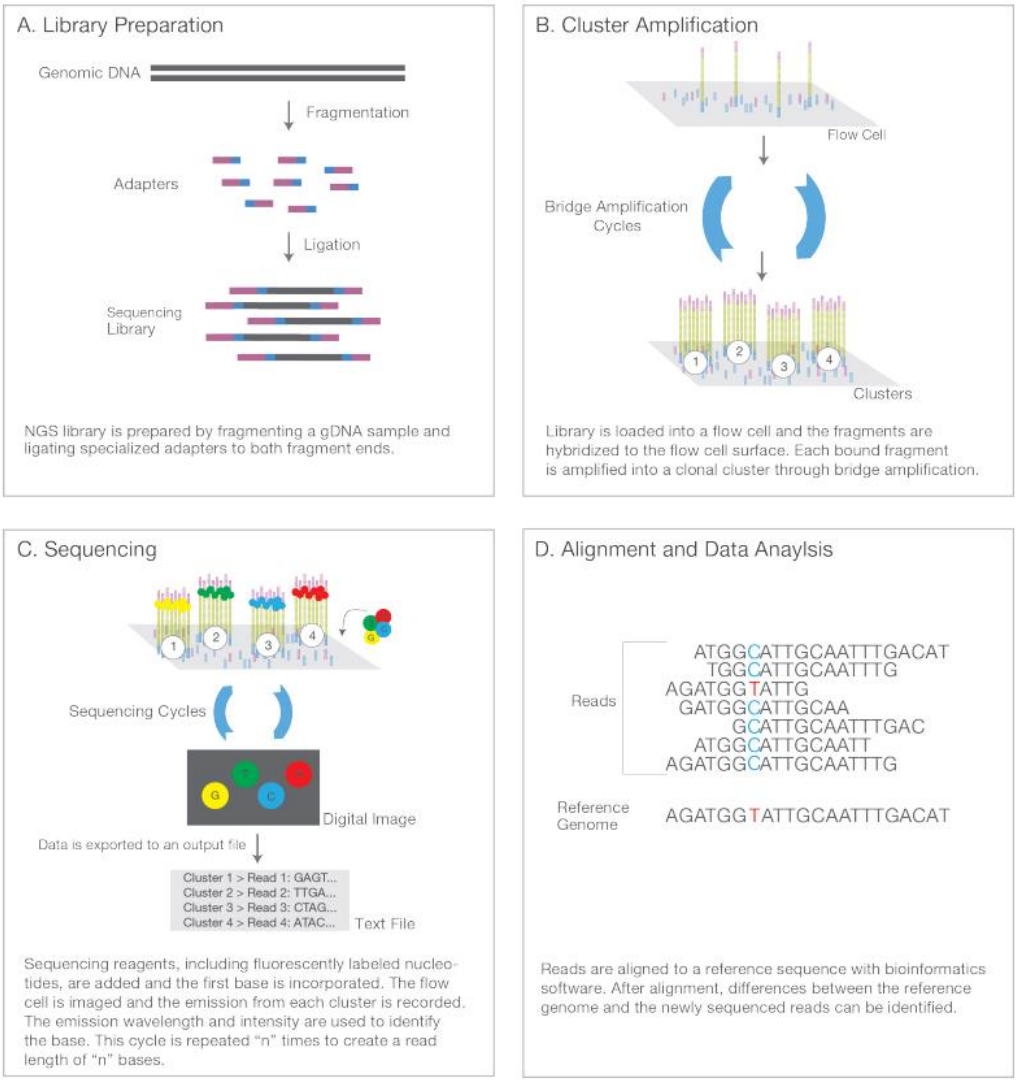


Figure 21: Whole-genome sequencing – an overview of the sequencing-by-synthesis. A: library preparation, B: cluster generation, C: sequencing, and D: alignment and data analysis. Adapted from Illumina [240].

Pros and cons of Illumina sequencing:

The method delivers high capacity and low turn-around time with thousands of genes sequenced simultaneously. The high genomic coverage and a high sensitivity to detect low-frequency variants is also ideal.

There is no need for specific primers in the process.

For use in comparative genomics there seems to be a problem with the contigs (see below) often ending with repetitive elements, affecting the possibility to study rearrangement and operons [140].

3. Assembling of the DNA sequence

The size of the bacterial genome varies greatly from species to species but is approximately 2,000,000 bp long. However, the sequencing techniques can only provide DNA fragments (reads) of 50 – 300,000 bp, depending on the sequencing method chosen (Illumina 50 - 300 bp) [139, 240]. The DNA fragments therefore have to be put together to an assembly. The reads are assembled into longer sequences (contigs) that put together represent the 'almost-complete' DNA-sequence – an assembly (Fig. 22).

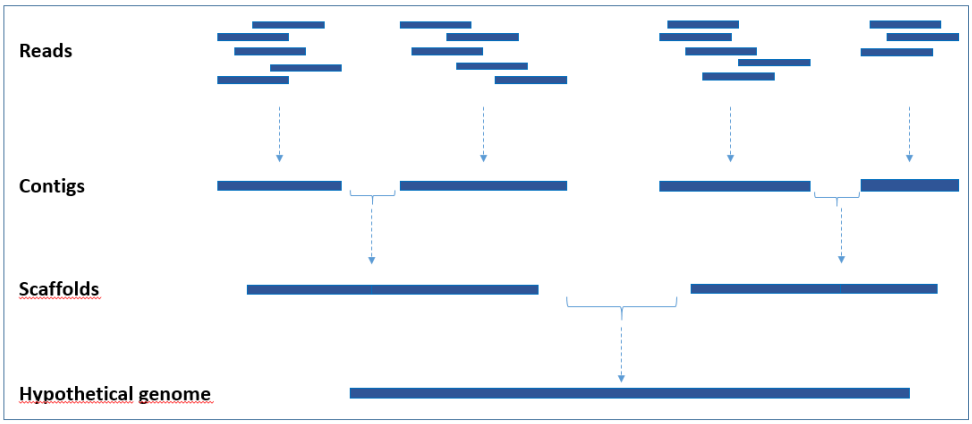


Figure 22: The process of assembling the hypothetical genome from reads to contigs and scaffolds.

In Sanger sequencing, the DNA fragment from each run must be assembled to obtain the entire DNA sequence.

In second generation sequencing, millions of overlapping DNA fragments of the entire DNA are created in the same run during the amplification process.

In both cases, the reconstruction is done by investigating how the nucleotides in the reads overlap each other – a bit like putting together a puzzle. It can be done either by alignment or by *de novo* assembling.

- *Alignment (reference genome assembling)*

If there already is a reference genome that can indicate what the DNA sequence should most likely look like and thus how the reads should be placed, this is called an alignment or reference genome assembling. It is similar to having a picture of the finished puzzle, and that the individual pieces must now be placed accordingly. Only an approximate matching can be done due to sequencing errors and natural variations, and the method can therefore be used for detecting assembly errors and biological differences [140].

There are many different algorithms used in different databases to carry out the comparison, and one of the most used is the heuristic Smith-Waterman algorithm.

The difference between sequences, also known as the distance, can be specified by either:

- Hamming distance: The number of substitutions to make the strain X identical with the strain Y, where X and Y have the same length
- Edit distance or Levenshtein distance: The number of changes (substitutions/insertions/deletions) to make the strain X identical with the strain Y. The strains X and Y do not have to be of the same length.

- *De novo assembling*

If a reference genome does not already exist, a *de novo* assembly is performed [139].
That is, there is no up-front picture of the final puzzle, so the pieces of the puzzle must be placed on a best effort basis.
Different methods can be used to find out which reads and contigs need to be assembled and whether gaps need to be inserted to put these together to obtain a near-complete sequence.
The different assembling algorithms that can be used for *de novo* assembling are: Naïve, Greedy, Overlap Layout Consensus (OLC) or deBruijn Graph assembling. Due to various challenges deBruijn Graph assembling is most often preferred.
The assembling can be done by various computer programs such as SKESA, SPAdes, Velvet, and Celera (Table 4) [140].

4. Quality control

After assembling the DNA, a quality check is performed on the full DNA strand, where base quality and coverage are found.

Base quality:

During the amplification and the subsequent DNA sequencing, several errors and several types of errors occur in the decoding of the DNA – e.g. the DNA polymerase is an enzyme that adds the complementary base when DNA is duplicated, and in this process errors may occur [240].
Using Illumina, the number of errors in base-calling during the sequencing is indicated by the base quality and can be given as a Phred-score (Q).
The Phred-score is given as a logarithmic scale ($Q= -10 * \log_{10} *p$), p=error probability:
Q_{10}: a risk of 1 error per 10 nucleotides
Q_{20}: a risk of 1 error per 100 nucleotides
Q_{30}: a risk of 1 error per 1,000 nucleotides
Low Q means a high risk of the base being wrong.
Q can be converted to an ASCE-II or Phred33 code used in the sequencing quality data information.

Coverage or depth:
The term ‘coverage’ is unfortunately used for different purposes in bioinformatics [241].
Coverage also known as ‘depth’ is an expression of the number of repetitions of the single base at the same position in each of all the reads that have been made (or the average number of aligned read fragments that cover a specific nucleotide at a specific place in the DNA sequence) [241].
Many overlaps allow for statistics to be used to calculate which nucleotide and thus which sequence is the most likely. The higher coverage the better because the probability of determine the correct nucleotide will increase.
The average coverage/depth can be determined for the whole genome as: the total number of all bases in all reads divided by the number of bases in the whole genome [241].
A lower limit is chosen for the average coverage/depth (often ≥ 30 if using an Illumina instrument) in terms of whether the DNA strand is found to be sufficiently valid for further use [240]. However, a different limit may be acceptable depending on the purpose of performing the WGS.
Another use of the term 'coverage' is as an expression of how large a percentage of the base pairs in the alignment sequence are detected in the test sequence - i.e. how large a match there is [241].

5. Interpretation
When the DNA sequence has been established, characterisation of the genome, comparison of bacteria isolates, and phylogenetic analysis can begin using different kinds of bioinformatic tools [139, 140].

The bacteria harbour genes that are conserved and genes with large variation. A conserved gene is a gene always present across species and at the same time with areas of strong variation between different species. If it is desired to use the genomic data for separating bacterial species from each other, it is necessary to use a gene that is present in all bacteria. Such a gene must be conserved and a gene encoding formation of the ribosome (rRNA) can therefore be used, e.g. the 16S rRNA gene. This gene is of 1,500 nucleotides/bases and contains areas that are variable between bacterial species [242].
If differences between the same species are to be investigated, other and more genes than 16S must be used. How much of the chromosome that is used for analysis depends on the end goal, the chosen analysis method, and the bacterial species (Fig. 23) [243].

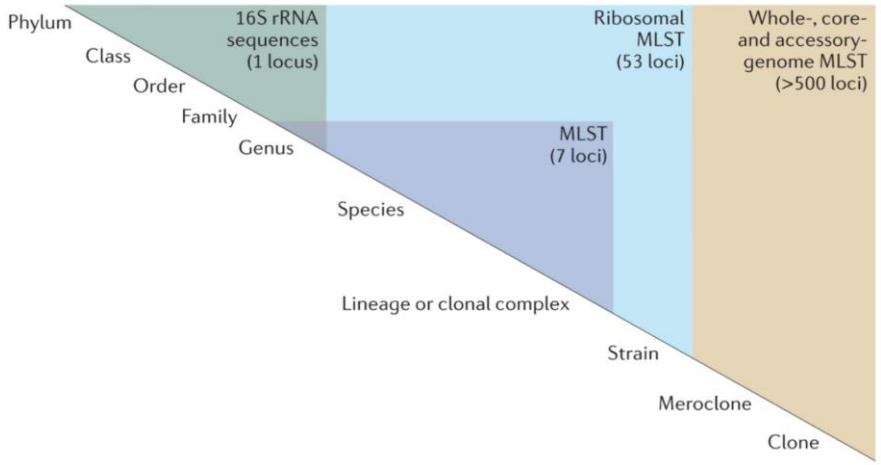


Figure 23: Sequencing data and the relation to nomenclature. Adapted from Maiden *et al.* [243].

a. Characterisation of the genome

The characterisation consist of the bacterial species identification and an indication of genes of clinical importance such as antimicrobial resistance and virulence [139, 140].
It is important to have in mind, that one thing is the genotype, another is the expression of the genome resulting in the phenotype of the bacterium.

Several databases can be used for the characterisation, of which the most common are:

- Species identification: NCBI BLAST, MLST web, and KmerFinder
- Virulence identification: VirulenceFinder
- Antimicrobial resistance: ResFinder, and PlasmidFinder

Some Web-based tools are pipelines containing both species-, virulence- and antimicrobial resistance identification [140]. The Center for Genomic Epidemiology (CGE) Bacterial Analysis Pipeline (BAP) are such a tool, developed by the Technical University of Denmark (DTU) [244].

b. Comparison of bacteria isolates

The comparison of bacteria isolates is used to determine genomic similarities and differences – most often between isolates of the same species (intra-species).
Some of the comparative methods used for intra-species investigations are among others single nucleotide polymorphism (SNP) based analysis, multilocus-sequence typing (MLST), ribosomal MLST (rMLST), core genome MLST (cgMLST), and whole-genome MLST (wgMLST) [140, 170, 239]. The methods differ in methodology, discriminatory power, and the ease with which they can be shared between laboratories. In rMLST the genes encoding ribosomal proteins are used as a subset of core genes, whereas wgMLST uses both core genes and accessory genes (Fig. 24) [243, 245].

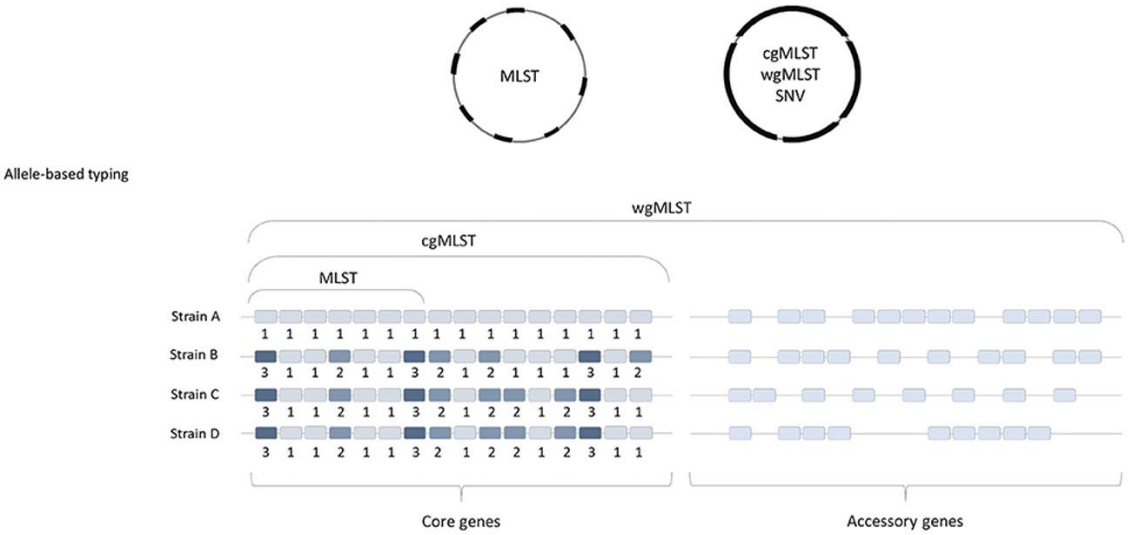


Figure 24: Allele-based typing and the difference in number of genes included for interpretation. SNV (single nucleotide variant) = SNP. Adapted and modified from Janezic *et al*. [245].

There are many pros and cons to consider before choosing which tools to use in a Department of Clinical Microbiology, and Quainoo *et al.* described these considerations very well in 2017 [140].

SNP (single nucleotide polymorphism)

The SNP analysis compares isolates by counting SNPs over the entire genome or part of it. It expresses the variation down to a single nucleotide in a specific position in the genomes.
The SNP method can be based on using either a reference genome or be non-reference based.
In case of recombination the divergent SNPs will be localised close to each other and lead to the number of differences in SNPs being 'falsely' high. This can be circumvented by using programs that discards/ignore altered SNPs less than a certain/fixed nucleotides distance apart from each other – a process called pruning.

The reference-based method:
A specific isolate is chosen as a reference genome. The number of differences in the base pairs mapped into this reference isolate, determines whether the isolates are alike or not. A threshold of ≤10-15 base-differences are often used, but a clear cut-off is not always present. Instead the proportion of differences can be given as a genetic distance [139].
There can be a problem in choosing which isolate to be the reference genome, and to ensure a high-quality reference genome. Elements that are not a part of or absent in the reference genome can lead to incorrect mapping [246].

The non-reference-based method:
When doing a reference-free SNPs calling, there is no reference genome helping to assign the base pairs to a specific and known genomic place [246].

SNPs between the isolates are identified by comparing their primary sequencing data – e.g. by aligning raw reads to assembled contigs [247]. The use of a non-reference-based method often suffer from misalignment, incompleteness, and errors in the raw reads and the assemblies [246].

Pros and cons:
SNP is considered to be the most accurate method due to the highest discriminatory power and are referred to as a Gold standard sequencing analysis method.
It does not focus exclusively on genes, but also includes the regions in-between.
Due to the counting system and the use of either a reference-based or non-reference-based method, it has some disadvantages as described above.
In an outbreak situation the collection of isolates is often a continuing process. If a new isolate is to be investigated for a match with all the existing different cases, all the different cases will have to be reanalysed together with the new isolate, and this makes it a time-consuming analysis.
Since no SNP databases exists and no uniform nomenclature is given the isolates, it is difficult to compare cases across hospitals and countries.
Finally, the operator will have to be well skilled.

MLST (multilocus sequence typing)

This method was introduced by Maiden *et al*. in 1998 and used for *Neisseria meningitides* as the first microorganism. MLST is a typing method based on DNA-sequence analysis of DNA fragments from several, but usually seven or eight housekeeping genes in the bacterium. The DNA sequences used for analysis can be obtained in two ways – either by WGS or by PCR amplification of each of the specific genes followed by sequencing of the PCR products. Instead of using the single nucleotide in the sequences for comparison, MLST uses alleles as unit [98].

Alleles:
From each of the selected housekeeping genes a specific DNA fragment of approx. 400 - 600 bp is used for analysis and named an allele. Each of the alleles is given a number and a MLST type is indicated by the number composition of the (seven or eight) genes. Together the alleles are described as the allelic profile of the isolate, e.g. for *E. faecium* [195]:
Isolate A: *gdh-4, purK-1, pstS-1, atpA-3, gyd-2, adk-1, ddl-1*
Isolate B: *gdh-4, purK-2, pstS-4, atpA-3, gyd-2, adk-1, ddl-1*

The allele-number or profile is converted to a clone type known as the sequence type (ST).
A change in the nucleotides of an allele, regardless the number of nucleotide-changes, is counted as an event, and results in a new allele number. This way of counting takes horizontal genetic transfer with insertion of DNA and thereby changes of many nucleotides into account [243].

A change in one of the alleles will result in a new ST-number. If no ST is provided by the bioinformatics tool used, the sequence can be submitted to the commercial company PubMLST.org, where a curator will provide the sequence with a new type/number.
The exact number of nucleotide differences between isolates with different MLST-number can be achieved by access to the sequence data.
Even though the housekeeping genes are conserved, there are variations in them creating many different alleles resulting in thousands of different allelic profiles. Because of the low mutation rate in the housekeeping genes the correlation between the mutation rate and time is expected to be constant.

It has to date not been possible to find a set of housekeeping genes that fits all bacterial species, but it is possible to create individual species typing schemes for almost all bacterial species. [98]. The first scheme was developed in 1998, and today schemes are available for many but not all species. The schemes are globally available through the web, which can ensure a uniform nomenclature and make it easy to compare isolates internationally [98, 248].
From the similarity of the allelic profile the STs can be grouped into clonal complexes (CC). CCs are groups of closely related isolates originating from the same ancestor, and sharing up to four identical alleles [98]. However, the use of CCs has almost disappeared after the introduction of cgMLST.

Pros and cons:
MLST provides a fixed ST-number which makes it is easy to use the method in a continuing process and exchange information between laboratories.
MLST is far less discriminative than SNP because the MLST analysis compares only DNA fragments from a few genes (seven genes in *E. faecium*). The method obscures the extent of recombination in the alleles with the same ST and can therefore hide a high degree of species diversity. This makes MLST inadequate for investigating nosocomial outbreaks [100, 140].

cgMLST

cgMLST has been developed as a highly discriminatory typing system based on WGS data and is an up-scaled or extended MLST [129]. Instead of using alleles of a handful of housekeeping genes for genotyping, this method uses alleles of hundreds to thousands of genes to create an allele type, called a cluster- or complex type (CT). The genes are selected if they are represented in the majority (95-99%) of the isolates of the specific species and include the genes used in MLST [100]. For each bacterial species, the number and which alleles to use for genotyping is indicated in an associated cgMLST scheme, e.g. for *E. faecium* 1,423 genes are used [100].
The first public software tool for cgMLST was launched in 2016. Today there are several providers of the cgMLST schemes (Fig. 25). cgMLST schemes for multiple species are under reconstruction, and the number of schemes keeps increasing. The schemes have so far been worldwide available.

Provider	Website	Publically accessible	Species
Enterobase	http://enterobase.warwick.ac.uk/	Yes	*Salmonella, Escherichia/Shigella, Clostridioides, Vibrio, Yersinia, Helicobacter, Moraxella*
Pasteur Institute	https://bigsdb.pasteur.fr/	Yes	*Klebsiella pneumoniae/ quasipneumoniae/variicola, Listeria, Bordetella, Corynebacterium diphtheriae, Yersinia, Leptospira Elizabethkingia anopheles/meningoseptica/miricola*
Ridom	https://cgMLST.org/ncs	Yes	*Acinetobacter baumannii, Brucella melitensis, Clostridioides difficile, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Klebsiella pneumoniae/variicola/quasipneumoniae, Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis/bovis/africanum/canettii, Mycoplasma gallisepticum, Staphylococcus aureus*
Applied Maths	http://www.applied-maths.com/applications/wgmlst	No	*Acinetobacter baumannii, Bacillus cereus, Bacillus subtilis, Burkholderia cepacia complex, Brucella* spp.
			Campylobacter coli - C. jejuni, Citrobacter spp., *Clostridium difficile, Cronobacter* spp., *Enterobacter cloacae, Enterococcus faecalis, Enterococcus faecium, Enterococcus raffinosus, Escherichia coli / Shigella, Francisella tularensis, Klebsiella aerogenes, Klebsiella oxytoca, Klebsiella pneumoniae, Legionella pneumophila, Listeria monocytogenes, Micrococcus* spp., *Mycobacterium bovis, Mycobacterium leprae, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus pseudointermedius, Streptococcus pyogenes*
INNUENDO/ chewBBACA	http://chewbbaca.online/	Yes	*Acinetobacter calcoaceticus/baumannii complex, Legionella pneumophila, Streptococcus pyogenes, Escherichia coli, Yersinia enterocolitica, Campylobacter jejuni, Salmonella*

Figure 25: Available cgMLST schemes and the providers. Adapted from Uelze *et al.* [249].

As with MLST, the differences between isolates are calculated in alleles instead of nucleotides. From species to species, a different threshold is used for the number of allelic differences needed to result in an epidemiologically unrelated isolate/a new CT. For each species an international stable threshold is available in the international schemes, whereas a self-selected threshold can be use locally. For *E. faecium* the international threshold is set to ≤20 allele differences [100]. cgMLST treats genetic mutation and recombination like MLST, and if a recombination affects multiple genes and thereby multiple alleles, it can cause a shift in the CT [100]. Because this scenario is high likely for species with a high recombination rate and due to the method used for CT name giving, CT-clusters have been established.

CT clusters:

CT clusters are a grouping of different CTs that have a distance less than a certain threshold between the nearest isolates with different CTs. It has been found necessary to invent these clusters due to the algorithm of CT creation, in which the order of the inclusion of isolates is important.

The CT-number is given by use of a founder and a threshold that triggers a new CT, e.g. ≤ 20 allele differences. If isolate A and isolate B has a distance with more than 20 alleles, they are given each its CT-number, e.g. ST80-CT1160 and ST80-CT2516.

When further isolates are detected and added to the data, it can turn out that isolate A and B are connected anyway but were considered different due to missing data/isolates (Fig. 26).

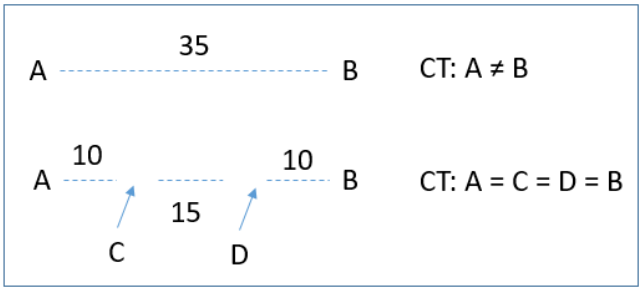


Figure 26: Allele distance between isolate A and B, and the assignment of cluster types (CT), depending on data from other isolates (isolate B and D).

Besides the problem with missing links, there can be a problem much alike with concerns the entry order of the submitted isolates. This is described by Ridom SeqSphere+ as shown in Figure 27.

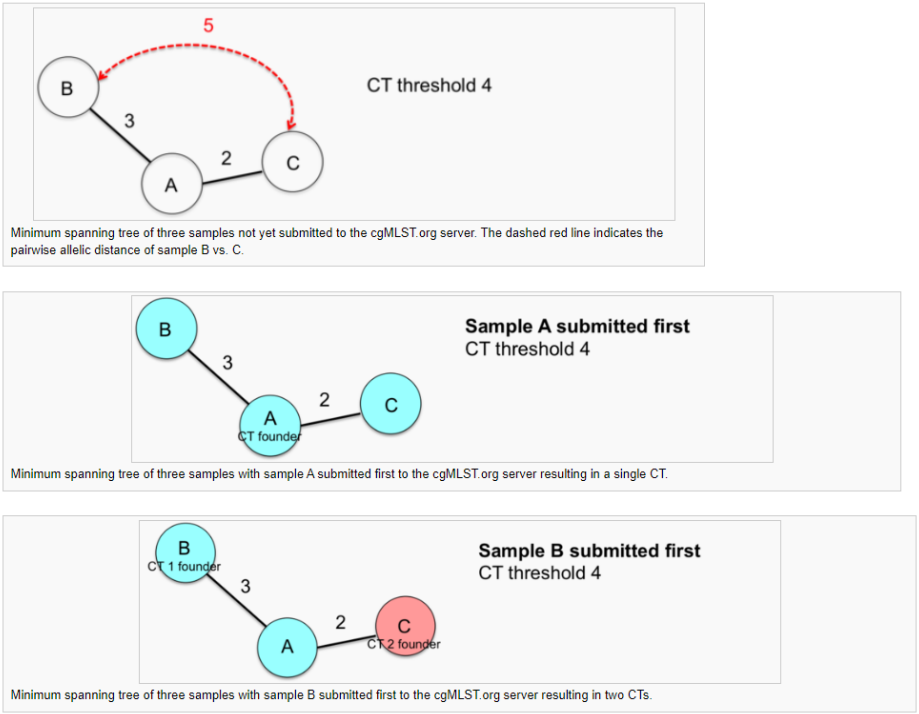


Figure 27: CT allocation and the importance of the order of submission of the isolates. Adapted from Ridom SeqSphere+ [250].

The difference between the MLST and cgMLST algorithms, consisting of a different number of allelic differences that trigger a change in type, results in isolates with the same CT most often, but unfortunately not always, having the same ST. An isolate can be given a ST but no assigned CT, if the complex type is either new or due to the requirements of a better DNA sequence [100]. The same accounts for an isolate given a CT but not an ST.
In a study from 2016, it was found that 25.3% of the *E. faecium* isolates required a repeated sequencing, due to low coverage and mixed cultures [141].
If no CT is assigned and the sequence is of good quality, it can be submitted to the commercial company that has provided the database e.g. Ridom SeqSphere+. The database will then assign the isolate a new CT.

Pros and cons:
Like the MLST method, it is easy to add new isolates to an already performed group of isolates in the cgMLST analysis software. Due to the CT number, cgMLST analysis makes it possible to compare types internationally, but also to create your own local types, though without a CT [140].

The high number of genes analysed has let to cgMLST being considered concordant to SNP analysis in evaluating the relatedness of bacteria. Together with the division of isolates in cluster types, cgMLST is expected to provide an effective and easy to use basis for rapid comparison of isolates, suitable for local outbreak investigation, infection surveillance both locally and from around the World [100, 129, 141, 251–254]. A disadvantage is, that it is only the core genome that is analysed. The method is therefore suitable for clonal but not necessarily plasmid-mediated outbreak investigations [255].

c. Molecular phylogenetic analysis

Phylogeny is the study of the evolutionary relatedness between species or organisms and are in the molecular phylogeny estimated from the genome characteristics. Phylogeny is used to cluster species or isolates and can be used to detect transmission networks.
Direct linkage can be estimated by using the ST-number, CT-number, or the SNP differences. Different algorithms are available for modelling the evolution, and the relationship can be visualised in branching diagrams having different tree-like structures (dendrograms). If the branch length is used to describe the evolution or changes the diagram are named a phylogram.

Phylogram – how to

The first step is to choose the sequences to be compared.
It is the one who indicates which sequences/isolates are to be used in the formation of the tree that must ensure that the data is sufficiently good for use - that it is the same species and that the number of errors in the amplification and sequencing are reasonable.
Next the sequences are aligned to secure that the sequences start at the same place before the differences are calculated.
Then a distance matrix is estimated, and the tree is clustered. This is done by preparing a distance matrix, in which the pairwise differences between the aligned sequences are converted to distances (Table 3):

- SNP: The number of differences in nucleotides across isolates are used as distance matrix
- MLST and cgMLST: The number of differences in alleles are used

Table 3: Distance matrix for the isolates S1 – Sn.
The numbers indicate the differences in base pairs between the sequences.

Isolate	S1	S2	S3	Sn
S1	-	4	9	xx
S2	4	-	12	yy
S3	9	12	-	zz
Sn	xx	yy	zz	-

Because it is not clear cut how much difference there can be in the number of alleles or SNPs for the isolates to remain identical, a genetic distance or the proportion of different alleles can be used instead: Genetic distance = allele differences/total number of genes shared by two sequences [139].
For investigating local linkages, it may be more convenient to use a threshold that differs from the standard, e.g.:

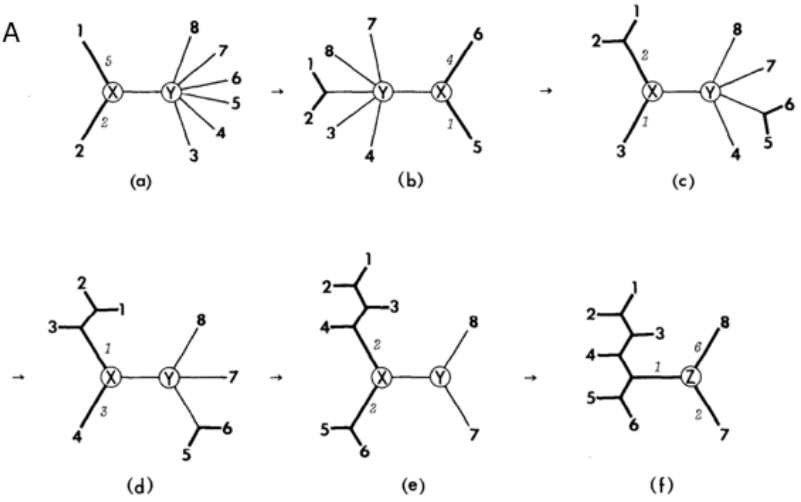
- A smaller threshold if transmission during a short period is suspected
- A larger threshold if transmission has taken place over a wide period and the microorganism has a high recombination rate

The distance matrix is used for hierarchical clustering.
There are several different ways to obtain hierarchical clusters: single-, competitive-, average- and centroid linkage. One of the most used in bacteriological phylogeny is the single-linkage clustering (SLC). In this type of clustering the distance between two clusters is defined as the minimum distance between members of the two clusters.
Using Ridom SeqSphere+ it is possible to create local SLC. The module allows to set a local minimum distance threshold used to create the CT clusters. This feature is appropriated in outbreak investigations where the distance is either to be smaller or larger due to the period of suspected transmission and the recombination rate of the bacterium.

Then the tree is to be rooted, by starting out deciding which kind of rooting that should be used. The nodes/or mark for an isolate can be placed in different ways in the tree, e.g. Outgroup, Midpoint, or unweighted pair group method with arithmetic mean (UPGMA).
The most common trees are created as neighbor-joining trees, minimum spanning trees, SNP trees, or nucleotide difference trees:

Neighbor-joining method/trees (NJTree):
This method uses the distance matrix and a Q matrix to continuously incorporate the isolate with the smallest distance matrix. The nodes are separated based on their average divergence from all other nodes (Fig. 28).

A



B

	OTU						
OTU	1	2	3	4	5	6	7
2 ..	7						
3 ..	8	5					
4 ..	11	8	5				
5 ..	13	10	7	8			
6 ..	16	13	10	11	5		
7 ..	13	10	7	8	6	9	
8 ..	17	14	11	12	10	13	8

Figure 28: A: Matrix distance and build of the associated neighbor-joining tree (NJTree). Italic numbers are the branch lengths. B: Distance matrix used for finding pairs of operational taxonomic units (OUTs = neighbors). Adapted and modified from Saitou and Nei [256].

Minimum spanning method/tree (MSTree):

A method that connects all nodes/isolates in a three-dimensional graph such that the sum of the lengths of the edges is minimised as much as possible. It can be created by using a founder and an allele threshold (Fig. 29) [257].

Different algorithms can be used to calculate the length, e.g. Kruskal´s algorithm and Prim-Jarnik´s algorithm.

Many different trees can be formed from the same data set, which is why MSTrees are not unique.

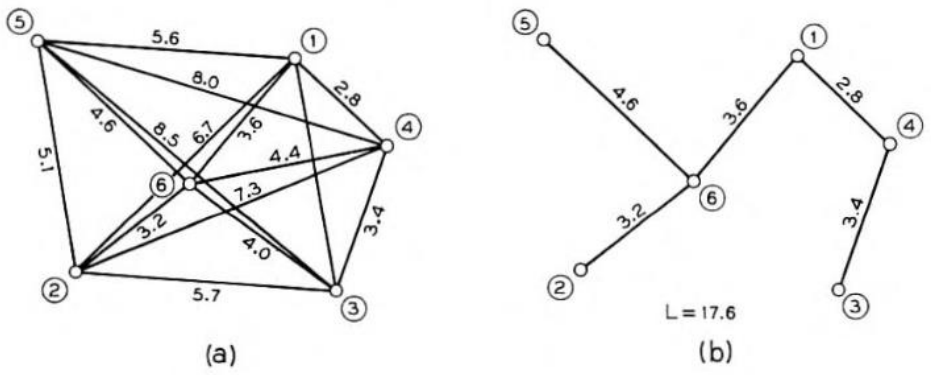


Figure 29: The build of the shortest spanning subtree of a complete labelled graph – an MSTree. (a) the distances between all isolates each, and (b) the shortest connection network. Adapted from Prim et al. [257].

Single nucleotide polymorphism tree (SNP tree):
Are made using the neighbor-joining method and SNPs for the matrix distance [140].

Nucleotide difference tree (NDTree):
This method uses k-mers. The genome is split into k-mers, which subsequently are mapped against a reference genome. Next the significance of each base call at each position are evaluated and z-scores calculated from the nucleotide differences in all positions. Then hierarchical clustering using matrix distance of the z-scores and UPGMA are used for building the tree [140, 258].

Phylogeny evolutionary algorithms
In the attempt to describe the changes during the evolutionary time, models are used to calculate the likelihood of phylogenetic events and create phylograms.
Many of the models are time reversible. This means that the models do not account for which sequence is the ancestor or descendant if the other parameters in the model are held constant.
Some of the used methods are as follows [259]:

Maximum likelihood estimation (MLE):
A method in which a sample is used to estimate the parameters of the probability distribution that generated the sample.

Bayesian methods:
A statistical theory describing the probability of an event. The probability is based on data and conditions related to the event.

Finally, the tree is drawn by using one of the many databases for this purpose, such as e.g. NEXUS, FigTree, MEGA or iTOL [140].

Outbreak analysis

An outbreak analysis can be done using all the different tools once at a time, to obtain species identification, resistance genes, and phylogeny but all-in-one software like BioNumerics, Ridom SeqSphere+ and the CGE BAP have been developed [140, 244]. It is also possible to program your own pipelines using both own and open-source programs. A challenge of using different bioinformatics pipelines including different sequencing platforms is that there is a risk of slightly different end results.

3.2 Upcoming genome sequencing methods

During the past four years the technology in this field has developed very fast, and third generation sequencing becomes available in many laboratories as well as the use of MALDI-TOF MS as a typing technique.

3rd generation sequencing

The third-generation sequencing techniques are known as single-molecule real-time (SMRT) sequencing but also as NGS like the second-generation sequencing methods. They create very long reads easing the process of assembling the DNA strand. This is highly effective in getting rid of the assembling problems of repetitive sequences, but unfortunately there is still up to 10% misreading that can lead to mismatches [260]. However, the technology is constantly being improved and misreading is being reduced [261].

The technology has a low turn-around time and are easy to use, but the equipment is still pricy. The technologies include the Oxford Nanopore Technologies (ONT) and the Pacific Bioscience SMRT technologies (PacBio).

Table 4: Pathway and sequencing tools from sample material to DNA analysis [139, 140, 238].

Name of the process/technique	Examples of tools	Algorithm
Bacterial extraction		Use of cell lysis buffer and magnetic beads
Amplification		PCR
WGS		
1. gen. sequencing ('shot-gun')	Sanger	Capillary electrophoresis sequencing also known as dideoxy chain-termination sequencing. Uses incorporation of fluorescent nucleotides.
2. gen. sequencing Next-generation sequencing (NGS) ('short-read')	454 pyrosequencing	Sequencing-by-synthesis method using registration of phosphate release when a nucleotide is built into the DNA. Registration is obtained by the enzyme luciferase emitting light when connected to phosphate. Registration happens by use of pico-titer plates. Reads up to 500 bp, fast.
	Illumina	Sequencing-by-synthesis or massively parallel sequencing. Uses barcodes on the fragments and incorporation of fluorescent nucleotides. Can be carried out as single- or paired-end sequencing.
	Ion Torrent Sequencing (ThermoFisher)	Sequencing-by-synthesis using the release of hydrogen-ions during the incorporation of nucleotides. The release is measured by pH changes in a fluid. Problems with detection of several identical nucleotide in a row.
3. gen. sequencing ('long-read')	Oxford Nanopore Minion	Single-molecule real-time (SMRT) sequencing. DNA is passed through a pore in a membrane. At the same time the resistance or current over the membrane are measured because each of the four specific nucleotide has its own ionic current. An advantage is very long reads at 50,000 – 100,000 bp, cheap, and fast. Disadvantage is the high error rate of 7%.
	Pacific Bioscience	SMRT sequencing obtained by feeding the DNA through an attached DNA-polymerase and use of fluorescent nucleotides. There is a high error rate at 10-15%, but because each position is sequenced multiple times, the overall error rate becomes less than 1%.
Assembling		
Reference based (alignment)	FASTA	Approximated Smith-Waterman algorithm that tolerates gaps in the aligned sequences.
	NCBI BLAST	BLAST (Basic Local Alignment Search Tool). Search for hits in the database that match the given sequence and is based on the Smith-Waterman algorithm.
De novo	Velvet/SPAdes/SKESA	De Bruijn graph-based assemblers (k-mer followed by Eulerian walk).
	Arachne/Celera/EDENA	Overlap Layout Consensus (OLC) framework (overlaps between reads identify contigs).
Quality Check		
	FastQC/BaseSpace	Base quality given by Phred-scores (Q).
Analysing data		
Identification		
	NCBI BLAST	Global align BLAST search.
	MLST web sever	Best matching of MLST alleles by using a BLAST-based ranking method.
	KmerFinder	Using k-mers.
Typing		
SNP calling	SeqSphere/BioNumerics/ CLC Genomic Workbench	Differences in the base pairs against a reference isolate, determines whether the isolates are alike or not. A threshold of ≤10-15 base-differences are often used.
MLST	PubMLST/SeqSphere/ BioNumerics	Allelic profile given by the composition of the chosen housekeeping genes. The allele-number or profile is converted to a clone type known as the sequence type (ST).
cgMLST	SeqSphere/BioNumerics	Allelic profile of hundreds to thousands of core genes named a cluster- or complex type (CT).
Phylogeny		
SNP	SNPtree/CSI phylogeny	Single nucleotide polymorphism (SNPs) and use of neighbor-joining method.
MLST	SeqSphere/BioNumerics	Minimum spanning trees or neighbor-joining trees.
cgMLST	SeqSphere/BioNumerics	Minimum spanning trees or neighbor-joining trees.
Resistance and virulence		
	ResFinder, PlasmidFinder, and VirulenceFinder (CGE website)	Detection of resistance genes. Detection of plasmid replicons from raw-reads or contigs. Detection of virulence by using the BLAST algorithm.

3.3 Which to choose and when

It can be difficult to choose the most optimal typing method and at the same time make sure to be cost-effective. To clarify the choice, Table 5 can hopefully be helpful.
For bacteria with a high recombination rate, the most ideal typing system may be the allele-based clustering techniques, because their systems take this into account.
For bacteria with low recombination rate, but with accumulated single mutations in multiple alleles, they appear to be as different as those with multiple recombination events. For these species, SNP analysis may be most appropriate [98].

Table 5: Typing methods and their characteristics. Adapted and modified from Zhou *et al*. [54].

Method	qPCR	PFGE	SNP	MLST	cgMLST	MALDI-TOF
Target	DNA	DNA (whole genome)	DNA (whole genome)	DNA (housekeeping genes)	DNA (core genome)	Proteins
Principle	+/- presence of a gene/genes	Macro- restriction analyses with +/- presence of DNA-fragments	Whole genome analysis of each nucleotide in the DNA	Allelic approach of 5-10 (7-8) housekeeping genes	Allelic approach of 1,300-2,000 core genes	Protein expression spectrums
Discriminatory power	Low	Medium/High	High	Medium	High	Low/Medium (developing)
Reproducibility	High	Medium	High	High	High	Low/Medium (developing)
Feed-back time	Hours	Days	Days	Days	Days	Hours
Data interpretation	Easy	Laborious	Easy	Easy	Easy	Laborious
Standardization	+	(+)	-	+	+	(developing)
Phylogeny	Fast	Slow	Slow	Fast	Fast	Fast
Ease of data exchange	Easy	Difficult	Difficult	Easy	Easy	Difficult
Needed databases	None	Local or curated databases	Local or curated databases	Curated databases	Curated databases	Local or curated databases
Cost in DKK[1]	Low	<500	500-1,000	500-1,000	500-1,000	Low

[1]The costs were estimated at the DCM, OUH in 2019.

3.4 Clinical data harvest

In Denmark, all citizens are assigned a unique identification number, which enables an unequivocal identification in administrative databases [262].

3.4.1 Clinical and screening samples

In studies investigating the clinical significance of a microorganism, it is important to distinguish between carrier status and infections.
Neither results from screenings nor clinical samples reflect whether there is an infection. However, it is more likely that an infection is present, if the microorganism is detected in a clinical specimen.

In Denmark, the isolates and associated sample material can be identified by using local and national databases, e.g. 'Mikrobiologisk Afdelings DataSystem (MADS, Aarhus, Denmark)' and 'The Danish Microbiology DataBase (MiBa)' [263, 264].

In the studies of this PhD, only clinical isolates were included.
A clinical isolate was defined as a VREfm/VVEfm isolate detected in any sample material, excluding isolates from rectal swabs, rectal wounds, and gastrointestinal stomas.

3.4.2 Clinical patient data

To investigate the clinical impact of the microorganism, each isolate must be linked to the clinical data of that individual.
The clinical data can be obtained in several ways, but is often achieved by extracting various parameters from databases, and more rarely by a personal review and assessment of each individual hospital stay.

In this PhD, the clinical data were retrieved by extraction from databases and personal examination of the electronic medical records (Cambio COSMIC (https://www.cambiogroup.com) and EPJ SYD) [265].

Mortality

Mortality is one of the most widely used parameters to indicate clinical significance. It is often reported as death within 30 days after the finding, referred to as 30-day mortality.
If the patient has several diseases at the same time, there may be problems with using the 30-day mortality as a measure, since it cannot be known which of the diseases the death is to be attributed to.

In this PhD, mortality of *E. faecium* was investigated using both the 30-day mortality and whether death could be attributed to *E. faecium.* The cause of death was investigated by examining the electronic medical records.

4. Results and discussion

The three studies in this PhD resulted in three papers (Fig. 20). A detailed description of the methods, results, and discussions can be found in the respective papers. This discussion will attempt to tie the findings of the three studies together.

4.1 Use of cgMLST for infection prevention and control

Why use cgMLST?

In 2017, an increasing number of VREfm at OUH raised the suspicion of an outbreak and spawned an IPC outbreak investigation. As part of the outbreak investigation, it was decided to try to implement WGS with cgMLST in our laboratory at OUH, with the aim of using it during the outbreak. This was to investigate whether the method, together with epidemiological data, could be used in real time as an aid in pointing out possible sources of the transmission and places in need of IPC interventions (Paper I) [1]. Only a limited number of studies had investigated the use of cgMLST during an outbreak for targeting IPC interventions, and mainly for post-outbreak research purposes [141, 266].

Could cgMLST in real time be used for IPC of VREfm transmission?

In Study I, using cgMLST in real time was found to be an important tool to narrow down the patients involved in an outbreak (Paper I) [1].

Together with epidemiological data, use of cgMLST further improved specifying the scope of the outbreak and targeted the IPC interventions in the outbreak management.

However, the execution of WGS and cgMLST required bioinformatic assistance and help to evaluate if the process and thus the data were valid. Furthermore, knowledge of the advantages and limitations of the method was needed. This need for technical and analytical skills has also been described by others as a bottleneck [267].

What were the pros and cons of using cgMLST?

In the investigation of phylogenetic relationships, cgMLST was easy to use, especially with regard to continuous inclusion of new isolates putatively involved in the VREfm outbreak (Paper I) [1]. The type-numbering (CTs) in cgMLST facilitated the communication regarding results internally and between collaborating hospitals compared to the use of SNP data. The CTs made it easy to use data from the national reference laboratory (SSI) to investigate for transmission from and subsequent spread to other collaborating hospitals in Denmark. Furthermore, it was easy to search for information about specific types from abroad, which is especially important in countries with many imported cases.

Because the *E. faecium* cgMLST scheme is used on *E. faecium* regardless of resistance profile, information on ST-CT must be supplemented with phenotype and gene content. In Denmark, the

ST1421–CT1134 *vanA E. faecium* was detected for the first time in the Capital Region in 2016. This type has been found in multiple places in the world, e.g. in Australia from 2015 [169, 228]. However, an important difference between the Danish and the Australian isolates is, that the Danish isolates are VVEfm, whereas the Australian are VREfm [268].

An important issue with using the cgMLST numbering is, that the exact number of the different CTs does not reflect how identical the sequences are, e.g. CT993 are not necessarily related to CT994, whereas CT880 may be related to CT5907. As explained in the method section, this is due to the CT numbering being sequential and thus determined by the time at which the genetically related isolates were submitted to the database. Ridom SeqSphere+ has acknowledged this problem, and they consider introducing a hierarchical CT concept, so that the similarity between the samples is indicated by the CTs in the future. However, this will not be implemented for the types that already exist, but only apply to new CTs (CTs that are given a high number) [250]. Isolates with different CTs can be highly similar, despite the different CT numbers. This is not only due to the sequential numbering method, but also due to the high recombination rate of *E. faecium*. Attempts have been made to solve the problem by introducing CT clusters, which can be helpful in the interpretation (Paper II and Paper III) [2, 3]. As far as I know, only a Danish national CT cluster scheme has been developed [47].
However, there are issues with connecting isolates into CTs and CT clusters. One is, if it is reasonable to connect isolate A and B, if they are connected by one or more isolates, and therefore have a pairwise distance far higher than the chosen threshold. In Study II, the ST117-CT24 *E. faecium* (VSE) and ST117-CT1180 *E. faecium* (VSE) should have been placed in the same CT cluster group, but due to a clear division of the groups and with only one single isolate with a distance of 19 alleles as the connection, it was chosen to separate them (Paper II) [2]. This is not an isolated case as the same problems were found in other investigations (personal communication, Anette M Hammerum, SSI). This indicates that any kind of sequencing data must be combined with epidemiological data in the interpretation for use in IPC.

To improve the interpretation of transmission, use of local SLC can be attempted. In Study II, it was only possible in a few putative transmission episodes to specify the epidemiological relationship by using local SLC with a reduced allele threshold compared to using CT clusters (Paper II) [2]. It may be difficult to estimate which allelic threshold is the most accurate to use, when also taking into account the recombination rate of this microorganism and the time span of the outbreak. This has also been found to apply if SNP distances are used [82, 101, 269]. This issue was even discussed back in the period when DNA restriction patterns obtained from PFGD were used [270]. Different outbreak studies have used different thresholds for defining *E. faecium* isolate relatedness. When using cgMLST for phylogeny, it might be necessary to have a floating threshold depending on the purpose of the investigation, the time span, and the recombination rate of the microorganism.

In the development of the cgMLST scheme by De Been *et al.,* a total of 40 VREfm isolates were used [100]. Due to the challenges raised above, it can be discussed whether the number of isolates was sufficiently large to cover the genetic differences of this species and thus sufficient for the selection of the number of genes and which to include in the typing scheme.

In previous studies, vancomycin resistance regarding the *vanA* gene was found to be located on a plasmid [172, 174]. When using cgMLST, spread of plasmids will not be detected, as the method does not include extrachromosomal DNA. Seemingly different isolates can harbour the same type of plasmid and thus be part of the same outbreak. If a clear relationship between the isolates is not demonstrated using cgMLST, it should therefore be considered to examine for matches in the extrachromosomal DNA.
In 2018, when Study I in this PhD was performed, only a few *vanA E. faecium* plasmids were fully characterised. Today, long-read WGS like ONT and PacBio can be used to overcome this obstacle, but at the time of study I, this was only in its infancy [267].
Another approach is to use the recently introduced target free k-mer based method 'split k-mer analysis' (SKA) on the CT cluster isolates [267, 271]. SKA uses isolate-to-isolate pairwise SNP distances, with SNP being defined as the number of k-mers detected in both samples, but where the middle base differs. A great advantage of SKA is the stable pairwise SNP distances over time [271]. However, this method also has some challenges, e.g. the threshold of the k-mer length.

4.2 Surveillance of vancomycin-susceptible *E. faecium* using cgMLST

Why investigate VSEfm with cgMLST?
When this PhD was initiated, the replacement of typing using PFGE with WGS supplemented with cgMLST for characterising *E. faecium* was well underway in Denmark. At the same time, the increasing prevalence of VREfm worldwide meant that studies of these were favoured over VSEfm, even though ampicillin-resistant *E. faecium* (AREfm) had previously given rise to epidemics [54, 272]. Due to this development, only few studies had characterised VSEfm using cgMLST, and information on the continued extent of transmission in hospitals including the types was scarce.
To study the development of VSEfm types, previous studies of AREfm can be used for comparison. These former studies have used other typing methods, of which MLST is the only method included in Study II. However, MLST does not provide sufficient discriminatory power for a detailed comparison.
Because VREfm/VVEfm arose from VSEfm, VREfm/VVEfm types can be used as representatives of the VSEfm types. However, since vancomycin resistance can be transferred horizontally with a possible subsequent clonal expansion, the detected VREfm types will most often only be the representative of some of the VSEfm types.

Which types of VSEfm were detected and were there changes from previous clones?

By using cgMLST, a high diversity of the VSEfm isolates was found (Paper II) [2]. This is in accordance with a Danish study from 2008 using PFGE and MLST for the investigation of AREfm [198]. Other European studies using PFGE and AFLP found a low diversity and a high clonality [195, 273].
Besides being a real difference, this divergence may be due to the number of included isolates and/or differences in the investigation methods.

CT clustering of the VSEfm revealed three large clusters in the investigated period - ST117-CT24, ST80-CT1160, and ST117-CT1180 (See Table 1 in Paper II) [2].
In Denmark, ST80 *E. faecium* (VSE) and ST117 *E. faecium* (VSE) were described for the first time in investigations including VSEfm and VREfm isolates from the period 2012-2015 [170, 198].
Both ST80 *E. faecium* (VSE) and ST117 *E. faecium* (VSE) have been described in studies from other countries, with ST80 being detected from 1997 and forward [207, 224, 274].
Of the former pandemic AREfm clones, the ST17 and ST18 were present [185, 198, 199, 224]. Both ST17 and ST18 were represented by several CTs in the beginning of the investigation period, but almost absent from 2017 and forward (See Supplementary in Paper II) [2].
However, only knowing the STs leaves doubt about the relationship between the STs identified in the past and the present. By using VREfm isolates investigated by cgMLST as representatives, both ST117-CT24 *vanA E. faecium* (VRE) and ST117-CT1180 *vanA E. faecium* (VRE) were detected in Denmark and Europe, whereas there was no data on ST80-CT1160 *E. faecium* (VSE and VRE) outside Denmark [275].

As a surprise, the sequencing of the VSEfm included in Study II revealed that some of the investigated isolates were phylogenetically clearly separated from the rest of the isolates. These 4.4% of the supposed VSEfm isolates turned out to be *E. lactis* (See Figure 1 in Paper II) [2]. This clear division of the isolates supports that *E. lactis* should be classified as its own or as a subpopulation of *E. faecium* [206].

Did transmission of VSEfm occur and in which departments?
In Study II, several clusters of VSEfm were found to be involved in putative transmission, particularly VSEfm belonging to ST117-CT24, ST80-CT1160, ST117-CT1180, and ST192-CT46. The putative transmissions occurred during the entire investigation period (Paper II) [2].
The most frequent localisations of transmission of VSEfm were in the Intensive Care Unit (ICU) and the Department of Haematology. This was also the case for the VREfm isolates investigated in the other studies (Papers I, II, and III) [1–3].
The wards involved is not a surprise. These wards have the most critically ill patients and a high consumption of broad-spectrum antibiotics, promoting an environment inside and outside the body where enterococci, regardless of vancomycin susceptibility, thrive. Furthermore, these departments and the associated diseases have been described as risk factors in several studies [158, 159, 161, 162, 164].

Can incidence of VSEfm number, CTs, CT clusters, or shifts be used to predict emergence of VREfm?
Previous studies have shown that widespread occurrence of VREfm is often preceded by a spread of the related vancomycin-susceptible ST-clones in the hospital environment [54, 170, 185, 276]. Furthermore, it has been suggested that the vancomycin resistance incorporates into strains of endemic AREfm [276]. Based on this, it was hypothesised, that the diversity of VSEfm ST-CTs would decrease before an introduction of VREfm/VVEfm, and/or that the number of single VSEfm ST-CTs would increase. This pattern would enable monitoring VSEfm and thus predict the risk of introduction of vancomycin resistance.

In Study II, a few dominating VSEfm ST-CTs were detected, but they were not found to be related to the emerging VREfm (Paper II) [2]. Furthermore, neither ST-CT diversity, incidence of specific ST-CTs, nor the total number of VSEfm prior to the introduction of VREfm/VVEfm were usable as predictors (Paper II) [2].
These results were not consistent with the above-mentioned studies. A reason for the divergent results may be, that the number of VREfm isolates included in Study II was small, and that only blood isolates of VSEfm and VREfm/VVEfm were used. Based on this, it can be debated whether surveillance should only include blood cultures, or whether it is more appropriate to monitor all sample materials. However, according to the national reference laboratory, SSI, the VREfm ST-CT types detected in Danish blood isolates are similar to the VREfm ST-CT types from isolates detected in other clinical sample materials (personal communication, Anette M Hammerum, SSI).

Due to the high prevalence of mainly four VSEfm ST-CT types localised in a few departments at OUH, it could be considered whether this was due to single events of introduction of each of the clones from another hospital followed by a clonal expansion or due to multiple introductions of each of the clones. A British study found multiple introductions of VREfm clones, whereas an introduction of a clone by a single index patient followed by an expansion in the entire hospital, has been described in an Australian study [82, 277]. The surveillance by DANMAP does not include VSEfm. However, of the four dominating VSEfm clones detected at OUH, the vancomycin-resistant counterparts (VREfm) were registered in the same years in the national surveillance. The ST117-CT24 *vanA E. faecium*, ST117-CT1180 *vanA E. faecium*, and ST192-CT46 *vanA E. faecium* were mostly registered in the Capital Region and Region Zealand, whereas the ST80-CT1160 *vanA E. faecium* was mostly detected in the Central Denmark Region (personal communication, Anette M Hammerum, SSI).

From many different places in the world, shifts in types and *van*-genes over time have be found, but the *van*-genes and types are not the same everywhere at the same point of time [172, 208, 211, 218, 277].
By investigating the Danish VSEfm during a five-year period in Study II, it was revealed that the most prevalent VSEfm were changing every second to third year (Paper II) [2]. Such a change may

explain differences in the most frequent STs between studies, due to differences in the investigation periods. As far as I know, periodic shifts have not previously been reported in relation to VSEfm, and periodic shifts of the most frequent VREfm/VVEfm types have only recently been described in a study from Denmark [220]. However, a recent Norwegian study has investigated the population structure of VREfm from 2010-2015, and it seems as similar shifts occur every second to third year as well [211].
A correlation between the changes in types of VSEfm and VREfm/VVEfm has to my knowledge not been described by any.

What could be gained from monitoring VSEfm?
Because transmission of VREfm/VVEfm and VSEfm ought to take place by the same transmission routes, conditions for transmission of VSEfm would also allow for transmission of the resistant counterpart if introduced - here VREfm/VVEfm. It was therefore hypothesised in this PhD that detection of transmission of VSEfm could be used as an indicator of the presence of risk factors that would also allow the spread of VREfm/VVEfm.

The investigation of VSEfm transmission at OUH using blood isolates revealed that putative transmission had taken place several times during the entire five-year investigation period (Paper II) [2].
Only a few cases of transmission of the same ST-CT of VSEfm and VREfm/VVEfm were detected, but a clear correlation of departments involved in transmission were found (Paper I-III) [1–3].

In a German study from 2017, daily molecular typing of MDROs was implemented and found effective for detection of pathogen clusters and faster IPC intervention [150].
This indicates that a surveillance of the antibiotic susceptible hospital adapted microorganisms can create a possibility to detect transmission and thereby intervene at a stage before an introduction of a multidrug-resistant microorganism, leaving the IPC one step ahead. Werner *et al.* stated 'descriptive is good, predictive would be better' [267].
The use of electronic surveillance systems of HAIs has been known for more than a decade [278, 279]. These systems should ease the data collection and thereby help in the interpretation of possible outbreaks. However, many of the systems need further development to become more optimal for use in IPC [280, 281]. The systems must be able to include all and not just some types of HAIs, the associated microorganisms, and at the same time have a low rate of false positives [49, 281].
Furthermore, to be used for transmission analyses, these systems must integrate epidemiological patient information (epi-data). The use of epi-data makes it possible not only to point out, who were involved in a transmission episode, but also to estimate where the transmission took place. In all three studies of this PhD, an increased benefit from using cgMLST data together with epidemiological patient information was found (Paper I-III) [1–3].

However, the results depend on the quality of the epi-data entered the system, understood as the fact that a given residential address is not always the same as the place where the patient actually is.
Furthermore, the transmission does not necessarily take place at the location from which the sample are requested. Patients are often transported inside the hospital for treatments that cannot take place in their ward. It has been found, that due to a high transfer of patients between departments within hospitals, patients can transmit microorganisms without the possibility of detection, leaving screening and targeted IPC interventions in only a few departments less relevant [82]. In some Danish hospitals, the individual departments are sized for fewer patients than present in peak periods. This gives rise to a widespread use of satellite/loan beds in other departments, and the patient is therefore moved between departments depending on where there is an available patient room. These stays and transports are difficult for an epidemiological system to include, and the sites of transmission may therefore not be found or misinterpreted.

4.3 Clinical impact of *E. faecium*

Why investigate the clinical significance of E. faecium?
A divergence between a high 30-day mortality from bacteraemia with *E. faecium* reported from several studies and a rare initiation of antibiotic treatment observed at OUH, generated a requirement to investigate the clinical relevance of *E. faecium* (Paper II and Paper III) [2, 3] . Due to the increased discriminatory power of cgMLST, it was investigated whether the clinical findings could be related to specific ST-CTs (Paper II and Paper III) [2, 3].
Patients with VSEfm from blood cultures were investigated in Study II, and patients with VREfm/VVEfm from all clinical sample materials (all-case) were investigated in Study III. To optimise the clinical investigation, clinical and para-clinical data from both microbiology databases and medical records were used. The patients were included in the studies with their first isolate of *E. faecium* in a clinical sample regardless of symptoms. The clinical impact was investigated afterwards. Individual symptoms were not used as parameters, but all the symptoms and para-clinical data were used in combination to give the full picture of the condition of the patient and assess the likelihood of infection and death due to *E. faecium*.

How did demographics compare to previous studies?
In agreement with most other studies, men were found more likely than women to have VSEfm or VREfm/VVEfm in blood cultures [162, 282, 283]. However, by including all sample materials with VREfm/VVEfm, more women than men were detected with a clinical VREfm/VVEfm isolate (Paper III) [3]. This appeared to correlate with the number of urine samples, and may be explained by bacteriuria occurring more frequently in women [284]. In the European guideline for urinalysis, the concentration of colony forming units (CFU) that justifies identification and susceptibility test regarding *E. faecium,* varies depending on the presence of a catheter [285]. This is probably due to an interpretation of *E. faecium* as a coloniser rather than a cause of infection. It can be debated

whether *E. faecium* in this sample material should always be interpreted as colonisation and perceived in line with a faecal sample. However, *E. faecium* detected in blood from an intravenous catheter or from a tissue sample can also be due to colonisation rather than infection, and in neither case it is known, if or when the microorganism leads to an infection.
In Study III, clinical samples from both in- and outpatients were used. All samples received from general practitioners were non-blood samples. Because of this, and the fact that patients examined by general practitioners must be assumed to be less ill than hospitalised patients, inclusion of this patient group may have had an impact on the results (Paper III) [3]. It can be considered to re-examine the data with exclusion of samples from general practitioners.

Did 30-day mortality of E. faecium correspond to the cause of death?
Most studies investigating the lethality of a microorganism uses the 30-day mortality. However, this can be a problematic parameter to use for low pathogenic microorganisms, especially if confounders of mortality are present. Because IPC units use mortality in their risk assessment for subsequent IPC management, it is of great importance that reported causes of mortality reflects reality.
In Study II and III, it was therefore investigated if the 30-day mortality of patients with *E. faecium* reflected whether the death was attributable to *E. faecium*.

The 30-day mortality was found to be 40% for VSEfm and 45.7% for VREfm/VVEfm bacteraemia cases (Paper II and Paper III) [2, 3]. The level was consistent with other studies, as were the higher mortality of VREfm/VVEfm compared to VSEfm [158, 159, 161, 162, 164]. However, a statistical comparison of the 30-day mortality of VREfm versus VSEfm was not carried out in this PhD. As described in the background section, it has been widely believed that VREfm has a significantly higher 30-day mortality rate than VSEfm. However, some studies find no statistical differences between 30-day mortality for VREfm/VVEfm and VSEfm bacteraemia cases if data are adjusted for confounders or APACHE II score [133, 172, 286]. This is supported by a national Danish study using a large data set of VSEfm and VRE/VVEfm from the same study period as the studies in this PhD [287].
In Study II and III, no adjustments for confounders were carried out in estimating the 30-day mortality. Instead, it was investigated if death was attributable to VSEfm or VREfm/VVEfm. For this, an algorithm to determine the cause of death was invented (Supplementary in Paper II) [2]. The patients were divided in to three categories (‘likely’, ‘possibly’, and ’unlikely’), depending on whether death within 30-days was attributable to *E. faecium*.
VSEfm was the ‘likely’ cause of death in only 6.3% of the VSEfm bacteraemia cases, and VREfm/VVEfm in 12.5% of the VREfm/VVEfm bacteraemia cases (Paper II and Paper III) [2, 3]. The number of VREfm/VVEfm bacteraemia cases was too small for meaningful statistical calculations. In Study III, including VREfm/VVEfm all-case patients, the 30-day mortality was 22.2%, and in 4.1% VREfm/VVEfm was the ‘likely’ cause of death.

It seemed like underlying diseases of the patients were confounders, making *E. faecium* appear to have a greater influence on a fatal outcome than it actually does. By doing an individual case evaluation of the cause of death, VREfm/VVEfm bacteraemia seemed more 'likely' to be attributable to death than VSEfm bacteraemia. This may be due to the low number of included VREfm/VVEfm isolates, or as previous mentioned due to patients with VREfm/VVEfm being more ill from underlying diseases than VSEfm cases and thus having a higher risk of being colonized with VREfm. The results therefore support the use of adjustment for confounders in investigating 30-day mortality.

An investigation of the attributable mortality has as far as I know not been investigated before regarding VSEfm, and only a few studies have investigated this for VREfm/VVEfm [288, 289]. In the Norwegian study from 2019, a separation between infection and colonisation was made by the reporting physicians for several multidrug-resistant microorganisms. The study revealed that only 9% of the VREfm cases had an infection. A low infection rate has also been described from several other studies and with a colonisation to infection ratio as high as 10:1 if screening samples were included [290–292].

In the Norwegian study, the 30-day mortality for the hospitalised patients with VREfm infection was 11%, and the 30-day mortality for VREfm bacteraemia cases 16%. These results are higher than detected in Study III, and can reflect several things, e.g. differences in patient population, treatment strategies, or the assessment of an infection. However, by including the group with 'possible' cases in the group of 'likely' cases in Study III, the results would be identical to the Norwegian – the 'likely' cause of death would be 11.3% for VREfm/VVEfm all-cases and 18.8% for VREfm/VVEfm bacteraemia cases (Paper III) [3].

The divergence between 30-day mortality and the attributable death may be due to combining death within 30 days and the detection of *E. faecium* through databases. Databases provide information on hard data, such as the presence of microbiological results or death. To date the databases have not been able to put the data into a clinical context and assess the correlation to an infection.

What was the need for treatment?

In both Study II and III, a high percentage of the bacteraemia cases had an arterial or intravenous catheter.

For VSEfm bacteraemia patients with a catheter and receiving a relevant *E. faecium* antibiotic treatment, the 30-day mortality was significantly lower if the catheter was removed or changed compared to not changing or removing it (Paper II) [2]. However, there was no impact on the 30-day mortality by removing or changing the catheter if a relevant *E. faecium* antibiotic treatment had already been initiated. For all-case patients with VREfm/VVEfm, the presence, removal or change of an intravascular catheter was not related to the 30-day mortality (Paper III) [3]. Furthermore, a VREfm/VVEfm active antibiotic treatment was significantly associated with an increased 30-day mortality (Paper III) [3]. This may reflect how critically ill the patients are and the assumption of patients with VREfm/VVEfm having more severe underlying diseases than patients

with VSEfm, and those patients in whom it is possible to remove the catheter being less ill than patients who cannot have their catheter changed or removed. This assumption is supported by the studies detecting a higher 30-day mortality for patients with VREfm than VSEfm bacteraemia [158, 159, 161, 162, 164]. This, and that VREfm infections themselves are predictors of mortality is consistent with a study of liver transplant patients from 1996 [289].
A limitation in Study II and III is, that only retrospective data from the medical records were used. This could be a problem due to incomplete documentation. The antimicrobial treatment was entered in a prescription module, and the para-clinical data in laboratory modules - both modules incorporated into the medical records. It is most likely that incomplete documentation may have only been a problem for the data collection of the placement and change/removal of a catheter, because this information had to be entered as a journal entry, unlike the other data used. However, the same medical record system was used in the entire investigation period, and without campaigns for improving the registration of catheter interventions. If there is a need to minimise this type of error, prospective studies must be used instead.

Was there any correlation between the ST-CT and the clinical findings?
There was no connection between specific ST-CT and age, gender, sample material, initiated antibiotic treatment, the presence of a catheter, or mortality detected for neither VSEfm nor VREfm/VVEfm (Paper II and Paper III) [2, 3].
In the study of VREfm/VVEfm, it seemed that *vanA E. faecium* (VRE) was more mortal than *vanB E. faecium* (VRE) and *vanA E. faecium* (VVE), but the number of included patients was too small for statistical calculation and further investigations with a larger numbers of patients are needed (Paper III) [3]. As far as I know, no other study has investigated this.

4.4 Impact of ending screening and isolation in a Danish university hospital

Why examine the impact of a cessation?
Screening, isolation, and disinfection are used in many hospitals as a supplement to standard precautions for controlling VREfm/VVEfm. As described in the background section, this approach increases the hospitals' expenses as well as the risk of poorer treatment of the patient who is placed in isolation. Studies from countries with a high VREfm/VVEfm incidence have shown that this practice can cease without major consequences for patient safety, but there is a lack of studies on whether this also applies to countries with a low incidence.
In RSD there are four hospitals, of which OUH is the largest and have a regional function for certain diseases. This collaboration results in a large exchange of patients with the other hospitals, thereby enabling transmission throughout the region.
In Study III, the impact of ending screening and isolation of VREfm/VVEfm patients at OUH was investigated. The impact on age, gender, specialities, site of infection, bacteraemia within 30-days after the primary infection, 30-day mortality, and VREfm/VVEfm attributable death for the

VREfm/VVEfm patients were registered. Furthermore, the impact on the collaborative hospitals in RSD was examined by investigating the burden of VREfm/VVEfm bacteraemia (Paper III) [3]. Before the cessation was initiated, a risk assessment was carried out and based, among other things, on the mortality of VREfm/VVEfm, the need for antibiotic treatment, and the risk of passing on the vancomycin resistance mechanism to other microorganisms.

What was the impact on various parameters of VREfm/VVEfm of a cessation?
After the end of screening and isolation the number of patients with a first time clinical VREfm/VVEfm sample (index patients) increased, but only very few differences in the other investigated parameters, including the impact on collaborating hospitals, were detected (Paper III) [3].
The most noticeable change was a significant shift in *van*-gene incidence - from *vanA E. faecium* (VVE clone) to *vanB E. faecium.* This change in *van*-gen type has been seen throughout Denmark and, as previously described, has taken place from 2020 onwards [47]. However, a calculation of the distribution of the various investigated parameters in the two periods in relation to each *van*-gene separately did not result in any striking differences, but it should be noted that the number of cases distributed in the categories was small.
The increase in numbers of VREfm isolates after ending screening and isolation has been described in studies from high-incidence countries. These studies found an increase during the following two to three years, after which a stabilisation took place without need for resuming the transmission-based precautions (TBP) [230–232].
In Study III, no stabilisation in number of index patients after the cessation was found, which could be due to the short follow-up period (Paper III) [3]. The development continues to be followed closely and has so far not given cause of concern.
Use of screening and isolation are widely debated. Some advocate for hospital-wide screening to control transmission in low-incidence countries [56]. Contrary to this, some studies detect no or limited benefit of screening, and recommend screening and isolation being restricted to specific groups of patients [230–232, 293]. Other studies investigating VREfm transmission recommend strengthening the IPC policies and compliance in general, and yet others challenge the rationale for use of more than just standard precautions [91, 229, 294].
Several attempts have been made to find the optimal screening procedure, but without a clear result [54–56, 141]. This gives rise to a discussion on whether sufficiently sensitive screening procedures are realistic to achieve, and further reviews are needed to clarify this.
In a recent review, the evidence for use of screening, hand hygiene, personal protective equipment, environmental cleaning, and managerial and organisation interventions were investigated [295]. The majority of the included studies concerned MRSA and revealed that universal screening followed by contact precautions were not necessarily cost-effective. Multimodal interventions to improve hand hygiene were cost-effective, as were environmental cleaning, and surveillance interventions. Furthermore, the interventions that aimed for more than one microorganism was more cost-effective than compared to MRSA interventions alone. The

major challenges in estimating the cost-effectiveness were the variation in practice, design, and costs of the interventions.
Standard precautions include cleaning as an important but often overlooked element. Several studies find that transmission of VREfm occurs via the environment and that reducing the VREfm/VVEfm occurrence in the environment can help prevent transmission [166, 224, 296]. Furthermore, a restrictive antibiotic policy is necessary to facilitate the reduction of MDRO [297]. As it appears, studies of the impact of screening and isolation and their cost-effectiveness are many and without a clear consensus on what is optimal.

Besides the short follow-up period, another limitation in investigating the impact of the cessation of VREfm/VVEfm precautions is, that it was carried out in the middle of the COVID pandemic. Due to a difference in the duration of the two periods used for comparison, a further comparison was made between the year 2021 and the year 2022. There was no changes in the data outcome by doing this (Paper III) [3]. In both periods, COVID restrictions were used, but with some of the restrictions being lifted during 2022. It is difficult to assess the significance of these restrictions. In the period 2020-2021, the COVID-19 restrictions may have led to a further decrease in the number of VREfm/VVEfm cases than would otherwise have been the case. On the other hand, it was observed during the busiest time of the pandemic that staff met less of the standard precautions outside the inpatient COVID-19 isolation rooms. During 2022, a general weariness was also seen in the population in relation to complying with the COVID-19 restrictions. If only the VREfm/VVEfm restrictions had been removed the number of VREfm/VVEfm cases might have been lower in 2022.

What impact did the cessation have on the collaborative hospitals in the region?
It has been shown that transmission not only takes place inside the hospitals, but also takes place between collaborating hospitals and other collaborating institutions such as nursing homes. Furthermore, this is a two-way transmission, which creates a need to monitor the development of the partners to be able to register the significance of the implemented changes, but also to take confounders into account [82–84, 292].
In Study III, the impact of ending the screening and isolation precautions at OUH was investigated in the collaborating hospitals. This was done by using the number of VREfm/VVEfm bacteraemia cases.
By this investigation, no significant increase of VREfm/VVEfm was detected at the collaborating hospitals. However, this could be due to the small number of VREfm/VVEfm bacteraemia cases (Paper III) [3].
During the investigation period, a decrease in admissions and an increase in the number of patients having a blood culture during the admission in all RSD hospitals were detected (Paper III) [3]. This may be a reflection of the changes in the Danish public healthcare system, where it is mainly the seriously ill patients who are admitted to the hospitals. This assumption is supported by the steady increase in number of invasive infections during the past ten years [47]. Furthermore, if more patients are blood cultured at the time of admission, and VREfm/VVEfm are acquired during

hospitalisation, this may lead to a reduced incidence. If the patients are more ill, there should be an increased number of patients treated with antibiotics during the stay which enhances the risk of acquisition of VREfm/VVEfm (Paper III) [3].

When investigating transmission, it is important to be aware of other initiated IPC precautions, including less obvious IPC changes such as the proportion of single-rooms in the hospital. In Study III, some of the collaborative hospitals underwent major or minor renovations during the study period, resulting in an increased number of single-rooms. This may have affected the results, as the risk of cross-infection between patients is reduced by the use of single-rooms [229].

5. Conclusion and perspectives

Almost 15 years ago, several studies and reviews of *E. faecium* were carried out by, e.g. Bonten M, Werner G, Cetinkaya Y, and Hammerum A [70, 133, 152, 173]. These studies formed the basic skeleton of an *E. faecium* puzzle. The years have passed with researchers trying to fill in the missing pieces, but they do not seem to have changed the outline of the picture.
This PhD was conducted aiming at investigating the puzzle piece of the real time use of cgMLST for IPC of *E. faecium* in a Danish university hospital. Furthermore, the clinical impact of *E. faecium* and the impact of ending screening and isolation of patients with VREfm/VVEfm was investigated.
As the studies provided answers to the questions asked, others emerged as new puzzle pieces to be added.

Can cgMLST be used in real time for IPC of VREfm transmission?

The use of cgMLST in real time was found to be a useful auxiliary tool in IPC to find the related patients in an outbreak.
However, as earlier described, there are challenges in using this method for species with a high recombination rate and if the relatedness lies is in the extrachromosomal DNA.

An issue with cgMLST is to ensure a uniform approach to CT clusters. It is advisable that national schemes for CT clusters describing which CTs each CT cluster includes, are developed in the nearest future.

As an increasing number of laboratories around the world begins to use cgMLST, it becomes easier to compare results and examine transmission patterns in and between countries. This also includes whether certain strains are more likely to cause widespread transmission than others and become pandemic. An international database with information of ST-CT, resistance-gene content, and transmission potential could be of great interest for the IPC unit. It can help the IPC units to be very specific in deciding whether a certain type of e.g. *E. faecium* should trigger an action, and what action it should be. The Ridom SeqSphere+ database was initially used as such a database. However, following the introduction of the 'General Data Protection Regulation' (GDPR), submission of place and time of detection of each isolate has been sparse. Hopefully, a way will be found in which this information can be collected and made widely available again, not only for E. *faecium*, but for all bacterial species.

The use of cgMLST was improved by implementing epidemiological data. Databases that can link sequencing data with epidemiological data already exist. However, such databases must also comply with GDPR, and a solution must be found so that the databases can be further developed, disseminated, and implemented for use in the IPC units.

As earlier described, HAIs are not just about cross-infections and environmental infections with microorganisms of the same type. In these cases, use of WGS for typing is an obvious strategy. It is speculated that in some cases, HAIs are infections with the patient's own microbial flora. However, these infections seem not to have been investigated using WGS for typing and the area in general is not well explored. It is important to investigate how many of the infections that are due to self-infections, as this has a bearing on the kind of focus IPC should have on the area.

The medical technological improvements are increasing steeply, creating possibilities only dreamt of. The hospital settings are changing in Denmark with a possible implication of the rate of HAIs. New hospitals are being built with an increased number of single-rooms and an increasing use of robots for operations, cleaning etc. An increasing proportion of the operations are performed without subsequent hospitalisation, and patients are discharged earlier from the hospital to smaller local nursing units or to their homes to recover. Systemic antibiotic treatment is given at home, and several specialised treatments are outsourced to general practitioners. These structural changes can lead to transmission being more difficult to detect, thus increasing the need for faster and high-discriminatory methods as WGS combined with interpretation methods like cgMLST.

The economic savings by using cgMLST to reduce transmission and HAIs due to VREfm/VVEfm and VSEfm were not investigated in this PhD. However, as described in the background section and with the reduction of wards requiring IPC interventions in Study I, the use of cgMLST is expected to have reduced total costs for OUH.

Is unrecognised transmission of VSEfm detected by use of cgMLST?

The investigations in this PhD have revealed, that there was a wide transmission of VSEfm in the hospital during the entire investigation period, indicating that IPC at the hospital could be optimised.
This finding may remind other hospitals that the less resistant bacterial species should also be monitored.
To find out whether and which VSEfm clones are endemic today, further studies from Denmark and other countries are needed.

Can cgMLST data of VSEfm be used to predict VREfm occurrence?

There was found no connection between the ST-CT types of VSEfm and the incidence of VREfm/VVEfm. However, the transmission of the VSEfm and VREfm/VVEfm took place in the same departments.
Furthermore, shifts in the dominating types were found every two to three years of VSEfm and VREfm/VVEfm.

cgMLST has the potential to be used in a daily surveillance of transmission without regard to antibiotic susceptibility, but studies of everyday use of this kind of surveillance program are lacking.
The extent of transmission of antibiotic-susceptible species calls for further attention, because by intervening against the susceptible strains, the risk of transmission with antibiotic-resistant strains ought to be reduced in advance. However, to find out which parameters of VSEfm that should be monitored to be able to predict an impending spread of VREfm/VVEfm, further studies are needed. It could be interesting to re-investigate the parameters used in Study II (the total incidence of VSEfm, the incidence of the ST-CTs, and the type of departments) with all kinds of sample materials included. Furthermore, it could be interesting to investigate if the incidence of different virulence factors in the VSEfm isolates, as well as the antibiotic consumption in the hospital and departments, could be used for the prediction of VREfm/VVEfm.

To demonstrate whether there is a correlation between the VSEfm and VREfm/VVEfm ST-CT cycles, further studies are needed. Studies investigating if the shifts observed in Denmark and possible shifts internationally are correlated would also be interesting. The results may be used for predicting what is to come. It can be recommended to include larger amounts of isolates from a period of at least 5 years, and that the investigations are performed in several countries.
In the investigation of shifts, it could be relevant to investigate for the correlation to the presence of plasmids and virulence factors such as the *purK* allele type.
It could also be interesting to carry out conjugation analyses to investigate how transferable the different plasmids containing *van*-genes are from VREfm/VVEfm to VSEfm, and if certain factors must be present in the isolates to give or receive a plasmid. Both large endemic clones and non-endemic types should be used.

To find out why some specific ST-CT clones ends up being large endemic clones, it could be important to investigate if they have more potent virulence factors than the non-endemic hospital clones. Furthermore, it would be interesting to study the potential diversity between the plasmids carried by the different clones.

What is the clinical relevance of VSEfm and VREfm/VVEfm?

In the conducted investigations both VSEfm and VREfm/VVEfm had some clinical relevance, but the treatment strategies were diverse and influenced by the presence of invasive catheters and sample material.
The 30-day mortality did not reflect the pathogenicity of this microorganism. The studies indicated that especially VREfm/VVEfm is a predictor of death, and that the patients died from underlying illness and not the *E. faecium* infection itself.

No specific ST-CT were found to be related to the clinical impact, but isolates containing a *vanA*-gene seemed to be more mortal than isolates containing *vanB* or VVEfm. However, the number included in the study was too small for statistical calculation.

The results in Study II and Study III indicated that treatment strategies for *E. faecium* infections were diverse and require revision. This, in combination with studies revealing a low frequency of VREfm infection compared to colonisation, indicates that there is an opportunity to optimise antibiotic stewardship [133, 288]. It should be investigated which of the *E. faecium* patients that should be offered antibiotic treatment and for how long, and it is important that the presence of invasive catheters is included in these studies, as these may influence the results. No studies investigating this have been found, but randomised controlled trials in this area are supposed to enable better personalised medication.
There are as far as I know, no studies examining the extent of infections in relation to colonisation with *E. faecium* in the urinary tract. This should be further investigated to clarify if or when a detection of *E. faecium* in the urine must be reported to the attending physician, and whether this sample material must be included in future studies of *E. faecium*.

There are many studies investigating VREfm mortality, including the mortality of VREfm versus VSEfm. However, most studies lack the level of detail that can be obtained by reviewing and assessing the clinical information in medical records. Because mortality is used in the IPC risk assessment, it is important to find an algorithm that can indicate the mortality of a microorganism, and at the same time describes whether death is attributable to the microorganism or not.

To investigate whether there is a different clinical impact in relation to *van*-gene content, further studies with more patients are required. It has been found that infection-derived *E. faecium* isolates are more enriched with putative virulence factors than non-infectious isolates [211, 298]. It could therefore be interesting to investigate whether the presence of specific virulence genes in *E. faecium* and or the types of plasmids in the hospital adapted VREfm/VVEfm have a clinical significance and thus an impact on the treatment strategy. Furthermore, it should be investigated whether some of these factors also cause different clinical impact in VSEfm cases. Maybe specific virulence factors and plasmids can be used to predict mortality.

What is the impact of ending a screening and isolation regime of VREfm/VVEfm patients at a Danish university hospital?

Use of screening and isolation can be a double-edged sword. These precautions may reduce the incidence of a microorganism, but they increase costs in hospitals, time consumption for the staff, and negative effects for the patients. The study of the impact of ending screening and isolation of VREfm/VVEfm patients revealed that the incidence increased but had no other negative effects in the short term.

Because hospitals have different settings, further research regarding the impact of cessation of VREfm/VVEfm screening and isolation in low-incident countries is required. The studies need to be in hospitals with different settings, e.g. demography and specialities, to confirm generalisability. They should also have a long follow-up period to detect whether there are negative consequences in the longer term.

Since no two hospitals are the same, it may not be appropriate to point out a single solution as the right one.
However, several factors described in the background section and discussion have to be approached in the choice of IPC precautions: optimal screening procedures is unlikely to be achieved, transmission may happen before the patient is recognised as a risk or as being at risk, patients does not only shed microorganisms in their room but all the time, including during transportation inside the hospital, *E. faecium* are capable of surviving harsh environments and achieve resistance to disinfectants, and whether death is attributable to VREfm/VVEfm can be questioned. Furthermore, it is important to remember the increased risk patients are exposed to when they are placed in an isolation room. It is not clear whether the studies investigating mortality of VREfm/VVEfm compared to VSEfm have considered that the isolation in-itself can be a confounder and cause of the increased mortality that was detected.
Due to all the above-mentioned factors, it may be appropriate to consider options that will not exclusively reduce the occurrence of VREfm/VVEfm. These options may include optimisation of the IPC organisation, training in infection prevention, antibiotic policies, and, in particular, adherence to the IPC recommendations.
I fully agree with my colleague professor Hans Jørn Kolmos who often states that the three main things to remember in the attempt to decrease the number of HAIs are to:

- make an effort to achieve better infection prevention and control
- use antibiotics wisely
- optimise diagnostics

The field of microbiology is dynamic, so settings with different demographics, health conditions, healthcare systems, and environments may have different IPC needs. We must therefore become better at considering whether the same IPC precautions suits everyone - perhaps even within the same country. To uncover this, we are welcoming the development taking place within DNA sequencing.

References

1. **Kjær Hansen S, Andersen L, Detlefsen M, Holm A, Roer L, *et al.*** Using core genome multilocus sequence typing (cgMLST) for vancomycin-resistant Enterococcus faecium isolates to guide infection control interventions and end an outbreak. *J Glob Antimicrob Resist* 2021;24:418–423.
2. **Hansen SGK, Roer L, Karstensen KT, Hoegh SV, Hansen F, *et al.*** Vancomycin-sensitive Enterococcus faecium bacteraemia - hospital transmission and mortality in a Danish University Hospital. *J Med Microbiol*;72. Epub ahead of print 12 July 2023. DOI: 10.1099/JMM.0.001731.
3. **Hansen SG., Klein K, Nymark A, Andersen L, Gradel KO, *et al.*** Vancomycin-resistant Enterococcus faecium - impact of ending screening and isolation in a Danish University Hospital. *J Hosp Infect (Submitted Publ*.
4. **Allegranzi B, Nejad B, Graafmans W, Phd A, Donaldson L, *et al.*** Articles Burden of endemic health-care-associated infection in developing countries: systematic review and meta-analysis. *Lancet* 2011;377:228–269.
5. **WHO**. Report on the Burden of Endemic Health Care-Associated Infection Worldwide Clean Care is Safer Care. *World Heal Organ* 2011;1–40.
6. **ECDC**. Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals 2016–2017. https://www.ecdc.europa.eu/en/publications-data/point-prevalence-survey-healthcare-associated-infections-and-antimicrobial-use-5 (accessed 29 August 2023).
7. **Rigsrevisionen**. Rigsrevisionens beretning om forebyggelse af hospitalsinfektioner afgivet til Folketinget med Statsrevisorernes bemaerkninger. www.rosendahls.dk (2017, accessed 29 August 2023).
8. **OUH**. Patientsikkerhedsstrategi 2015-2018. OUH. Region Syddanmark. http://ipaper.ipapercms.dk/RegionSyddanmark/OUH/Direktion_og_Stabe/Kvalitet__Forskning__Innovation_og_Uddannelse/127465/#/ (accessed 6 February 2020).
9. **Statens Serum Institut**. Healthcare-Associated Infections Database - HAIBA. https://www.esundhed.dk/Emner/Patienter-og-sygehuse/HAIBA (accessed 29 August 2023).
10. **Umscheid CA, Mitchell MD, Doshi JA, Agarwal R, Williams K, *et al.*** Estimating the proportion of healthcare-associated infections that are reasonably preventable and the related mortality and costs. *Infect Control Hosp Epidemiol* 2011;32:101–114.
11. **Scott RD**. *The direct medical costs of healthcare-associated infections in U.S. hospitals and the benefits of prevention*. CDC. https://stacks.cdc.gov/view/cdc/11550 (2009, accessed 29 August 2023).
12. **Zimlichman E, Henderson D, Tamir O, Franz C, Song P, *et al.*** Health care-associated infections: a meta-analysis of costs and financial impact on the US health care system. *JAMA Intern Med* 2013;173:2039–2046.
13. **Dik JWH, Dinkelacker AG, Vemer P, Lo-Ten-Foe JR, Lokate M, *et al.*** Cost-analysis of seven nosocomial outbreaks in an academic hospital. *PLoS One*;11. Epub ahead of print 2016. DOI: 10.1371/journal.pone.0149226.
14. **Riu M, Chiarello P, Sala M, Garcia-Alzorriz E, Castells X, *et al.*** Incremental cost of nosocomial bacteremia according to the focus of infection and antibiotic sensitivity of the

causative microorganism in a university hospital. *Medicine (Baltimore)*. Epub ahead of print 2017. DOI: 10.1097/MD.0000000000006645.
15. **Hughes JM**. Study on the efficacy of nosocomial infection control (Senic project): Results and implications for the future. *Chemotherapy* 1988;34:553–561.
16. **Enhed for Infektionshygiejne C, Serum Institut S**. Nationale Infektionshygiejniske Retningslinjer Generelle forholdsregler for sundhedssektoren Central Enhed for Infektionshygiejne | 1. udgave 2017.
17. Life expectancy - Statistics Denmark. https://www.dst.dk/en/Statistik/emner/borgere/befolkning/middellevetid (accessed 12 July 2023).
18. **WHO**. WHO publishes list of bacteria for which new antibiotics are urgently needed. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed 22 October 2019).
19. **Haley RW, Culver DH, White JW, Morgan WM, Emori TG, *et al.*** The efficacy of infection surveillance and control programs in preventing nosocomial infections in US hospitals. *Am J Epidemiol* 1985;121:182–205.
20. **Storr J, Twyman A, Zingg W, Damani N, Kilpatrick C, *et al.*** Core components for effective infection prevention and control programmes: New WHO evidence-based recommendations. *Antimicrob Resist Infect Control*;6. Epub ahead of print 2017. DOI: 10.1186/s13756-016-0149-9.
21. **Dhar S, Cook E, Oden M, Kaye KS**. Building a Successful Infection Prevention Program Key Components, Processes, and Economics THE EMERGENCE AND DEVELOPMENT OF REGULATION AND REQUIREMENTS OF INFECTION PREVENTION AND CONTROL. *Infect Dis Clin N Am* 2016;30:567–589.
22. **Zingg W, Holmes A, Dettenkofer M, Goetting T, Secci F, *et al.*** Hospital organisation, management, and structure for prevention of health-care-associated infection: A systematic review and expert consensus. *The Lancet Infectious Diseases* 2015;15:212–224.
23. **Bryant KA, Harris AD, Gould C V, Humphreys E, Lundstrom T, *et al.*** Necessary Infrastructure of Infection Prevention and Healthcare Epidemiology Programs: A Review HHS Public Access. *Infect Control Hosp Epidemiol* 2016;37:371–380.
24. **Pedersen KM, Kolmos HJ**. *Hospitalsinfektioners økonomi, Health Economics Papers 2007:4, University of Southern Denmark*. 2007.
25. **Pappas G, Kiriaze IJ, Falagas ME**. Insights into infectious disease in the era of Hippocrates. *International Journal of Infectious Diseases* 2008;12:347–350.
26. **Jensen K**. *Bekaempelse af infektionssygdomme*. www.ssi.dk (2002, accessed 6 February 2020).
27. **Yapijakis C**. Hippocrates of Kos, the father of clinical medicine, and asclepiades of Bithynia, the father of molecular medicine. *In Vivo* 2009;23:507–514.
28. **Yourcenar M**. *Kejser Hadrians erindringer*.
29. **March J**. *Dictionary of Classical Mythology*. Cassell; 1998.
30. **Shampo MA, Kyle RA**. Medical mythology: Hygeia. *Mayo Clin Proc* 1993;68:441.
31. Hygieia, the Goddess of Health - World History Encyclopedia. https://www.worldhistory.org/article/253/hygieia-the-goddess-of-health/ (accessed 14 December 2023).
32. **Ariatti A, Mandrioli P**. Lazzaro spallanzani: A blow against spontaneous generation.

Aerobiologia (Bologna) 1993;9:101–107.
33. **Weed LA**. John Tyndall and his contribution to the theory of spontaneous generation. *Ann Med Hist* 1942;4:55.
34. **Semmelweis I**. *The Ethiology, Concept, and Prophylaxis of Childbed Fever Translated by K. Codell Carter*. University of Wisconsin Press; 1983.
35. **Lister J**. On the Antiseptic Principle in the Practice of Surgery. *Br Med J* 1867;2:246.
36. **Conti AA**. Quarantine Through History. *International Encyclopedia of Public Health* 2008;454.
37. **Tognotti E**. Lessons from the History of Quarantine, from Plague to Influenza A. *Emerg Infect Dis* 2013;19:254.
38. **Lee G, Clark AM, Thompson DR**. Florence Nightingale--never more relevant than today. *J Adv Nurs* 2013;69:245–246.
39. **Skinhøj P BI**. Vaccination i historisk perspektiv | Ugeskriftet.dk. *Ugeskr Læger*. https://ugeskriftet.dk/videnskab/vaccination-i-historisk-perspektiv (2021, accessed 3 November 2023).
40. **CDC**. Our History - Our Story | About | CDC. https://www.cdc.gov/about/history/index.html (accessed 9 February 2020).
41. **ECDC**. About ECDC. https://www.ecdc.europa.eu/en/about-ecdc (accessed 12 January 2024).
42. **Central Enhed for Infektionshygiejne SSI**. Hvad er infektionshygiejne ? https://hygiejne.ssi.dk/organisering/hvad-er-infektionshygiejne (accessed 6 February 2020).
43. **Central Enhed for Infektionshygiejne SSI**. *NATIONALE INFEKTIONSHYGIEJNISKE RETNINGSLINJER OM GENERELLE FORHOLDSREGLER I SUNDHEDSSEKTOREN Central Enhed for Infektionshygiejne 1. udgave 2017*. 2017.
44. Danish Health Authority. https://www.sst.dk/en/english (accessed 6 November 2023).
45. **DANMAP**. About DANMAP -. https://www.danmap.org/da/about-danmap (accessed 3 January 2024).
46. **Central Enhed for Infektionshygiejne SSI**. Surveillance_and preparedness. https://en.ssi.dk/surveillance-and-preparedness/infection-control (accessed 13 January 2024).
47. **DANMAP**. Reports. https://www.danmap.org/reports (accessed 22 November 2022).
48. **Central Enhed for Infektionshygiejne SSI**. Overvågning af infektioner. https://hygiejne.ssi.dk/overvaagning (accessed 28 November 2023).
49. **Woeltje KF**. Moving into the future: Electronic surveillance for healthcare-associated infections. *Journal of Hospital Infection* 2013;84:103–105.
50. **CDC**. Transmission-Based Precautions | Basics | Infection Control | CDC". https://www.cdc.gov/infectioncontrol/basics/transmission-based-precautions.html (accessed 3 January 2024).
51. **Central Enhed for Infektionshygiejne SSI**. *NATIONALE INFEKTIONSHYGIEJNISKE RETNINGSLINJER OM SUPPLERENDE FORHOLDSREGLER VED INFEKTIONER OG BÆRERTILSTAND I SUNDHEDSSEKTOREN*. 2019.
52. **Birgand G, Moore LSP, Bourigault C, Vella V, Lepelletier D, *et al.*** Measures to eradicate multidrug-resistant organism outbreaks: How much do they cost? *Clin Microbiol Infect* 2016;22:162.e1-162.e9.
53. **ECDC**. *Economic evaluations of interventions to prevent healthcare-associated infections*

Literature review www.ecdc.europa.eu. Epub ahead of print 2017. DOI: 10.2900/4617.
54. **Zhou X, Willems RJL, Friedrich AW, Rossen JWA, Bathoorn E**. Enterococcus faecium: From microbiological insights to practical recommendations for infection control and diagnostics. *Antimicrob Resist Infect Control*;9. Epub ahead of print 10 August 2020. DOI: 10.1186/s13756-020-00770-1.
55. **Gast KB, van Oudheusden AJG, Murk JL, Stohr JJJM, Buiting AG, *et al.*** Successful containment of two vancomycin-resistant Enterococcus faecium (VRE) outbreaks in a Dutch teaching hospital using environmental sampling and whole-genome sequencing. *J Hosp Infect* 2021;111:132–139.
56. **Frakking FNJ, Bril WS, Sinnige JC, Klooster JE va. t., de Jong BAW, *et al.*** Recommendations for the successful control of a large outbreak of vancomycin-resistant Enterococcus faecium in a non-endemic hospital setting. *J Hosp Infect* 2018;100:e216–e225.
57. **Stelfox HT, Bates DW, Redelmeier DA**. Safety of patients isolated for infection control. *JAMA* 2003;290:1899–1905.
58. **Evans HL, Shaffer MM, Hughes MG, Smith RL, Chong TW, *et al.*** Contact isolation in surgical patients: A barrier to care? *Surgery* 2003;134:180–188.
59. **Martin EM, Bryant B, Grogan TR, Rubin ZA, Russell DL, *et al.*** Noninfectious Hospital Adverse Events Decline After Elimination of Contact Precautions for MRSA and VRE. *Infect Control Hosp Epidemiol* 2018;39:788.
60. **Purssell E, Gould Di, Chudleigh J**. Impact of isolation on hospitalised patients who are infectious: systematic review with meta-analysis. *BMJ Open* 2020;10:30371.
61. **Central Enhed for Infektionshygiejne. Statens Serum Institut**. Nationale infektionshygiejniske retningslinjer (NIR) for desinfektion i sundhedssektoren, 1. udgave. 2014.
62. **Boyce JM**. Modern technologies for improving cleaning and disinfection of environmental surfaces in hospitals. *Antimicrob Resist Infect Control*;5. Epub ahead of print 2016. DOI: 10.1186/s13756-016-0111-x.
63. **Maillard JY, Pascoe M**. Disinfectants and antiseptics: mechanisms of action and resistance. *Nat Rev Microbiol* 2024;22:4–17.
64. **Mims C, Playfair J, Roitt I, Wakelin D, Williams R**. *Medical Microbiology*. 2nd editio. Mosby International; 1998.
65. **Van 't Veen A, Van Der Zee A, Nelson J, Speelberg B, Kluytmans JAJW, *et al.*** Outbreak of Infection with a Multiresistant Klebsiella pneumoniae Strain Associated with Contaminated Roll Boards in Operating Rooms. *J Clin Microbiol* 2005;43:4961–4967.
66. **Tornieporth NG, Roberts RB, John J, Hafner A, Riley LW**. Risk Factors Associated with Vancomycin-Resistant Enterococcus faecium Infection or Colonization in 145 Matched Case Patients and Control Patients. *Clin Infect Dis* 1996;23:767–772.
67. **Shay DK, Maloney SA, Montecalvo M, Banerjee S, Wormser GP, *et al.*** Epidemiology and mortality risk of vancomycin-resistant enterococcal bloodstream infections. *J Infect Dis* 1995;172:993–1000.
68. **Cosgrove SE, Qi Y, Kaye KS, Harbarth S, Karchmer AW, *et al.*** The Impact of Methicillin Resistance in Staphylococcus aureus Bacteremia on Patient Outcomes: Mortality, Length of Stay, and Hospital Charges. *Infect Control Hosp Epidemiol* 2005;26:166–174.
69. **Boyce JM, Opal SM, Chow JW, Zervos MJ, Poyter-bynoe G, *et al.*** *Outbreak of Multidrug-Resistant Enterococcus faecium with Transferable vanB Class Vancomycin Resistance*. 1994.

70. **Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, *et al.*** Emergence and spread of vancomycin resistance among enterococci in Europe. *Euro surveillance : bulletin européen sur les maladies transmissibles = European communicable disease bulletin*;13. Epub ahead of print 2008. DOI: 10.2807/ese.13.47.19046-en.
71. **Kolmos HJ**. *Hygiejne for klinikere*. Munksgaard; 2021.
72. **Kluytmans J, Van Belkum A, Verbrugh H**. Nasal carriage of Staphylococcus aureus: Epidemiology, underlying mechanisms, and associated risks. *Clinical Microbiology Reviews* 1997;10:505–520.
73. **Hota B**. Contamination, Disinfection, and Cross-Colonization: Are Hospital Surfaces Reservoirs for Nosocomial Infection? *Clin Infect Dis* 2004;39:1182–1189.
74. **Dancer SJ**. Review Importance of the environment in meticillin-resistant Staphylococcus aureus acquisition: the case for hospital cleaning. *Lancet*;8. Epub ahead of print 2008. DOI: 10.1016/S1473.
75. **Weber DJ, Anderson D, Rutala WA**. The role of the surface environment in healthcare-associated infections. *Curr Opin Infect Dis* 2013;26:338–344.
76. **Weber DJ, Rutala WA, Miller MB, Huslage K, Sickbert-Bennett E**. Role of hospital surfaces in the transmission of emerging health care-associated pathogens: Norovirus, Clostridium difficile, and Acinetobacter species. *Am J Infect Control*;38. Epub ahead of print 2010. DOI: 10.1016/j.ajic.2010.04.196.
77. **Boyce JM**. Environmental contamination makes an important contribution to hospital infection. *J Hosp Infect* 2007;65 Suppl 2:50–54.
78. **Godijk NG, Bootsma MCJ, Bonten MJM**. Transmission routes of antibiotic resistant bacteria: a systematic review. *BMC Infect Dis*;22. Epub ahead of print 1 December 2022. DOI: 10.1186/S12879-022-07360-Z.
79. **Souverein D, Houtman P, Euser SM, Herpers BL, Kluytmans J, *et al.*** Costs and benefits associated with the MRSA search and destroy policy in a hospital in the Region Kennemerland, The Netherlands. *PLoS One*;11. Epub ahead of print 2016. DOI: 10.1371/journal.pone.0148175.
80. **CDC**. Prevention and Control | MDRO Management | Guidelines Library | Infection Control | CDC. https://www.cdc.gov/infectioncontrol/guidelines/mdro/prevention-control.html (accessed 18 September 2023).
81. **Authority DH and M**. Guidance on Preventing the Spread of MRSA - The Danish Health Authority. https://www.sst.dk/en/english/publications/2022/guidance-on-preventing-the-spread-of-mrsa (accessed 18 September 2023).
82. **Raven KE, Gouliouris T, Brodrick H, Coll F, Brown NM, *et al.*** Complex routes of nosocomial vancomycin-resistant Enterococcus faecium transmission revealed by genome sequencing. *Clin Infect Dis* 2017;64:886–893.
83. **Brodrick HJ, Raven KE, Harrison EM, Blane B, Reuter S, *et al.*** Whole-genome sequencing reveals transmission of vancomycin-resistant Enterococcus faecium in a healthcare network. *Genome Med*;8. Epub ahead of print 12 January 2016. DOI: 10.1186/S13073-015-0259-7.
84. **Donker T, Henderson KL, Hopkins KL, Dodgson AR, Thomas S, *et al.*** The relative importance of large problems far away versus small problems closer to home: Insights into limiting the spread of antimicrobial resistance in England. *BMC Med*;15. Epub ahead of print 27 April 2017. DOI: 10.1186/s12916-017-0844-2.

85. **Hammerum AM, Lester CH, Heuer OE**. Antimicrobial-resistant enterococci in animals and meat: a human health hazard? *Foodborne Pathog Dis* 2010;7:1137–1146.
86. **ECDC**. Principles of Epidemiology: Lesson 6, Section 1|Self-Study Course SS1978|CDC. https://www.cdc.gov/csels/dsepd/ss1978/lesson6/section1.html (accessed 4 March 2020).
87. **Gandra S, Barter DM, Laxminarayan R**. Economic burden of antibiotic resistance: How much do we really know? *Clinical Microbiology and Infection* 2014;20:973–980.
88. **Birgand G, Leroy C, Nerome S, Binh L, Nguyen L, *et al.*** Costs associated with implementation of a strict policy for controlling spread of highly resistant microorganisms in France. *BMJ Open* 2016;6:9029.
89. **Neidell MJ, Cohen B, Furuya Y, Hill J, Jeon CY, *et al.*** Costs of healthcare- and community-associated infections with antimicrobial-resistant versus antimicrobial-susceptible organisms. *Clin Infect Dis* 2012;55:807–815.
90. **Puchter L, Chaberny IF, Schwab F, Vonberg RP, Bange FC, *et al.*** Economic burden of nosocomial infections caused by vancomycin-resistant enterococci. *Antimicrob Resist Infect Control*;7. Epub ahead of print 2018. DOI: 10.1186/S13756-017-0291-Z.
91. **Edmond MB, Ober JF, Weinbaum DL, Pfaller MA, Hwang T, *et al.*** Vancomycin-Resistant enterococcus faecium bacteremia: Risk factors for infection. *Clin Infect Dis* 1995;20:1126–1133.
92. **Jakobsen M, Marie C, Skovgaard B, Jensen ML, Trap R, *et al.*** Omkostninger ved husdyr-MRSA for sundhedsvaesenet i Danmark. 2015;1–64.
93. **Chowers M, Carmeli Y, Shitrit P, Elhayany A, Geffen K**. Cost analysis of an intervention to prevent methicillin-resistant Staphylococcus aureus (MRSA) transmission. *PLoS One*;10. Epub ahead of print 2015. DOI: 10.1371/journal.pone.0138999.
94. **Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA**. *Medical microbiology*. 3rd Editio. Mosby; 1998.
95. Biology 101: Prokaryotic vs. Eukaryotic Cells - Owlcation. https://owlcation.com/stem/Biolog-101-Cells (accessed 20 December 2023).
96. Gram Positive vs Gram Negative | Technology Networks. https://www.technologynetworks.com/immunology/articles/gram-positive-vs-gram-negative-323007 (accessed 20 December 2023).
97. **Ryzhov V, Fenselau C**. Characterization of the protein subset desorbed by MALDI from whole bacterial cells. *Anal Chem* 2001;73:746–750.
98. **Maiden MCJ**. Multilocus Sequence Typing of Bacteria. *Annu Rev Microbiol* 2006;60:561–588.
99. **Berrazeg M, Diene SM, Drissi M, Kempf M, Richet H, *et al.*** Biotyping of Multidrug-Resistant Klebsiella pneumoniae Clinical Isolates from France and Algeria Using MALDI-TOF MS. *PLoS One*;8. Epub ahead of print 2013. DOI: 10.1371/journal.pone.0061428.
100. **De Been M, Pinholt M, Top J, Bletz S, Mellmann A, *et al.*** Core genome multilocus sequence typing scheme for high-resolution typing of enterococcus faecium. *J Clin Microbiol* 2015;53:3788–3797.
101. **Pinholt M, Larner-Svensson H, Littauer P, Moser CE, Pedersen M, *et al.*** Multiple hospital outbreaks of vanA Enterococcus faecium in Denmark, 2012-13, investigated by WGS, MLST and PFGE. *J Antimicrob Chemother* 2015;70:2474–2482.
102. **Bello-López JM, Cabrero-Martínez OA, Ibáñez-Cervantes G, Hernández-Cortez C, Pelcastre-Rodríguez LI, *et al.*** Horizontal Gene Transfer and Its Association with Antibiotic Resistance

in the Genus Aeromonas spp. *Microorganisms*;7. Epub ahead of print 1 September 2019. DOI: 10.3390/MICROORGANISMS7090363.
103. **C Reygaert W**. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol* 2018;4:482–501.
104. **Martinez JL**. General principles of antibiotic resistance in bacteria. *Drug Discov Today Technol* 2014;11:33–39.
105. **Norman A, Hansen LH, Sørensen SJ**. Conjugative plasmids: Vessels of the communal gene pool. *Philos Trans R Soc B Biol Sci* 2009;364:2275–2289.
106. **Muñoz-López M, García-Pérez JL**. DNA Transposons: Nature and Applications in Genomics. *Curr Genomics* 2010;11:115.
107. **Domingues S, Silva GJ da, Nielsen KM**. Integrons: Vehicles and pathways for horizontal dissemination in bacteria. *Mob Genet Elements* 2012;2:211.
108. **Mahillon J, Chandler M**. Insertion Sequences. *Microbiol Mol Biol Rev* 1998;62:725.
109. **Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA, *et al.*** Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in Klebsiella pneumoniae, an urgent threat to public health. *Proc Natl Acad Sci U S A* 2015;112:E3574–E3581.
110. **Malachowa N, Deleo FR**. Mobile genetic elements of Staphylococcus aureus. *Cell Mol Life Sci* 2010;67:3057–3071.
111. **Huddleston JR**. Infection and Drug Resistance Dovepress Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. *Infect Drug Resist* 2014;7–167.
112. **Peterson JW**. Bacterial Pathogenesis, Chapter 7, Medical Microbiology - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK8526/ (accessed 19 September 2023).
113. **Shapiro-Ilan DI, Fuxa JR, Lacey LA, Onstad DW, Kaya HK**. Definitions of pathogenicity and virulence in invertebrate pathology. *J Invertebr Pathol* 2005;88:1–7.
114. **Forland F, Kleinkauf N, Monnet DL, Todd Weber J, Giesecke J, *et al.*** *European Centre for Disease Prevention and Control. Risk assessment on the spread of carbapenemase-producing Enterobacteriaceae (CPE) through patient transfer between healthcare facilities, with special emphasis on cross-border transfer*. 2011. Epub ahead of print 2011. DOI: 10.2900/59034.
115. **Tamma PD, Goodman KE, Harris AD, Tekle T, Roberts A, *et al.*** Comparing the Outcomes of Patients With Carbapenemase-Producing and Non-Carbapenemase-Producing Carbapenem-Resistant Enterobacteriaceae Bacteremia. *Clin Infect Dis* 2017;64:257–264.
116. **Wyllie DH, Crook DW, Peto TEA**. Mortality after Staphylococcus aureus bacteraemia in two hospitals in Oxfordshire, 1997-2003: cohort study. *BMJ Br Med J* 2006;333:281.
117. **Bai AD, Lo CKL, Komorowski AS, Suresh M, Guo K, *et al.*** Staphylococcus aureus bacteraemia mortality: a systematic review and meta-analysis. *Clin Microbiol Infect* 2022;28:1076–1084.
118. **ReAct**. Antibiotic use in humans – Part 2 – ReAct. https://www.reactgroup.org/antibiotic-resistance/course-antibiotic-resistance-the-silent-tsunami/part-2/antibiotic-use-in-humans/ (accessed 21 January 2024).
119. **Mandell GL, Bennett JE DR**. *Mandell, Douglas, and Bennett's principles and practice of infectious diseases, 7th edition*. Elsevier Health Sciences; 2010.
120. **Rice LB**. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens:

No ESKAPE. *J Infect Dis* 2008;197:1079–1081.
121. **Kramer A, Schwebke I, Kampf G**. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Diseases*;6. Epub ahead of print 16 August 2006. DOI: 10.1186/1471-2334-6-130.
122. **Wagenvoort JHT, De Brauwer EIGB, Penders RJR, Willems RJ, Top J, *et al.*** Environmental survival of vancomycin-resistant Enterococcus faecium. *J Hosp Infect* 2011;77:282–283.
123. **Otter JA, Vickery K, Walker JT, Delancey Pulcini E, Stoodley P, *et al.*** Surface-attached cells, biofilms and biocide susceptibility: implications for hospital cleaning and disinfection. *J Hosp Infect* 2015;89:16–27.
124. **Wilson JW, Schurr MJ, LeBlanc CL, Ramamurthy R, Buchanan KL, *et al.*** Mechanisms of bacterial pathogenicity. *Postgraduate Medical Journal* 2002;78:216–224.
125. **DiazGranados CA, Zimmer SM, Klein M, Jernigan JA**. Comparison of mortality associated with vancomycin-resistant and vancomycin-susceptible enterococcal bloodstream infections: a meta-analysis. *Clin Infect Dis* 2005;41:327–33.
126. **McBride SJ, Upton A, Roberts SA**. Clinical characteristics and outcomes of patients with vancomycin- susceptible Enterococcus faecalis and Enterococcus faecium bacteraemia-a five-year retrospective review. *Eur J Clin Microbiol Infect Dis* 2010;29:107–114.
127. **Vrioni G, Tsiamis C, Oikonomidis G, Theodoridou K, Kapsimali V, *et al.*** MALDI-TOF mass spectrometry technology for detecting biomarkers of antimicrobial resistance: current achievements and future perspectives. *Ann Transl Med* 2018;6:240.
128. **Sabat AJ, Budimir A, Nashev D, Sá-Leão R, van Dijl JM, *et al.*** Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Eurosurveillance* 2013;18:20380.
129. **Mellmann A, Andersen PS, Bletz S, Friedrich AW, Kohl TA, *et al.*** High Interlaboratory Reproducibility and Accuracy of Next-Generation-Sequencing-Based Bacterial Genotyping in a Ring Trial. *J Clin Microbiol*;55. Epub ahead of print 2017. DOI: 10.1128/JCM.02242-16.
130. **Wendt C, Grunwald WJ**. Polyclonal bacteremia due to gram-negative rods. *Clin Infect Dis* 2001;33:460–465.
131. **Willemse-Erix HFM, Jachtenberg J, Barutçi H, Puppels GJ, Van Belkum A, *et al.*** Proof of principle for successful characterization of methicillin-resistant coagulase-negative staphylococci isolated from skin by use of Raman spectroscopy and pulsed-field gel electrophoresis. *J Clin Microbiol* 2010;48:736–740.
132. **Bouchami O, Machado M, Carriço JA, Melo-Cristino J, Lencastre H de, *et al.*** Spontaneous Genomic Variation as a Survival Strategy of Nosocomial Staphylococcus haemolyticus. *Microbiol Spectr*;11. Epub ahead of print 13 April 2023. DOI: 10.1128/SPECTRUM.02552-22.
133. **Cetinkaya Y, Falk P, Mayhall CG**. Vancomycin-resistant enterococci. *Clinical Microbiology Reviews* 2000;13:686–707.
134. **Patangia D V., Anthony Ryan C, Dempsey E, Paul Ross R, Stanton C**. Impact of antibiotics on the human microbiome and consequences for host health. *Microbiologyopen*;11. Epub ahead of print 1 February 2022. DOI: 10.1002/MBO3.1260.
135. **EUSTM -European Society for Translational Medicine**. *Translational medicine: Tools And Techniques*. 1st ed. Elsevier, Academic Press (2016); 2015.
136. **Watson JD, Crick FHC**. Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. *Nat 1953 1714356* 1953;171:737–738.
137. **Sanger F, Coulson AR**. A rapid method for determining sequences in DNA by primed

synthesis with DNA polymerase. *J Mol Biol*;94. Epub ahead of print 25 May 1975. DOI: 10.1016/0022-2836(75)90213-2.
138. **Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, *et al.*** Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. *Science* 1995;269:496–512.
139. **Deurenberg RH, Bathoorn E, Chlebowicz MA, Couto N, Ferdous M, *et al.*** Application of next generation sequencing in clinical microbiology and infection prevention. *J Biotechnol* 2017;243:16–24.
140. **Quainoo S, Coolen JPM, van Hijum SAFT, Huynen MA, Melchers WJG, *et al.*** Whole-genome sequencing of bacterial pathogens: The future of nosocomial outbreak analysis. *Clinical Microbiology Reviews* 2017;30:1015–1063.
141. **Mellmann A, Bletz S, Böking T, Kipp F, Becker K, *et al.*** Real-Time Genome Sequencing of Resistant Bacteria Provides Precision Infection Control in an Institutional Setting. *J Clin Microbiol* 2016;54:2874–2881.
142. **Fox JM, Saunders NJ, Jerwood SH**. Economic and health impact modelling of a whole genome sequencing-led intervention strategy for bacterial healthcare-associated infections for England and for the USA. *Microb Genomics*;9. Epub ahead of print 1 August 2023. DOI: 10.1099/MGEN.0.001087.
143. **Singhal N, Kumar M, Kanaujia PK, Virdi JS**. MALDI-TOF mass spectrometry: An emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology*;6. Epub ahead of print 2015. DOI: 10.3389/fmicb.2015.00791.
144. **Clark AE, Kaleta EJ, Arora A, Wolk DM**. Matrix-Assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. *Clin Microbiol Rev* 2013;26:547–603.
145. **Suarez S, Ferroni A, Lotz A, Jolley KA, Guérin P, *et al.*** Ribosomal proteins as biomarkers for bacterial identification by mass spectrometry in the clinical microbiology laboratory. *J Microbiol Methods* 2013;94:390–396.
146. **Mlaga KD, Dubourg G, Abat C, Chaudet H, Lotte L, *et al.*** Using MALDI-TOF MS typing method to decipher outbreak: the case of Staphylococcus saprophyticus causing urinary tract infections (UTIs) in Marseille, France. *Eur J Clin Microbiol Infect Dis* 2017;36:2371–2377.
147. **Østergaard C, Hansen SGK, Møller JK**. Rapid first-line discrimination of methicillin resistant Staphylococcus aureus strains using MALDI-TOF MS. *Int J Med Microbiol* 2015;305:838–847.
148. **Lasch P, Fleige C, Stämmler M, Layer F, Nübel U, *et al.*** Insufficient discriminatory power of MALDI-TOF mass spectrometry for typing of Enterococcus faecium and Staphylococcus aureus isolates. *J Microbiol Methods* 2014;100:58–69.
149. **Griffin PM, Price GR, Schooneveldt JM, Schlebusch S, Tilse MH, *et al.*** Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify vancomycin-resistant enterococci and investigate the epidemiology of an outbreak. *J Clin Microbiol* 2012;50:2918–2931.
150. **Mutters NT, Heeg K, Späth I, Henny N, Günther F**. Improvement of infection control management by routine molecular evaluation of pathogen clusters. *Diagn Microbiol Infect Dis* 2017;88:82–87.
151. **Steensels D, Deplano A, Denis O, Simon A, Verroken A**. MALDI-TOF MS typing of a nosocomial methicillin-resistant Staphylococcus aureus outbreak in a neonatal intensive

care unit. *Acta Clin Belgica Int J Clin Lab Med* 2017;72:219–225.
152. **Arias CA, Murray BE**. The rise of the Enterococcus: Beyond vancomycin resistance. *Nat Rev Microbiol* 2012;10:266–278.
153. **Fiore E, Van Tyne D, Gilmore MS**. Pathogenicity of Enterococci. *Microbiol Spectr*;7. Epub ahead of print 2019. DOI: 10.1128/microbiolspec.gpp3-0053-2018.
154. **Selleck EM, Van Tyne D, Gilmore MS**. Pathogenicity of Enterococci. *Microbiol Spectr*. Epub ahead of print 2019. DOI: 10.1128/microbiolspec.GPP3-0053-2018.
155. **Horner C, Mushtaq S, Allen M, Hope R, Gerver S, *et al.*** Replacement of Enterococcus faecalis by Enterococcus faecium as the predominant enterococcus in UK bacteraemias. *JAC-Antimicrobial Resist*;3. Epub ahead of print 2021. DOI: 10.1093/jacamr/dlab185.
156. **Nørgaard M, Larsson H, Pedersen G, Schønheyder HC, Rothman KJ, *et al.*** Short-term mortality of bacteraemia in elderly patients with haematological malignancies. *Br J Haematol* 2006;132:25–31.
157. **Nørgaard M, Larsson H, Pedersen G, Schønheyder HC, Sørensen HT**. Haematological malignancies--a predictor of a poor outcome in patients with bacteraemia. *J Infect* 2006;53:190–198.
158. **Prematunge C, MacDougall C, Johnstone J, Adomako K, Lam F, *et al.*** VRE and VSE Bacteremia Outcomes in the Era of Effective VRE Therapy: A Systematic Review and Meta-analysis. *Infect Control Hosp Epidemiol* 2016;37:26–35.
159. **López-Luis BA, Sifuentes-Osornio J, Lambraño-Castillo D, Ortiz-Brizuela E, Ramírez-Fontes A, *et al.*** Risk factors and outcomes associated with vancomycin-resistant Enterococcus faecium and ampicillin-resistant Enterococcus faecalis bacteraemia: A 10-year study in a tertiary-care centre in Mexico City. *J Glob Antimicrob Resist* 2021;24:198–204.
160. **Austin ED, Sullivan SS, Macesic N, Mehta M, Miko BA, *et al.*** Reduced mortality of staphylococcus aureus bacteremia in a retrospective cohort study of 2139 patients: 2007-2015. *Clin Infect Dis* 2020;70:1666–1674.
161. **Ali GA, Goravey W, Najim MS, Shunnar KM, Ibrahim SI, *et al.*** Cohort Study Epidemiology, microbiological and clinical characteristics of Enterococcus species bloodstream infections: A 10-year retrospective cohort study from Qatar. *Ann Med Surg* 2022;80:104258.
162. **Bright M, Parfitt EC, Pasquill K, Steele L, Laupland KB**. Occurrence and determinants of enterococcal bloodstream infections: a population-based study. *Infect Dis (Auckl)* 2020;52:638–643.
163. **Feldman SF, Temkin E, Wullfhart L, Nutman A, Schechner V, *et al.*** A nationwide population-based study of Escherichia coli bloodstream infections: incidence, antimicrobial resistance and mortality. *Clin Microbiol Infect* 2022;28:879.e1-879.e7.
164. **Rottier WC, Arpi M, Blank SN, Nabuurs-Franssen MH, Ruijs GJHM, *et al.*** Attributable mortality of vancomycin resistance in ampicillin-resistant Enterococcus faecium bacteremia in Denmark and the Netherlands: A matched cohort study. *Infect Control Hosp Epidemiol* 2022;43:719–727.
165. **Gorrie C, Higgs C, Carter G, Stinear TP, Howden B**. Genomics of vancomycin-resistant enterococcus faecium. *Microb Genomics*;5. Epub ahead of print 1 July 2019. DOI: 10.1099/mgen.0.000283.
166. **Hayden MK, Bonten MJM, Blom DW, Lyle EA, Van De Vijver DAMC, *et al.*** Reduction in acquisition of vancomycin-resistant enterococcus after enforcement of routine environmental cleaning measures. *Clin Infect Dis* 2006;42:1552–1560.

167. **Gurk-Turner C**. Quinupristin/dalfopristin: the first available macrolide-lincosamide-streptogramin antibiotic. *Proc (Bayl Univ Med Cent)* 2000;13:83.
168. **García-Solache M, Rice LB**. The Enterococcus: a Model of Adaptability to Its Environment. *Clin Microbiol Rev*;32. Epub ahead of print 1 April 2019. DOI: 10.1128/CMR.00058-18.
169. **Wagner TM, Janice J, Schulz M, Ballard SA, da Silva AG, *et al.*** Reversible vancomycin susceptibility within emerging ST1421 Enterococcus faecium strains is associated with rearranged vanA-gene clusters and increased vanA plasmid copy number. *Int J Antimicrob Agents*;62. Epub ahead of print 1 July 2023. DOI: 10.1016/J.IJANTIMICAG.2023.106849.
170. **Pinholt M**. *Hospital-acquired vancomycin-resistant Enterococcus faecium in Denmark. PhD Thesis. University of Copenhagen. Faculty of Health and Medical Science.* https://www.forskningsdatabasen.dk/en/catalog/2349367931 (2016, accessed 7 February 2020).
171. **Xavier BB, Coppens J, de Koster S, Rajakani SG, van Goethem S, *et al.*** Novel vancomycin resistance gene cluster in Enterococcus faecium ST1486, Belgium, June 2021. *Eurosurveillance*;26. Epub ahead of print 9 September 2021. DOI: 10.2807/1560-7917.ES.2021.26.36.2100767.
172. **Werner G, Neumann B, Weber RE, Kresken M, Wendt C, *et al.*** Thirty years of VRE in Germany - 'expect the unexpected': The view from the National Reference Centre for Staphylococci and Enterococci. *Drug Resist Updat*;53. Epub ahead of print 1 December 2020. DOI: 10.1016/J.DRUP.2020.100732.
173. **Bonten MJ, Willems R, Weinstein RA**. Vancomycin-resistant enterococci: why are they here, and where do they come from? *Lancet Infect Dis* 2001;1:314–325.
174. **Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A**. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. *Clin Microbiol Infect* 2010;16:541–554.
175. **McGuinness WA, Malachowa N, DeLeo FR**. Focus: Infectious Diseases: Vancomycin Resistance in Staphylococcus aureus. *Yale J Biol Med* 2017;90:269.
176. **Périchon B, Courvalin P**. VanA-Type Vancomycin-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother* 2009;53:4580.
177. **Statens Serum Institut**. Stafylokok rapporter. https://antibiotika.ssi.dk/overvaagning-og-forskning/overvaagning-af-mrsa-i-dk/stafylokok-rapporter (accessed 3 October 2023).
178. **Murray BE**. Diversity among multidrug-resistant enterococci. *Emerg Infect Dis* 1998;4:37–47.
179. **Cattoir V, Leclercq R**. Twenty-five years of shared life with vancomycin-resistant enterococci: Is it time to divorce. *J Antimicrob Chemother* 2013;68:731–742.
180. **Leclercq R, Derlot E, Duval J, Courvalin P**. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. *N Engl J Med* 1988;319:157–161.
181. **Uttley AHC, Collins CH, Naidoo J, George RC**. VANCOMYCIN-RESISTANT ENTEROCOCCI. *Lancet* 1988;331:57–58.
182. **Morris JG, Shay DK, Hebden JN, McCarter RJ, Perdue BE, *et al.*** Enterococci resistant to multiple antimicrobial agents, including vancomycin: Establishment of endemicity in a University Medical Center. *Ann Intern Med* 1995;123:250–259.
183. **CDC**. Nosocomial enterococci resistant to vancomycin--United States, 1989-1993. *MMWR Morb Mortal Wkly Rep* 1993;42:597–599.
184. **Treitman AN, Yarnold PR, Warren J, Noskin GA**. Emerging Incidence of Enterococcus

faecium among Hospital Isolates (1993 to 2002). *J Clin Microbiol* 2005;43:462–463.
185. **Coque TM, Willems RJL, Fortún J, Top J, Diz S, *et al.*** Population Structure of Enterococcus faecium Causing Bacteremia in a Spanish University Hospital: Setting the Scene for a Future Increase in Vancomycin Resistance? *Antimicrob Agents Chemother* 2005;49:2693–2700.
186. **Bates J, Jordens Z, Selkon JB**. Evidence for an animal origin of vancomycin-resistant enterococci. *Lancet (London, England)* 1993;342:490–491.
187. **Hammerum AM**. Enterococci of animal origin and their significance for public health. *Clin Microbiol Infect* 2012;18:619–625.
188. **Aarestrup FM**. Occurrence of glycopeptide resistance among Enterococcus faecium isolates from conventional and ecological poultry farms. *Microb Drug Resist* 1995;1:255–257.
189. **Bager F, Aarestrup FM, Madsen M, Wegener HC**. Glycopeptide resistance in Enterococcus faecium from broilers and pigs following discontinued use of avoparcin. *Microb Drug Resist* 1999;5:53–56.
190. **McDonald LC, Kuehnert MJ, Tenover FC, Jarvis WR**. Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. *Emerg Infect Dis* 1997;3:311.
191. **O'Driscoll T, Crank CW**. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. *Infect Drug Resist* 2015;8:217–230.
192. **ECDC**. Surveillance Atlas of Infectious Diseases. https://atlas.ecdc.europa.eu/public/index.aspx?Dataset=27&HealthTopic=4 (accessed 6 October 2023).
193. **World Health Organization**. WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed. https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed 7 February 2020).
194. **Skjøt-Arkil H, Mogensen CB, Lassen AT, Johansen IS, Chen M, *et al.*** Carrier prevalence and risk factors for colonisation of multiresistant bacteria in Danish emergency departments: A cross-sectional survey. *BMJ Open*;9. Epub ahead of print 2019. DOI: 10.1136/bmjopen-2019-029000.
195. **Homan WL, Tribe D, Poznanski S, Li M, Hogg G, *et al.*** Multilocus Sequence Typing Scheme for Enterococcus faecium. *J Clin Microbiol* 2002;40:1963–1971.
196. **Willems RJL, Top J, Van den Braak N, Van Belkum A, Endtz H, *et al.*** Host specificity of vancomycin-resistant Enterococcus faecium. *J Infect Dis* 2000;182:816–823.
197. **Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG**. eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. *J Bacteriol* 2004;186:1518.
198. **Lester CH, Sandvang D, Olsen SS, Schønheyder HC, Jarløv JO, *et al.*** Emergence of ampicillin-resistant Enterococcus faecium in Danish hospitals. *J Antimicrob Chemother* 2008;62:1203–1206.
199. **Galloway-Peña JR, Nallapareddy SR, Arias CA, Eliopoulos GM, Murray BE**. Analysis of clonality and antibiotic resistance among early clinical isolates of enterococcus faecium in the United States. *J Infect Dis* 2009;200:1566–1573.
200. **Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, *et al.*** Restricted gene flow among hospital subpopulations of Enterococcus faecium. *MBio*;3. Epub ahead of print 2012. DOI: 10.1128/mBio.00151-12.
201. **Leavis HL, Bonten MJ, Willems RJ**. Identification of high-risk enterococcal clonal complexes:

global dispersion and antibiotic resistance. *Curr Opin Microbiol* 2006;9:454–460.
202. **Turner KM, Hanage WP, Fraser C, Connor TR, Spratt BG**. Assessing the reliability of eBURST using simulated populations with known ancestry. *BMC Microbiol*;7. Epub ahead of print 2007. DOI: 10.1186/1471-2180-7-30.
203. **Wurster JI, Saavedra JT, Gilmore MS**. Impact of Antibiotic Use on the Evolution of Enterococcus faecium. *J Infect Dis* 2016;213:1862–1865.
204. **Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE**. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of enterococcus faecium. *PLoS One*;7. Epub ahead of print 2012. DOI: 10.1371/journal.pone.0030187.
205. **Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, *et al.*** Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. *MBio*;4. Epub ahead of print 2013. DOI: 10.1128/mBio.00534-13.
206. **Viviana Belloso Daza M, Almeida-Santos AC, Novais C, Read A, Alves V, *et al.*** Distinction between Enterococcus faecium and Enterococcus lactis by a gluP PCR-Based Assay for Accurate Identification and Diagnostics. *Microbiol Spectr*;XX. Epub ahead of print December 2022. DOI: 10.1128/SPECTRUM.03268-22.
207. **Top J, Willems R, Van Der Velden S, Asbroek M, Bonten M**. Emergence of Clonal Complex 17 Enterococcus faecium in The Netherlands. *J Clin Microbiol* 2008;46:214–219.
208. **van Hal SJ, Willems RJL, Gouliouris T, Ballard SA, Coque TM, *et al.*** The global dissemination of hospital clones of Enterococcus faecium. *Genome Med*;13. Epub ahead of print 2021. DOI: 10.1186/s13073-021-00868-0.
209. **Rosvoll TCS, Pedersen T, Sletvold H, Johnsen PJ, Sollid JE, *et al.*** PCR-based plasmid typing in Enterococcus faecium strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunol Med Microbiol* 2010;58:254–68.
210. **Freitas AR, Pereira AP, Novais C, Peixe L**. Multidrug-resistant high-risk Enterococcus faecium clones: can we really define them? *Int J Antimicrob Agents*;57. Epub ahead of print 1 January 2021. DOI: 10.1016/J.IJANTIMICAG.2020.106227.
211. **Rubaye MAL, Janice J, Bjørnholt JV, Kacelnik O, Haldorsen BC, *et al.*** The population structure of vancomycin-resistant and -susceptible Enterococcus faecium in a low-prevalence antimicrobial resistance setting is highly influenced by circulating global hospital-associated clones. *Microb genomics*;9. Epub ahead of print 1 December 2023. DOI: 10.1099/MGEN.0.001160.
212. **Montealegre MC, Singh K V., Murray BE**. Gastrointestinal Tract Colonization Dynamics by Different Enterococcus faecium Clades. *J Infect Dis* 2016;213:1914–1922.
213. **Grønnemose RB, Rosenlund K, Kemp M, Andersen TE, Pedersen RM**. High intestinal colonisation capacity of the vanA Enterococcus faecium ST1421-CT1134 may explain its epidemic spread: data from a close to physiological in vitro model. https://portal.findresearcher.sdu.dk/en/publications/high-intestinal-colonisation-capacity-of-the-vana-enterococcus-fa (2022, accessed 22 November 2023).
214. **Howden CP, Holt BP, Lam KE, Seemann M, Ballard T, *et al.*** Genomic insights to control the emergence of vancomycin-resistant enterococci. *MBio* 2013;4:412–425.
215. **Leigh RJ, McKenna C, McWade R, Lynch B, Walsh F**. "Comparative genomics and pangenomics of vancomycin resistant and susceptible Enterococcus faecium from Irish

hospitals across 20 years". *J Med Microbiol* 2022;71:2021.11.22.469549.
216. **van Hal SJ, Ip CLC, Ansari MA, Wilson DJ, Espedido BA, *et al.*** Evolutionary dynamics of Enterococcus faecium reveals complex genomic relationships between isolates with independent emergence of vancomycin resistance. *Microb genomics*;2. Epub ahead of print 2016. DOI: 10.1099/mgen.0.000048.
217. **Jensen LB, Garcia-Migura L, Valenzuela AJS, Løhr M, Hasman H, *et al.*** A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* 2010;80:25–43.
218. **Hammerum AM, Baig S, Kamel Y, Roer L, Pinholt M, *et al.*** Emergence of vanA Enterococcus faecium in Denmark, 2005-15. *J Antimicrob Chemother* 2017;72:2184–2190.
219. **Pinholt M, Bayliss SC, Gumpert H, Worning P, Jensen VVS, *et al.*** WGS of 1058 Enterococcus faecium from Copenhagen, Denmark, reveals rapid clonal expansion of vancomycin-resistant clone ST80 combined with widespread dissemination of a vanA-containing plasmid and acquisition of a heterogeneous accessory genome | Journal. *J Antimicrob Chemother* 2019;74:1776–1785.
220. **Hammerum A, Karstensen K, Roer L, Kaya H, Porsbo L, *et al.*** "Surveillance of vancomycin-resistant enterococci reveals shift in dominating clusters from vanA Enterococcus faecium clusters to vanB E. faecium clusters, Denmark 2015-2022". *Eurosurveillance (Submitted Publ*.
221. **Folkhälsomyndigheten**. Vancomycinresistenta enterokocker (VRE) – sjukdomsstatistik — Folkhälsomyndigheten. https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/vancomycinresistenta-enterokocker-vre/?tab=tab-report (accessed 10 January 2024).
222. **Eichel V, Klein S, Bootsveld C, Frank U, Heeg K, *et al.*** Challenges in interpretation of WGS and epidemiological data to investigate nosocomial transmission of vancomycin-resistant Enterococcus faecium in an endemic region: incorporation of patient movement network and admission screening. *J Antimicrob Chemother*. Epub ahead of print 2020. DOI: 10.1093/jac/dkaa122.
223. **Robert Koch Institut**. *Epidemiologisches Bulletin Jahresbericht Vancomycin-resistente Enterokokken in Deutschland*. https://ars.rki.de (2023, accessed 10 January 2024).
224. **Cassone M, Wang J, Lansing BJ, Mantey J, Gibson KE, *et al.*** Diversity and persistence of MRSA and VRE in skilled nursing facilities: environmental screening, whole-genome sequencing and development of a dispersion index. *J Hosp Infect* 2023;138:8–18.
225. **Falgenhauer L, Fritzenwanker M, Imirzalioglu C, Steul K, Scherer M, *et al.*** Near-ubiquitous presence of a vancomycin-resistant Enterococcus faecium ST117/CT71/vanB -clone in the Rhine-Main metropolitan area of Germany. *Antimicrob Resist Infect Control*;8. Epub ahead of print 29 July 2019. DOI: 10.1186/s13756-019-0573-8.
226. **Hughes A, Ballard S, Sullivan S, Marshall C**. An outbreak of vanA vancomycin-resistant Enterococcus faecium in a hospital with endemic vanB VRE. *Infect Dis Heal* 2019;24:82–91.
227. **McCracken M, Wong A, Mitchell R, Gravel D, Conly J, *et al.*** Molecular epidemiology of vancomycin-resistant enterococcal bacteraemia: results from the Canadian Nosocomial Infection Surveillance Program, 1999-2009. *J Antimicrob Chemother* 2013;68:1505–1509.
228. **Hammerum AM, Justesen US, Pinholt M, Roer L, Kaya H, *et al.*** Surveillance of vancomycin-resistant enterococci reveals shift in dominating clones and national spread of a vancomycin-variable vanA Enterococcus faecium ST1421-CT1134 clone, Denmark, 2015 to

March 2019. *Euro Surveill*;24. Epub ahead of print 22 August 2019. DOI: 10.2807/1560-7917.ES.2019.24.34.1900503.
229. **Browning S, Davis JS, Mitchell BG**. Have gloves and gowns had their day? An Australian and New Zealand practice and attitudes survey about contact precautions for MRSA and VRE colonisation. *Infect Dis Heal* 2023;28:221–225.
230. **Cho SY, Kim HM, Chung DR, Choi JR, Lee MA, *et al.*** The impact of vancomycin-resistant Enterococcus (VRE) screening policy change on the incidence of healthcare-associated VRE bacteremia. *Infect Control Hosp Epidemiol*. Epub ahead of print 2021. DOI: 10.1017/ice.2021.189.
231. **Lemieux C, Gardam M, Evans G, John M, Suh KN, *et al.*** Longitudinal Multicenter Analysis of Outcomes After Cessation of Control Measures for Vancomycin-Resistant Enterococci. *Infect Control Hosp Epidemiol* 2017;38:24–30.
232. **Popiel KY, Miller MA**. Evaluation of vancomycin-resistant enterococci (VRE)-associated morbidity following relaxation of VRE screening and isolation precautions in a tertiary care hospital. *Infect Control Hosp Epidemiol* 2014;35:818–825.
233. **Kleyman R, Cupril-Nilson S, Robinson K, Thakore S, Haq F, *et al.*** Does the removal of contact precautions for MRSA and VRE infected patients change health care-associated infection rate?: A systematic review and meta-analysis. *Am J Infect Control* 2021;49:784–791.
234. **Martin EM, Colaianne B, Bridge C, Bilderback A, Tanner C, *et al.*** Discontinuing MRSA and VRE contact precautions: Defining hospital characteristics and infection prevention practices predicting safe de-escalation. *Infect Control Hosp Epidemiol*;43. Epub ahead of print 1 November 2022. DOI: 10.1017/ICE.2021.457.
235. **Marra AR, Edmond MB, Schweizer ML, Ryan MPH GW, Diekema DJ**. Discontinuing contact precautions for multidrug-resistant organisms: A systematic literature review and meta-analysis. *Am J Infect Control* 2018;46:333–373.
236. **Schwartz DC, Cantor CR**. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 1984;37:67–75.
237. **CDC**. Outbreak Detection | PulseNet | CDC. https://www.cdc.gov/pulsenet/outbreak-detection/index.html (accessed 3 January 2024).
238. **Shendure J, Ji H**. Next-generation DNA sequencing. *Nat Biotechnol* 2008;26:1135–1145.
239. Illumina | Sequencing and array-based solutions for genetic research. https://www.illumina.com/ (accessed 7 February 2020).
240. **Illumina**. *An introduction to Next-Generation Sequencing Technology*. www.illumina.com/technology/next-generation-sequencing.html (accessed 20 December 2023).
241. **Sims D, Sudbery I, Ilott NE, Heger A, Ponting CP**. Sequencing depth and coverage: key considerations in genomic analyses. *Nat Rev Genet* 2014;15:121–132.
242. **Johnson JS, Spakowicz DJ, Hong BY, Petersen LM, Demkowicz P, *et al.*** Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat Commun 2019 101* 2019;10:1–11.
243. **Maiden MCJ, Jansen Van Rensburg MJ, Bray JE, Earle SG, Ford SA, *et al.*** MLST revisited: the gene-by-gene approach to bacterial genomics Europe PMC Funders Group. *Nat Rev Microbiol* 2013;11:728–736.
244. **Thomsen MCF, Ahrenfeldt J, Cisneros JLB, Jurtz V, Larsen MV, *et al.*** A bacterial analysis

platform: An integrated system for analysing bacterial whole genome sequencing data for clinical diagnostics and surveillance. *PLoS One*;11. Epub ahead of print 1 June 2016. DOI: 10.1371/journal.pone.0157718.
245. **Janezic S, Rupnik M**. Development and Implementation of Whole Genome Sequencing-Based Typing Schemes for Clostridioides difficile. *Front public Heal*;7. Epub ahead of print 24 October 2019. DOI: 10.3389/FPUBH.2019.00309.
246. **Li Y, Lin Y**. Kmer2SNP: reference-free SNP calling from raw reads based on matching. *bioRxiv*. Epub ahead of print 17 May 2020. DOI: 10.1101/2020.05.17.100305.
247. **Leggett RM, MacLean D**. Reference-free SNP detection: dealing with the data deluge. *BMC Genomics*;15 Suppl 4. Epub ahead of print 2014. DOI: 10.1186/1471-2164-15-S4-S10.
248. **Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, *et al.*** Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* 1998;95:3140–3145.
249. **Uelze L, Grützke J, Borowiak M, Hammerl JA, Juraschek K, *et al.*** Typing methods based on whole genome sequencing data. *One Heal Outlook 2020 21* 2020;2:1–19.
250. Ridom SeqSphere+ Core_Genome_MLST_Cluster_Type.html. https://www.ridom.de/seqsphere/ug/v40/Core_Genome_MLST_Cluster_Type.html (accessed 12 December 2023).
251. Ridom SeqSphere+ - Overview. https://www.ridom.de/seqsphere/ (accessed 7 February 2020).
252. Ridom SeqSphere + Software is designed for distributed work-groups (client/server model) and allows automatic processing and analyzing of NGS (e.g. www.ridom.de (2019, accessed 7 February 2020).
253. **Moura A, Criscuolo A, Pouseele H, Maury MM, Leclercq A, *et al.*** Whole genome-based population biology and epidemiological surveillance of Listeria monocytogenes. *Nat Microbiol* 2016;2:16185.
254. **Pearce ME, Alikhan NF, Dallman TJ, Zhou Z, Grant K, *et al.*** Comparative analysis of core genome MLST and SNP typing within a European Salmonella serovar Enteritidis outbreak. *Int J Food Microbiol* 2018;274:1–11.
255. **Schürch AC, Arredondo-Alonso S, Willems RJL, Goering R V**. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. *Clinical Microbiology and Infection* 2018;24:350–354.
256. **Saitou N, Nei M**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–425.
257. **Prim RC**. Shortest connection networks and some generalizations. *Bell Syst Tech J* 1957;36:1389–1401.
258. **Leekitcharoenphon P, Nielsen EM, Kaas RS, Lund O, Aarestrup FM**. Evaluation of whole genome sequencing for outbreak detection of Salmonella enterica. *PLoS One*;9. Epub ahead of print 4 February 2014. DOI: 10.1371/JOURNAL.PONE.0087991.
259. **Holder M, Lewis PO**. Phylogeny estimation: traditional and Bayesian approaches. *Nat Rev Genet* 2003;4:275–284.
260. **World Health Organization**. Whole genome sequencing for foodborne disease surveillance. *Whole genome sequencing for foodborne disease surveillance* 2018;1–42.
261. **Zhang T, Li H, Ma S, Cao J, Liao H, *et al.*** The newest Oxford Nanopore R10.4.1 full-length

16S rRNA sequencing enables the accurate resolution of species-level microbial community profiling. *Appl Environ Microbiol*;89. Epub ahead of print 1 October 2023. DOI: 10.1128/AEM.00605-23.
262. **Schmidt M, Pedersen L, Sørensen HT**. The Danish Civil Registration System as a tool in epidemiology. *Eur J Epidemiol* 2014;29:541–549.
263. **Voldstedlund M, Haarh M, Mølbak K**. The danish microbiology database (MIBA) 2010 to 2013. *Eurosurveillance* 2014;19:20667.
264. **Moller JK**. A microcomputer-assisted bacteriology reporting and information system. *Acta Pathol Microbiol Immunol Scand B* 1984;92:119–126.
265. **Systematic.com**. Systematic Columna CIS. https://systematic.com/en-gb/industries/healthcare/hospital-care/columna-cis/#6af214b4-6126-44ff-b38f-6b70094cc97c (accessed 26 June 2023).
266. **Woksepp H, Ryberg A, Berglind L, Schön T, Söderman J**. Epidemiological characterization of a nosocomial outbreak of extended spectrum β-lactamase Escherichia coli ST-131 confirms the clinical value of core genome multilocus sequence typing. *APMIS* 2017;125:1117–1124.
267. **Werner G, Couto N, Feil EJ, Novais A, Hegstad K, *et al.*** Taking hospital pathogen surveillance to the next level. *Microb Genomics* 2023;9:1.
268. **Lee RS, Gonçalves Da Silva A, Baines SL, Strachan J, Ballard S, *et al.*** The changing landscape of vancomycin-resistant Enterococcus faecium in Australia: a population-level genomic study. *J Antimicrob Chemother* 2018;73:3268–3278.
269. **Gouliouris T, Coll F, Ludden C, Blane B, Raven KE, *et al.*** Quantifying acquisition and transmission of Enterococcus faecium using genomic surveillance. *Nat Microbiol* 2021;6:103–111.
270. **Tenover FC, Arbeit RD, Goering R V, Mickelsen PA, Murray BE, *et al.*** GUEST COMMENTARY Interpreting Chromosomal DNA Restriction Patterns Produced by Pulsed-Field Gel Electrophoresis: Criteria for Bacterial Strain Typing. *J Clin Microbiol* 1995;33:2233–2239.
271. **Higgs C, Sherry NL, Seemann T, Horan K, Walpola H, *et al.*** Optimising genomic approaches for identifying vancomycin-resistant Enterococcus faecium transmission in healthcare settings. *Nat Commun*;13. Epub ahead of print 1 December 2022. DOI: 10.1038/s41467-022-28156-4.
272. **Boyce JM, Opal SM, Pot1rer-Bynoe G, Laforge RG, Zervos MJ, *et al.*** Emergence and Nosocomial Transmission of Ampicillin-Resistant Enterococci. *Antimicrob Agents Chemother* 1992;36:1032–1039.
273. **Jureen R, Top J, Mohn SC, Harthug S, Langeland N, *et al.*** Molecular characterization of ampicillin-resistant Enterococcus faecium isolates from hospitalized patients in Norway. *J Clin Microbiol* 2003;41:2330–2336.
274. **Tedim AP, Ruíz-Garbajosa P, Rodríguez MC, Rodríguez-Baños M, Lanza VF, *et al.*** Long-term clonal dynamics of Enterococcus faecium strains causing bloodstream infections (1995-2015) in Spain. *J Antimicrob Chemother* 2017;72:48–55.
275. **Egan SA, Kavanagh NL, Shore AC, Mollerup S, Samaniego Castruita JA, *et al.*** Genomic analysis of 600 vancomycin-resistant Enterococcus faecium reveals a high prevalence of ST80 and spread of similar vanA regions via IS1216E and plasmid transfer in diverse genetic lineages in Ireland. *J Antimicrob Chemother* 2022;77:320–330.
276. **Suppola JP, Kolho E, Salmenlinna S, Tarkka E, Vuopio-Varkila J, *et al.*** vanA and vanB incorporate into an endemic ampicillin-resistant vancomycin-sensitive Enterococcus

faecium strain: effect on interpretation of clonality. *J Clin Microbiol* 1999;37:3934–3939.
277. **Pratama R, Beukers AG, McIver CJ, Keighley CL, Taylor PC, *et al.*** A vanA vancomycin-resistant Enterococcus faecium ST80 outbreak resulting from a single importation event. *J Antimicrob Chemother* 2021;77:31–37.
278. **Freeman R, Moore LSP, García Álvarez L, Charlett A, Holmes A**. Advances in electronic surveillance for healthcare-associated infections in the 21st Century: A systematic review. *J Hosp Infect* 2013;84:106–119.
279. **Abat C, Chaudet H, Colson P, Rolain JM, Raoult D**. Real-time microbiology laboratory surveillance system to detect abnormal events and emerging infections, Marseille, France. *Emerg Infect Dis* 2015;21:1302–1310.
280. **Grota PG, Stone PW, Jordan S, Pogorzelska M, Larson E**. Electronic surveillance systems in infection prevention: Organizational support, program characteristics, and user satisfaction. *Am J Infect Control* 2010;38:509–514.
281. **Roel Streefkerk HA, Roel Verkooijen PAJ, Wichor Bramer M, Henri Verbrugh A**. Electronically assisted surveillance systems of healthcare-associated infections: a systematic review. *Euro Surveill*;25. Epub ahead of print 16 January 2020. DOI: 10.2807/1560-7917.ES.2020.25.2.1900321.
282. **Pinholt M, Østergaard C, Arpi M, Bruun NE, Schønheyder HC, *et al.*** Incidence, clinical characteristics and 30-day mortality of enterococcal bacteraemia in Denmark 2006-2009: A population-based cohort study. *Clin Microbiol Infect* 2014;20:145–151.
283. **Correa-Martínez CL, Schuler F, Kampmeier S**. Sex differences in vancomycin-resistant enterococci bloodstream infections—a systematic review and meta-analysis. *Biol Sex Differ*;12. Epub ahead of print 2021. DOI: 10.1186/s13293-021-00380-5.
284. **Nicolle LE**. Asymptomatic Bacteriuria and Bacterial Interference. *Microbiol Spectr*;3. Epub ahead of print 4 September 2015. DOI: 10.1128/MICROBIOLSPEC.UTI-0001-2012.
285. **Aspevall O, Hallander H, Gant V, Kouri T**. European guidelines for urinalysis: a collaborative document produced by European clinical microbiologists and clinical chemists under ECLM in collaboration with ESCMID. *Clin Microbiol Infect* 2001;7:173–178.
286. **Dubler S, Lenz M, Zimmermann S, Richter DC, Weiss KH, *et al.*** Does vancomycin resistance increase mortality in Enterococcus faecium bacteraemia after orthotopic liver transplantation? A retrospective study. *Antimicrob Resist Infect Control* 2020;9:1–10.
287. **Bager P, Kähler J, Andersson M, Holzknecht B, Hansen S, *et al.*** "Comparison of morbidity and mortality after bloodstream infection with vancomycin-resistant versus –susceptible Enterococcus faecium: A nationwide cohort study in Denmark, 2010-2019". *Emerg Microbes Infect (Submitted Publ*.
288. **Elstrøm P, Astrup E, Hegstad K, Samuelsen Ø, Enger H, *et al.*** The fight to keep resistance at bay, epidemiology of carbapenemase producing organisms (CPOs), vancomycin resistant enterococci (VRE) and methicillin resistant Staphylococcus aureus (MRSA) in Norway, 2006 - 2017. *PLoS One*;14. Epub ahead of print 1 February 2019. DOI: 10.1371/JOURNAL.PONE.0211741.
289. **GA P, BR M, J M, MH M, W L, *et al.*** Nosocomial infections with vancomycin-resistant Enterococcus faecium in liver transplant recipients: risk factors for acquisition and mortality. *Clin Infect Dis* 1996;23:760–766.
290. **Jordens JZ, Bates J, Griffiths DT**. Faecal carriage and nosocomial spread of vancomycin-resistant Enterococcus faecium. *J Antimicrob Chemother* 1994;34:515–528.

291. **Montecalvo MA, de Lencastre H, Carraher M, Gedris C, Chung M, *et al.*** Natural history of colonization with vancomycin-resistant Enterococcus faecium. *Infect Control Hosp Epidemiol* 1995;16:680–685.
292. **Brennen C, Wagener MM, Muder RR**. Vancomycin-resistant Enterococcus faecium in a long-term care facility. *J Am Geriatr Soc* 1998;46:157–160.
293. **Hertz FB, Nielsen KL, Olsen MH, Ebdrup SR, Nielsen C, *et al.*** Vancomycin-resistant Enterococcus faecium: should we screen on admission? *APMIS* 2022;130:657–660.
294. **Wilson J, Prieto J**. Re-visiting contact precautions - 25 years on. *J Infect Prev* 2021;22:242–244.
295. **Rice S, Carr K, Sobiesuo P, Shabaninejad H, Orozco-Leal G, *et al.*** Economic evaluations of interventions to prevent and control health-care-associated infections: a systematic review. *Lancet Infect Dis* 2023;23:e228–e239.
296. **Dancer SJ**. The role of environmental cleaning in the control of hospital-acquired infection. *J Hosp Infect* 2009;73:378–385.
297. **Remschmidt C, Schröder C, Behnke M, Gastmeier P, Geffers C, *et al.*** Continuous increase of vancomycin resistance in enterococci causing nosocomial infections in Germany - 10 years of surveillance. *Antimicrob Resist Infect Control*;7. Epub ahead of print 24 April 2018. DOI: 10.1186/S13756-018-0353-X.
298. **Freitas AR, Tedim AP, Novais C, Coque TM, Peixe L**. Distribution of putative virulence markers in Enterococcus faecium: towards a safety profile review. *J Antimicrob Chemother* 2018;73:306–319.

Papers

Paper I

Journal of Global Antimicrobial Resistance 24 (2021) 418–423

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Short Communication

Using core genome multilocus sequence typing (cgMLST) for vancomycin-resistant *Enterococcus faecium* isolates to guide infection control interventions and end an outbreak☆

Sanne Kjær Hansen[a,b,*], Lise Andersen[c], Mette Detlefsen[c], Anette Holm[a,c], Louise Roer[b], Panagiotis Antoniadis[a], Marianne Nielsine Skov[a,c], Anette M. Hammerum[b], Michael Kemp[a]

[a] *Research Unit for Department of Clinical Microbiology, Odense University Hospital, University of Southern Denmark, Odense, Denmark*
[b] *Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Copenhagen S, Denmark*
[c] *Department of Clinical Microbiology, Odense University Hospital, Odense C, Denmark*

ARTICLE INFO

Article history:
Received 5 September 2020
Received in revised form 11 December 2020
Accepted 5 February 2021
Available online 19 February 2021

Keywords:
cgMLST
VRE
Epidemiology
Transmission
Outbreak
Infection control

ABSTRACT

Objectives: Until July 2016, vancomycin-resistant*Enterococcus faecium* (VREfm) was sporadically detected in Odense University Hospital, Denmark. After July 2016, the number of VREfm cases increased. This study aimed to apply a core genome multilocus sequence typing (cgMLST) scheme for *E. faecium* to type and analyse VREfm isolates collected at a single Danish hospital and to compare the results with cgMLST data from other regions of Denmark to trace transmission.

Methods: A total of 38 VREfm clinical isolates from inpatients at the hospital in the period January 2014 through June 2017 were included in the study and analysed using whole-genome sequencing. Use of SeqSphere + software was initiated from the beginning of June 2017 to obtain MLST, cgMLST and epi curves. Admission histories were incorporated and national surveillance data on cgMLST were used to identify transmission routes.

Results: Six different sequence types (STs) were identified, the most frequent being ST80, ST117 and ST203. cgMLST subdivided the 38 isolates into 18 different complex types (CTs) with 13 isolates (34%) belonging to ST80-CT993. Epi curves indicated transmission of ST80-CT993 in several departments. Transmission from patients transferred from other hospitals was not identifiable. Infection control interventions launched in one department ended the outbreak.

Conclusion: The high resolution of cgMLST allowed for detailed interpretation with evidence of nosocomial transmission of specific CTs. cgMLST made it easy to compare our local isolates with national findings, thereby clarifying transmission routes. Supplemented with admission histories, cgMLST targeted the epidemiological investigation and delineated the expensive and time-consuming infection control interventions.

1. Introduction

Vancomycin resistance in *Enterococcus faecium* has been known since the end of the 1980s, and in human clinical isolates it is primarily due to *vanA* or *vanB* genes [1,2]. Vancomycin-resistant *E. faecium* (VREfm) thrive very well in the hospital environment and may cause hospital-acquired infections. VREfm have for the last decade been rising in number worldwide, and infections caused by these isolates are difficult to treat owing to their inherent resistance to many antimicrobials [3,4].

For many years, the prevalence of clinical VREfm found in blood cultures and spinal fluids in Denmark was ~1%, but in 2012 large outbreaks in two of the five Danish regions began. More than 1000 patients were infected or colonised during the period 2012–2015 and the transmission is still ongoing, leaving the Danish prevalence of these clinical VREfm above 10% in 2018 [5]. Multilocus sequence

☆ These data have been presented in part as an abstract and poster at the 11th Healthcare Infection Society (HIS) International Conference, 26–28 November 2018, Liverpool, UK.

* Corresponding author at: Department of Clinical Microbiology, Odense University Hospital, J.B. Winsløwsvej 21, 2nd floor, DK-5000 Odense, Denmark.
E-mail address: sanne.groenvall.kjaer.hansen@rsyd.dk (S. Kjær Hansen).

http://dx.doi.org/10.1016/j.jgar.2021.02.007

typing (MLST) of clinical VREfm from all kind of materials revealed that several sequence types (STs) were involved in the outbreaks, in particular ST80, ST117 and ST203. In 73% of the VREfm isolates, resistance to vancomycin was due to the *vanA* gene [5–7]. Initially these outbreaks did not affect hospitals in the Region of Southern Denmark despite transfer of patients from affected hospitals. At Odense University Hospital in Region of Southern Denmark, five to six patients were identified with VREfm each year in this period. However, in 2016 the number of patients with VREfm increased, and in the first half of 2017 an outbreak was suspected [5].

The traditional method used for outbreak investigation in Denmark had for many years been based on *Sma*I macrorestriction analysis through pulsed-field gel electrophoresis (PFGE). More recently, single nucleotide polymorphism (SNP)-based mapping of short-read (Illumina) data against a reference genome has been applied for outbreak investigations, but this method has now been substituted with core genome MLST (cgMLST). Typing based on cgMLST, with the designation of a complex type (CT), has a high discriminatory power and studies have found the method concordant with the SNP-based mapping mentioned above in evaluating the relatedness of bacteria [8–11]. Due to the availability of easy-to-use software solutions, we implemented the cgMLST method in the Department of Clinical Microbiology at Odense University Hospital in June 2017 and used it for investigation of the local rise of VREfm.

The aim of this study was to apply a cgMLST scheme for *E. faecium* implemented in SeqSphere + software to type and analyse the VREfm isolates collected at a single Danish hospital over a period of 4 years, to compare the results with cgMLST data of VREfm isolates identified in other regions of Denmark as a part of the national VREfm surveillance, and to use these data to trace transmission.

2. Materials and methods

2.1. Demographic data

Denmark is divided into five regions (NUTs, level 2), of which the Region of Southern Denmark (RSD) covers approximately one-fifth (1.2 million) of the Danish population [12]. The biggest hospital in RSD is Odense University Hospital (OUH), with approximately 1000 beds.

All samples collected from patients admitted to OUH are analysed in the Department of Clinical Microbiology of the hospital.

2.2. Bacterial isolates

Consecutive VREfm isolates from clinical samples (non-screening) from hospitalised patients in the period 1 January 2014 to 1 July 2017 were analysed. Only the first isolate from each patient was included in this study. Isolates from the start of June 2017 onwards were analysed in real time, while the rest were sequenced and analysed retrospectively.

Bacterial identification was performed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Microflex LT; Bruker Daltonik GmbH, Bremen, Germany).

Resistance to vancomycin was detected by agar disk diffusion susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using 4-mm Mueller–Hinton agar plates and 5 mg Vancomycin Neo-Sensitabs™ (Rosco, Taastrup, Denmark).

All isolates were tested for the presence of the *vanA* and *vanB* genes using an in-house real-time PCR. The *vanA* primer and probe sequences were based on the study of Fang et al. [13]. The *vanB* primer and probe sequences were designed using Primer Express 3.0 (Thermo Scientific) and consisted of the following: forward primer GGRAACGAGGATGATTTGATTG; reverse primer CGTGGCTCARCCGGATT; and probe VIC-CGG CGAAGTGGATC-MGB. Detection was performed in a 25 μL reaction volume containing 12.5 μL of *Taq*Man™ FAST Universal PCR Master Mix (Applied Biosystems), 1000 nM of each primer, 200 nM of the probes and 5 μL of template DNA using a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycling parameters: 95 °C for 20 s; and 45 cycles of 95 °C for 3 s and 60 °C for 30 s.

VREfm screening was based on culturing a single rectal swab from each patient. Culturing was carried out on a 5% blood agar plate [Statens Serum Institut (SSI), Denmark] read after 24 h and 48 h of incubation at 35 °C, followed by visual inspection for enterococcal growth, bacterial identification with MALDI-TOF/MS, and vancomycin susceptibility testing as described above. If the isolate was found to be resistant to vancomycin or single colonies were found in the clear zone near the vancomycin tablet, the colonies in the zone or a scrape from the edge of the growth nearest the vancomycin tablet was tested for the presence of *vanA* and *vanB* using the in-house method described above.

2.3. Whole-genome sequencing (WGS) data analysis

WGS was carried out on a MiSeq platform (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions to obtain paired-end reads of 2 × 150 bp in length.

Draft genomes were de novo assembled using SKESA v.2.2 in the Bifrost pipeline at SSI. A quality control has been performed on the raw reads using the Bifrost pipeline (https://github.com/ssi-dk/bifrost) with accepted avg. coverage >30.

The draft genome sequences of all isolates were analysed using SeqSphere + software v.5.0 (Ridom GmbH, Germany) (*E. faecium* cgMLST scheme v.1.1;1423 loci) by which sequence types (STs), complex types (CTs) and cluster arrangement were obtained. The parameter 'pairwise ignoring missing values' was activated and the cluster distance threshold for the core genome was ≤20 allele differences [10].

2.4. Epidemiology and relatedness to other regions in Denmark

Admission history within a period of 1 year from the VREfm finding of the individual patient was extracted from the patient journal system Cambio COSMIC (https://www.cambiogroup.com/our-solutions/cambio-cosmic/). Collection date and sample material were extracted from the Danish microbiological department database system 'MADS' (www.madsonline.dk). Epidemic curves (epi curves) of the cgMLST results were constructed using SeqSphere + software. All cases were examined for prior VREfm findings.

The local data were compared with the national data on VREfm from clinical samples, which have undergone WGS since 2015 [5]. In cases where the patient had been transferred from another region, the local Department of Clinical Microbiology in the region in question was contacted for cgMLST and/or MLST results.

3. Results

A total of 38 VREfm isolates from 38 patients were included in the study. A comprehensive list of characteristics of the isolates, including sample type, site of isolation, collection date, *vanA*/*B*, sequencing results and admission histories, is given in Table 1.

3.1. Phylogenetic analysis and resistance genes

The 38 isolates belonged to six different STs, with the most frequent being ST80, ST117 and ST203.

Table 1
Characterisation of the vancomycin-resistant *Enterococcus faecium* (VREfm) isolates at Odense University Hospital (OUH), Region of Southern Denmark, in the period 1 January 2014 to 1 July 2017 (n = 38) and appertaining epidemiological data in a 12-month period prior to the sample date.

Number	Sample date (month/year)	MLST	cgMLST	*van* gene	Sample	Department number at OUH where sample was collected	Prior admissions in a 12-month period before sample date at OUH	Admissions in other regions of Denmark and other countries	Prior VREfm type
01	03/2014	ST80	CT14	*vanA*	Urine	1			
02	03/2014	ST80	CT2499	*vanA*	Urine	2	6		
03	04/2014	ST117	CT24	*vanA*	Urine from catheter	2	3		
04	05/2014	ST192	CT17	*vanA*	Blood culture	3		CR	ST192-CT17
05	05/2014	ST117	CT24	*vanA*	Swab from abscess	3	2		
06	06/2014	ST18	CT864	*vanA*	Liquid from abdominal drain	4	6, 2		
07	05/2015	ST80	CT866	*vanA*	Urine	5		CR	
08	06/2015	ST78	CT1438	*vanA*	Liquid from abdominal drain	6		RZ	
09	08/2015	ST80	CT880	*vanB*	Blood culture	7	13		
10	09/2015	ST117	CT1834	*vanA*	Blood culture	3			
11	10/2015	ST80	CT14	*vanA*	Urine	1	6		
12	02/2016	ST80	CT14	*vanA*	Ascites	3			
13	05/2016	ST203	CT859	*vanA*	Urine from catheter	2	8	CR	
14	05/2016	ST80	CT993	*vanA*	Urine	7	1		
15	06/2016	ST80	CT880	*vanB*	Urine	8			
16	08/2016	ST80	CT993	*vanA*	Blood culture	1	2, 14		
17	10/2016	ST80	CT993	*vanA*	Urine	9	15		
18	11/2016	ST80	CT993	*vanA*	Urine from catheter	7	16, 17		
19	11/2016	ST80	CT993	*vanA*	Liquid from abdominal drain	2	9		
20	11/2016	ST80	CT993	*vanA*	Urine	10	6		
21	12/2016	ST80	CT993	*vanA*	Urine	2	9		
22	01/2017	ST203	CT1144	*vanA*	Urine from catheter	11	18	RZ	
23	02/2017	ST203	CT1143	*vanA*	Urine	1	18		
24	02/2017	ST80	CT993	*vanA*	Abdominal wound swab	2	3, 6, 19		
25	02/2017	ST80	CT993	*vanA*	Urine from catheter	1			
26	02/2017	ST203	CT859	*vanA*	Urine	7	13, 1		
27	02/2017	ST80	CT993	*vanA*	Urine	9			
28	03/2017	ST203	CT859	*vanA*	Urine	7	13		
29	03/2017	ST80	CT993	*vanA*	Urine from catheter	2	9		
30	03/2017	ST80	CT1839	*vanA*	Urine from catheter	12		CRD	ST80-CT997
31	04/2017	ST117	CT1182	*vanA*	Urine from catheter	2	9		
32	05/2017	ST80	CT32	*vanB*	Urine from catheter	2		Germany	Unknown
33	05/2017	ST80	CT866	*vanA*	Urine	7	20	CR	Unknown
34	05/2017	ST80	CT1508	*vanA*	Urine from catheter	8			
35	05/2017	ST80	CT993	*vanA*	Sputum	9	2		
36	06/2017	ST80	CT1508	*vanA*	Urine	8			
37	06/2017	ST80	CT1545	*vanA*	Blood culture, catheter	6	1, 2, 3, 11, 12		
38	06/2017	ST80	CT993	*vanA*	Tissue, ischial tuberosity	9	8		

MLST, multilocus sequence typing; cgMLST, core genome multilocus sequence typing; ST, sequence type; CT, complex type; CR, Capital Region of Denmark; RZ, Region Zealand; CRD, Central Denmark Region.

Of the 38 isolates, 26 (68%) belonged to ST80, 4 belonged to ST117 and 5 belonged to ST203, while the remaining three STs (ST18, ST78 and ST192) comprised 1 isolate each.

cgMLST analysis of the 38 isolates revealed 18 different CTs (Table 1). The ST80 group was subdivided into nine different clusters, with ST80-CT993 being the dominant type.

The ST80-CT993 isolates had an allele difference of 0–25 among the isolates. The second largest ST80 group was CT14, which consisted of three isolates with 7–24 allele differences among the isolates, while the remaining six ST80 clusters consisted of only one or two isolates each.

Using cgMLST, two of the four ST117 isolates showed no allele differences and belonged to CT24. Likewise, for ST203 three of the five isolates appeared related and belonged to CT859, with allele differences of 2–3. The remaining CTs were all singletons (Table 1).

Vancomycin resistance in 35 of the 38 isolates was found to be related to the *vanA* gene, while only 3 isolates contained *vanB*.

3.2. Epidemiological data and transmission

Use of cgMLST data for an epi curve illustrated some small clusters at the beginning of 2014, the end of the investigation period in 2017, and a larger cluster during the last year of the period (Fig. 1).

Before the actual rise of VREfm in mid-2016, only two small clusters of ST117-CT24 and ST80-CT14 were detected. Patient admission histories for the two patients with ST117-CT24 isolates revealed that transmission was likely to have occurred in Department 1 or 2 (Table 1). Two of the three patients with ST80-CT14 had been admitted to the same ward, but with 1.5 years in between, and the last patient had not been on this ward at all. Isolates from patients admitted to the same ward had an allele difference of 24, and taking the admission dates into account, transmission was rejected. Two of the patients had isolates differing by 7 alleles, but no connection regarding their hospitalisation was found.

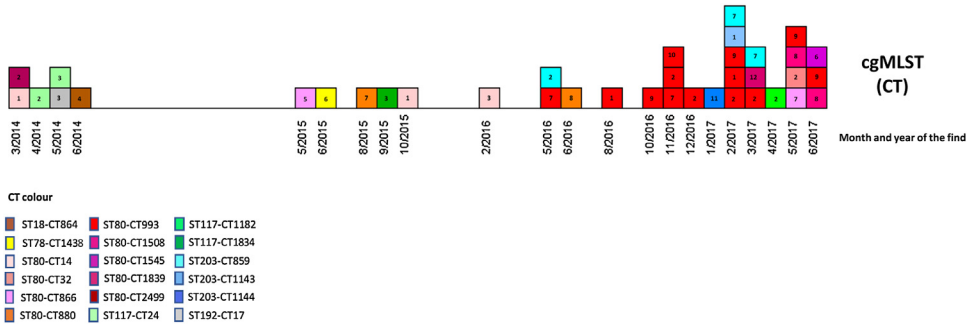


Fig. 1. Epidemic curves (epi curves) for vancomycin-resistant *Enterococcus faecium* isolates detected at Odense University Hospital, Region of Southern Denmark, during 1 January 2014 to 1 July 2017. The epi curves were based on multilocus sequence typing (MLST) with creation of sequence types (STs), and core genome MLST (cgMLST) with creation of complex types (CTs). Each box represents one specific isolate (1–38). Colours correspond to the ST-CT number. The number in the box correlates to the department number where the sample was collected.

Two isolates of ST80-CT866 and ST80-CT880, respectively, were found in the period 2015–2017. The patients with ST80-CT880 had not been admitted to the same ward, and the ST80-CT866 isolates were found with >1 year apart. The allele difference in both cases was <20, but due to the distance in time and admission histories, transmission was rejected. Three isolates of ST203-CT859 were found in mid-2016 and the beginning of 2017. The isolates from the two patients detected in 2017 had an allele difference at 2. The patients had been admitted to the same ward and transmission was found to be most likely.

In May 2017, two isolates of ST80-CT1508 appeared. These two patients had been admitted to the same ward within 1 month, suggesting transmission (Table 1). The allele difference was 34, leaving the connection weaker.

From May 2016 through June 2017, a total of 13 isolates of ST80-CT993 were continuously detected and matched the rise in number of VREfm detected at OUH. For each specific isolate, an allele difference at 0–5 to the nearest neighbour was found. At the time of sampling, the patients were distributed in 5 departments but with prior admissions in a total of 13 departments in the past 12 months. Seven of the patients had been admitted to Department 9 and four of these patients had also stayed in Department 2, where a further two patients with ST80-CT993 had been admitted. Three of the four patients found in Department 2 had been previously admitted to Department 9, while only one patient prior to admission in Department 9 had been in Department 2, and was in addition found late in the investigation period.

3.3. Relatedness to other regions in Denmark

In 7 of the 38 VREfm cases, patients were transferred from Danish hospitals located in high-incident regions. Three of these seven patients had prior to admission to OUH been found harbouring VREfm, with two of them being singletons and one with no sequencing data available (Table 1).

The other four patients were not known to be colonised before admission to OUH, but according to our results one of these four patients was found to harbour an ST203-CT859 *vanA E. faecium* and one patient an ST80-CT866 *vanA E. faecium*. These types were highly prevalent in the Capital Region and the Central Denmark Region, from where the patients were transferred, and the patients might have been colonised during hospitalisation in these regions [5–7].

The remaining two patients were colonised with ST203-CT1144 and ST78-CT1438 strains (Table 1). Both types had been rarely found in Denmark—in our own region (RSD) and in the Capital Region [5].

A single patient was transferred from a German hospital and was colonised with a ST80-CT32 *vanB*-positive isolate, which to our knowledge has never been detected in Denmark [5].

On a national level, 11 (~3%) of 422 collected clinical VREfm isolates in 2016 belonged to ST80-CT993 *vanA E. faecium* [5]. Of these 11 patients, we found that 7 patients had been at OUH. Three patients had been hospitalised in the same region as OUH but at a minor hospital, and one patient had been found in the Central Denmark Region. One of the patients from the minor hospital and the patient from the Central Denmark Region were detected with an ST80-CT993 *vanA E. faecium* strain before this type appeared at OUH. None of the patients with a ST80-CT993 that were diagnosed at OUH in 2016–2017 had been hospitalised outside the Region of Southern Denmark (Table 1). No connection was found between the index patient at OUH and other hospitals inside the region.

3.4. Infection control interventions

Based on cgMLST analysis and the admission histories, an epidemiological link was established among 7 of the 13 patients with ST80-CT993. Transmission had most likely happened in Department 2 or 9. Due to the highest number of cases in Department 9, and since three of the four patients carrying ST80-CT993 in Department 2 also had been admitted to Department 9, infection control interventions were launched in the latter department at the end of June 2017.

To reveal whether there was a large ongoing outbreak in Department 9, we initiated screening samples from all currently hospitalised patients in this department on 30 June 2017. No further screening criteria besides being hospitalised in Department 9 on the screening day was used. A total of 18 patients were included and all samples were negative using culture-based screening.

An audit in Department 9 revealed the need for improved compliance with standard precautions and suggested transmission through environmental surfaces. A tidying up of the entire department, including rinsing, storage and staff rooms, took place before cleaning the environment. This was followed by non-touch automated disinfection with hydrogen peroxide or manual disinfection with chlorine.

After completion of the infection control interventions in Department 9, only one additional case of ST80-CT993 colonisation occurred in the following 6 months and therefore no further infection control interventions or screening tests were initiated in the hospital departments.

4. Discussion

Analysis of cgMLST data indicated that multiple clones of VREfm were introduced at OUH during the period 1 January 2014 to 1 July 2017 and that transmission occurred between patients within the hospital.

Use of cgMLST on VREfm allowed for a detailed interpretation of the diversity, thereby indicating transmission of only certain complex types and not all isolates with an identical sequence type. This confirms the results from other clinical studies with other micro-organisms in this field [11,14,15].

cgMLST in combination with allele differences and admission histories revealed transmissions of three minor (ST203-CT859, ST117-CT24 and ST80-CT1508) and a single larger clone of ST80-CT993, representing the first outbreak of VREfm in the Region of Southern Denmark.

The patients with ST80-CT993 VREfm clone had connections to a total of 13 departments, but transmission had most likely happened in 1 or 2 departments. Infection control interventions were launched in Department 9, which stopped the outbreak.

Initial transmission from outside the region or from a regional hospital into OUH could not be established. Transmission could however have occurred through an unknown carrier, and it is most likely that transmission occurred between hospitals in Southern Denmark owing to the higher number of patient transfers. Screening samples from patients in Department 9 were all found negative using culturing. This may be explained by the large turnover of patients in the department, with the risk that we did not test the right patients, or due to an inefficient screening method for colonisation with a low number of VREfm, by culturing. Today, this may be solved by using a molecular diagnostic method and enterococcus selective growth media for detecting colonisation.

It has not been possible to find any descriptions of the ST80-CT993 *vanA* clone from outside Denmark by searching www.pubmed.gov and www.cgMLST.org.

Transmission inside OUH with clones from patients transferred from regions with ongoing outbreaks was not identified. This was a surprise, especially for the ST203-CT859 *vanA* VREfm, which had a high prevalence in the other regions, accounting for 51–61% of the Danish VREfm in 2015–2017 [5]. The ST80-CT14 and ST80-CT866 were both frequent clones in 2015 but were reduced to ~4% in 2017. An analysis of which factors prevented these transmissions could prove valuable.

A single patient was transferred from a German hospital and harboured an ST80-CT32 *vanB* VREfm. This clone has been detected several times in Germany [16]. ST80, together with ST117 and ST203, are the most frequent sequence types both in Germany and Denmark, but in contrast to the Danish isolates the vancomycin resistance in the German clones is often mediated by *vanB* [17].

cgMLST has been described for outbreak investigation but, as far as we know, there is only a limited number of outbreak descriptions where cgMLST has been used during the investigation to target hygiene interventions [14,18]. In this present study, we demonstrated the ability of cgMLST to identify outbreak strains, to assess whether VREfm was introduced from outside the region, and to help decrease the number of departments where infection control interventions were introduced to terminate transmission. We experienced that cgMLST results were easy to use for non-bioinformaticians, but it is a field that should be investigated further in order to achieve an enhanced use of the system in the frontline.

In conclusion, we found that cgMLST was useful in local characterisation of VREfm, distinguishing sporadic clones from outbreak strains. Use of cgMLST made it easy to compare our local isolates with the national findings, thereby clarifying transmission routes. In combination with admission histories, cgMLST targeted possible outbreaks and located the specific wards involved. This limited the outbreak and saved us from expensive and time-consuming infection control interventions.

Funding

None.

Competing interests

None declared.

Ethical approval

The Danish Patient Safety Authority has approved the collection from all the databases used in this paper [Ref. number: 3-3013-2554/1].

Acknowledgments

Thanks to all of the Danish Departments of Clinical Microbiology who have permitted us to obtain the sequencing types for the VREfm from patients admitted in their regions prior to the Odense University Hospital (OUH), admission. The authors also wish to thank E. Knudsen at the Department of Clinical Microbiology of OUH for her execution of laboratory work, and H. Hasman at Statens Serum Institut for his excellent technical assistance.

References

[1] Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant enterococci. Clin Microbiol Rev 2000;13:686–707, doi:http://dx.doi.org/10.1128/CMR.13.4.686-707.2000.
[2] Gorrie C, Higgs C, Carter G, Stinear TP, Howden B. Genomics of vancomycin-resistant *Enterococcus faecium*. Microb Genomics 2019;5:e000283, doi:http://dx.doi.org/10.1099/mgen.0.000283.
[3] Wagenvoort JHT, De Brauwer EIGB, Penders RJR, Willems RJ, Top J, Bonten MJ. Environmental survival of vancomycin-resistant *Enterococcus faecium*. J Hosp Infect 2011;77:282–3, doi:http://dx.doi.org/10.1016/j.jhin.2010.11.008.
[4] Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis 2006;6:130, doi:http://dx.doi.org/10.1186/1471-2334-6-130.
[5] DANMAP. *Reports*. https://www.danmap.org/reports. [Accessed 8 July 2020].
[6] Hammerum AM, Baig S, Kamel Y, Roer L, Pinholt M, Gumpert H, et al. Emergence of *vanA Enterococcus faecium* in Denmark, 2005–15. J Antimicrob Chemother 2017;72:2184–90, doi:http://dx.doi.org/10.1093/jac/dkx138.
[7] Pinholt M. Hospital-acquired vancomycin-resistant Enterococcus faecium in Denmark [PhD thesis]. Copenhagen, Denmark: University of Copenhagen, Faculty of Health and Medical Science; 2016.
[8] Quainoo S, Coolen JPM, van Hijum SAFT, Huynen MA, Melchers WJG, van Schaik W, et al. Whole-genome sequencing of bacterial pathogens: the future of nosocomial outbreak analysis. Clin Microbiol Rev 2017;30:1015–63, doi:http://dx.doi.org/10.1128/CMR.00016-17.
[9] Mellmann A, Andersen PS, Bletz S, Friedrich AW, Kohl TA, Lilje B, et al. High interlaboratory reproducibility and accuracy of next-generation-sequencing-based bacterial genotyping in a ring trial. J Clin Microbiol 2017;55:908–13, doi:http://dx.doi.org/10.1128/JCM.02242-16.
[10] De Been M, Pinholt M, Top J, Bletz S, Mellmann A, Van Schaik W, et al. Core genome multilocus sequence typing scheme for high-resolution typing of *Enterococcus faecium*. J Clin Microbiol 2015;53:3788–97, doi:http://dx.doi.org/10.1128/JCM.01946-15.
[11] Pearce ME, Alikhan NF, Dallman TJ, Zhou Z, Grant K, Maiden MCJ. Comparative analysis of core genome MLST and SNP typing within a European *Salmonella* serovar Enteritidis outbreak. Int J Food Microbiol 2018;274:1–11, doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2018.02.023.
[12] Hammerum AM, Justesen US, Pinholt M, Roer L, Kaya H, Worning P, et al. Surveillance of vancomycin-resistant enterococci reveals shift in dominating clones and national spread of a vancomycin-variable *vanA Enterococcus faecium* ST1421-CT1134 clone, Denmark, 2015 to March 2019. Euro Surveill 2019;24:1900503, doi:http://dx.doi.org/10.2807/1560-7917.ES.2019.24.34.1900503.
[13] Fang H, Ohlsson AK, Jiang GX, Ullberg M. Screening for vancomycin-resistant enterococci: an efficient and economical laboratory-developed test. Eur J Clin Microbiol Infect Dis 2012;31:261–5, doi:http://dx.doi.org/10.1007/s10096-011-1304-0.
[14] Woksepp H, Ryberg A, Berglind L, Schön T, Söderman J. Epidemiological characterization of a nosocomial outbreak of extended spectrum β-lactamase *Escherichia coli* ST-131 confirms the clinical value of core genome multilocus sequence typing. APMIS 2017;125:1117–24, doi:http://dx.doi.org/10.1111/apm.12753.
[15] Van Walle I, Björkman JT, Cormican M, Dallman T, Mossong J, Moura A, et al. Retrospective validation of whole genome sequencing-enhanced surveillance of listeriosis in Europe, 2010 to 2015. Euro Surveill 2018;23:1700798, doi:http://dx.doi.org/10.2807/1560-7917.ES.2018.23.33.1700798.
[16] *Enterococcus faecium* cgMLST. Search strains. https://www.cgmlst.org/ncs/schema/991893/searchstrain/?f=ct&v=32. [Accessed 23 August 2020].

[17] Eichel V, Klein S, Bootsveld C, Frank U, Heeg K, Boutin S, et al. Challenges in interpretation of WGS and epidemiological data to investigate nosocomial transmission of vancomycin-resistant *Enterococcus faecium* in an endemic region: incorporation of patient movement network and admission screening. J Antimicrob Chemother 2020;75:1716–21, doi:http://dx.doi.org/10.1093/jac/dkaa122.

[18] Mellmann A, Bletz S, Böking T, Kipp F, Becker K, Schultes A, et al. Real-time genome sequencing of resistant bacteria provides precision infection control in an institutional setting. J Clin Microbiol 2016;54:2874–81, doi:http://dx.doi.org/10.1128/JCM.00790-16.

Paper II

JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Hansen *et al.*, *Journal of Medical Microbiology* 2023;72:001731

DOI 10.1099/jmm.0.001731

MICROBIOLOGY SOCIETY

OPEN ACCESS

Vancomycin-sensitive *Enterococcus faecium* bacteraemia – hospital transmission and mortality in a Danish University Hospital

Sanne Groenvall Kjaer Hansen[1,2,3,*], Louise Roer[3], Kasper Thystrup Karstensen[3], Silje Vermedal Hoegh[1,2], Frank Hansen[3], Kasper Klein[1,2], Flemming S. Rosenvinge[1,2], Anette Holm[1,2], Marianne N. Skov[1,2], Anette M. Hammerum[3] and Henrik Hasman[3]

Abstract

Introduction. The emergence of vancomycin-resistant *Enterococcus faecium* (VREfm) has left the vancomycin-sensitive *E. faecium* (VSEfm) strains almost unnoticed.

Hypothesis. Molecular characteristics, hospital transmission patterns and clinical impact of VSEfm have changed, and VSEfm is a predictor of VREfm introduction.

Aim. We wanted to do a molecular characterization of VSEfm to identify hospital transmissions and links between VSEfm and VREfm, and to investigate the demographics, treatment and impact on mortality of VSEfm bacteraemia.

Methodology. VSEfm and VREfm blood culture isolates from Odense University Hospital, Denmark, from 2015 to 2019 were characterized using whole-genome sequencing and core-genome multilocus sequence typing (cgMLST). Clonal shifts and diversity of the VREfm isolates were compared to the VSEfm isolates. Hospital records were used for clinical data and transmission investigation of VSEfm cases.

Results. Six-hundred and thirty VSEfm isolates from 599 patients belonged to 42 sequence types (STs) and 131 complex types (CTs) in several clusters. Multiple types were involved in putative transmission, occurring over the entire period. Twenty-seven VREfm bacteraemia cases were included. No correlation between the VSEfm and VREfm clones was identified. The 30 day mortality was 40%, but only in 6.3% of the cases, VSEfm bacteraemia was the likely cause of death.

Conclusion. The molecular types of VSEfm bacteraemia isolates are changing and diverse. No direct correlation between VSEfm and the introduction of VREfm was found, but widespread hospital transmission indicates a presence of risk factors that could facilitate transmission of other micro-organisms as well. VSEfm bacteraemia is rarely the cause of death, indicating that 30 day mortality does not reflect the cause of death.

INTRODUCTION

Enterococcus faecium is a Gram-positive bacterium that comprises a small amount of the human microbiota in the gut [1, 2]. It is found in hospitals all over the world, where it thrives very well in the environment, belonging to the group of hospital-adapted

Received 06 February 2023; Accepted 20 June 2023; Published 12 July 2023

Author affiliations: [1]Department of Clinical Microbiology and Infection Control, Odense University Hospital, 2nd Floor, J.B. Winsløws Vej 21, 5000 Odense, Denmark; [2]Research Unit of Clinical Microbiology, Department of Clinical Research, Odense University Hospital, 2nd Floor, J.B. Winsløws Vej 21, 5000 Odense, Denmark; [3]Department of Bacteria, Parasites and Fungi, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen S, Denmark.
***Correspondence:** Sanne Groenvall Kjaer Hansen, sanne.groenvall.kjaer.hansen@rsyd.dk;sannedsimonsen@yahoo.dk

Keywords: bacteraemia; *Enterococcus faecium*; infection control ; mortality; transmission; VSE.

Abbreviations: ACVC, arterial or central venous catheter; CC, clonal complex; cgMLST, core-genome MLST; CT, complex type; ICU, Intensive Care Unit; MADS, Microbiology Departments Data System; MLST, multilocus sequence typing; OUH, Odense University Hospital; rMLST, ribosomal MLST; SLC, single-linkage clustering; SNP, single nucleotide polymorphism; ST, sequence type; VREfm, vancomycin-resistant *Enterococcus faecium*; VSEfm, vancomycin-sensitive *Enterococcus faecium*; VVEfm, vancomycin-variable *Enterococcus faecium*; WGS, whole-genome sequencing.

The GenBank/EMBL/DDBJ BioProject accession numbers for the sequences of the *Enterococcus faecium* are PRJEB63070, PRJEB63096 and PRJEB38219.

One supplementary table and other supplementary material are available with the online version of this article.

bacteria with the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) and causing hospital-associated infections [3–5].

Decades ago, *E. faecium* was investigated extensively due to its acquisition of ampicillin-resistance. However, after the widespread introduction of vancomycin resistance, both the ampicillin-resistant and the vancomycin-sensitive *E. faecium* (VSEfm) were almost forgotten [6–12]. In Denmark, only a few per cent of the *E. faecium* isolates have remained susceptible to ampicillin, and the subdivisions of the species are today referred to as vancomycin-sensitive *E. faecium* (VSEfm) and vancomycin-resistant *E. faecium* (VREfm) [13].

By use of multilocus sequence typing (MLST), clonal relatedness of *E. faecium* clones was found to split into clades. In one branch (clade A), the isolates containing ampicillin-resistance were genetically related, and associated with epidemic hospital strains (clade A1) or sporadic human infection strains (clade A2) [12]. In the other branch (clade B), the ampicillin-susceptible isolates represented the commensals, having a high clonal diversity, and a low prevalence in hospitals [10, 12, 14, 15]. Based on molecular investigations, a recent study suggests that all the isolates in clade B should be reclassified as *Enterococcus lactis* [16]. Nowadays, whole-genome sequencing (WGS) is the gold standard for bacterial strain typing supplemented with core-genome MLST (cgMLST) or single nucleotide polymorphism (SNP) analysis. In 2015, de Been *et al*. developed a cgMLST scheme for *E. faecium*, thereby transferring the SNP diversity into a standardized allele system that could overcome the inter-laboratory surveillance exchange [17]. The scheme has been used worldwide, and although mostly for VREfm, it also creates an opportunity to gain new and more detailed information about VSEfm [18].

Furthermore, studies have suggested that VREfm emerge from the circulating VSEfm by transposon gain events [19, 20]. At Odense University Hospital (OUH), Denmark, we detected the first cases of VREfm infection in 2014, and until mid-2018 only sporadic findings were detected. The dominating types were ST80, ST117 and ST203, all harbouring a *vanA* gene. In 2016, the vancomycin-variable *E. faecium* (VVEfm) clone ST1421-CT1134 was detected for the first time in Denmark. This VVEfm was characterized by its containing the *vanA-vanX* gene complex but being phenotypically susceptible to vancomycin [21, 22]. This VVEfm was introduced at OUH in 2018 and caused transmission on a larger scale in the hospital during the following years [13, 23]. Since enterococci thrive in the environment, and transmission has been described to follow the same pathways in hospitals regardless of the susceptibility, it is also of interest to investigate whether VSEfm can be used as an indicator of risk factors that contributes to the spread of VREfm [4, 5, 24, 25]. If so, VREfm transmission can be prevented at an earlier stage by use of infection control measures.

Another important topic to address in relation to the above is the clinical impact of *E. faecium* bacteraemia. Several studies have reported a high 30 day mortality of 24–66% of *E. faecium* bacteraemia [26, 27]. The reported 30 day mortality is different for VREfm (40–56%) and VSEfm (29–32%) bacteraemia, but both are correlated to severe underlying illness [28–30]. This points to these patients having a poor state of health before the onset of infection, which raises the questions of: it is the underlying disease and extensive use of antibiotics that facilitates the growth of *E. faecium*, and whether the patient dies of or with the *E. faecium* bacteraemia. Therefore, we need to investigate the demographics, treatment and impact on mortality of VSEfm bacteraemia, to determine whether VSEfm bacteraemia is an indicator of severe disease rather than the cause of death.

Therefore, we conducted a descriptive study to analyse VSEfm isolated from patients with bloodstream infections at OUH in Denmark, in the period 2015 to 2019, by using cgMLST and hospital records, in order to do: (i) a molecular characterization of the isolates, (ii) an investigation of transmission, (iii) an investigation of prevalence, types and diversity of VSEfm as a predictor for VREfm introduction, and (iv) an investigation of the demographics, treatment and impact on mortality.

METHODS

Bacterial isolates

All VSEfm and VREfm isolates detected from blood cultures at the Department of Clinical Microbiology, OUH, Denmark, from January 2015 through December 2019, were included in the study. Isolates were stored at −80 °C and identified by data harvest in the laboratory information system, Microbiology Departments Data System (MADS) (www.madsonline.dk). Consecutive isolates from the same patient were included if there was more than 1 month between the collection dates, in accordance with the case definition of a new bacteraemia episode in the national database, Healthcare-Associated Infections Database (HAIBA) (https://miba.ssi.dk/overvaagningssystemer/haiba/casedefinitioner/bakteriaemi).

Each isolate was cultured on a 5% blood agar plate (SSI Diagnostica) for 48 h at 35 °C. From this agar plate, one colony was chosen and re-cultured on a new 5% blood agar plate and afterwards used for bacterial identification and WGS. Bacterial identification was performed with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) (Microflex LT; Bruker Daltonik).

Susceptibility testing

Susceptibility to vancomycin was tested according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) guidelines. In cases of uncertainty, in-house PCR was applied for detecting the presence of the vancomycin-resistance genes *vanA*, *vanB* and *vanX* [23]. Successively, presence of vancomycin-resistance genes was crosschecked against the WGS data (see below) and for VSEfm used for discarding isolates found false susceptible by the EUCAST susceptibility test or PCR.

WGS analysis

Genomic DNA was extracted using a MagNA Pure 96 DNA and Viral NA kit (Roche) or Chemagic 360 CMG-1091 (PerkinElmer), and library preparation was performed using a Nextera XT kit (Illumina), according to the manufacturer's instructions. Paired-end fragments of at least 2×150 bp were sequenced on a NextSeq system (Illumina), and quality control, genome assembly (SKESA v. 2.2), detection of resistance genes, as well as species identification, were carried out using the Bifrost pipeline (https://github.com/ssi-dk/bifrost). All the included VSEfm and VREfm isolates were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/). In case of doubt regarding species identification or questionable read-quality parameters, PubMLST – rMLST (ribosomal MLST) (https://pubmlst.org/species-id) was used to define the species.

Clonal complexes (CCs), sequence types (STs) and complex types (CTs) were determined using SeqSphere⁺ software (Ridom) (version 8.3.1,) [31]. Core-genome distance matrices were submitted anonymously to cgMLST.org for isolates with an unknown CT for generating and retrieving the new CT numbers. In case of an unknown ST, the WGS result was submitted to the *Enterococcus faecium* Typing Database (https://pubmlst.org/bigsdb?db=pubmlst_efaecium_seqdef).

Cluster groups of CTs were generated by using a maximum distance threshold at 20 alleles between the nearest isolates with different CTs and named by the lowest ST-CT in the group. Minimum spanning trees and epicurves of CT cluster groups were visualized by use of SeqSphere⁺ software. Minimum spanning trees were created by using the parameter 'pairwise ignoring missing values' and SNP-allele distance matrices with a maximum distance threshold at 20 alleles for the core genome [17].

Local single-linkage clustering (SLC) was calculated in SeqSphere⁺ in order to enhance our study on putative transmissions. We defined the maximum allelic distance for the SLC clusters by investigating the maximum allelic distances in each of the two largest putative transmission episodes from our dataset.

Clinical impact and transmission

The number of all cause hospitalizations was reported by the Data Section at OUH, and the total number of patients having a blood culture during admission was extracted from MADS. The number of VSEfm bacteraemia episodes was extracted from MADS. Demographic and clinical data were gathered for all patients with VSEfm bacteraemia, with each patient included with the first VSEfm isolate. If the patient had more than one bacteraemia episode, the latest isolate was used for the investigation of correlation between the molecular characteristics and 30 day mortality and cause of death. Gender, age, collection date, requisition ward and data on intravenous or arterial catheters were extracted from MADS, as well as the hospital records (Cambio COSMIC – https://www.cambiogroup.com).

The antibiotic treatment for the individual patient, and the date of death were extracted from the hospital records in June 2022. Because removal or change of arterial or central venous catheters (ACVCs) with a single dose of vancomycin is a treatment strategy at OUH, we extracted these events from the hospital records as well.

Cause of death due to VSEfm for patients who died within 30 days was divided into the groups 'likely', 'possible', 'unlikely' and 'unknown', based on an algorithm developed by the authors. The algorithm required access to the hospital records and can be found in the supplementary material (available with the online version of this article). All cases were investigated by a medical doctor who was a specialist in clinical microbiology. The cases allocated in the possible group were reviewed by a further algorithm by a second medical doctor who was a specialist in clinical microbiology (see the supplementary material). If there were any discrepancies between the doctors' assessments, the worst-case scenario was selected.

Putative transmission was determined by combining the SeqSphere⁺ data with the date of requisition and ward for each patient included in the study. At least two patients with the same CT cluster group and related to the same ward within a month from the VSEfm detection had to be present to register a possible transmission.

Statistical analysis

The data is described by median, mean and proportions. For each year, the prevalence of the specific CTs and STs was calculated and directly compared. The diversity was calculated for each year as the total number of specific CTs, and directly compared between the years. Chi-square test for contingency tables and Fisher's exact test were used for calculation of statistical significance [32].

Table 1. Number of patients admitted to the hospital, blood cultures and VSEfm blood isolate distribution in patients, STs and CTs during the period 2015–2019, at OUH, Denmark

Characteristic	Year					
	2015 (*n*)	**2016 (*n*)**	**2017 (*n*)**	**2018 (*n*)**	**2019 (*n*)**	**Total (*n*)**
No. of hospital admissions	113560	97519	95737	93322	91030	491168
No. of admitted patients with a blood culture	13356 (11.8%)	13382 (13.7%)	13958 (14.6%)	14790 (15.8%)	14804 (16.3%)	70290 (14.3%)
No. of blood cultured patients with VSE bacteraemia	133 (1.0%)	122 (0.9%)	118 (0.85%)	117 (0.79%)	109 (0.74%)	599 (0.85%)
No. of VSE isolates	137	130	125	123	115	630
VSE singletons	10	16	18	21	20	85
No. of different VSE STs	11	14	12	15	16	42
No. of different VSE CTs	25	39	38	44	44	129
New VSE STs compared to previous years	–	7	6	10	8	–
New VSE CTs compared to previous years	–	28	25	32	19	–
New VSE CT cluster groups compared to previous years	–	20	20	30	18	–
Most prevalent VSE type (*n*=%)	ST117-CT24 (77=56%)	ST117-CT24 (42=32%)	ST80-CT1160 (43=34%)	ST117-CT1180 (32=26%)	ST117-CT1180 (31=27%)	–
Second most prevalent VSE type(s) (*n*=%)	ST192-CT46 (14=10%)	ST80-CT1160 (25=19%)	ST117-CT1180 (17=14%)	ST80-CT1160 (17=14%)	ST80-CT1160 (7) and ST203-CT1513 (7=6%)	–

RESULTS

Isolates

A total of 630 VSEfm isolates from 599 patients in the period 2015 to 2019 was included in the study. The number of VSEfm isolates was stable with 115 to 137 isolates and 109 to 133 patients per year; also when compared to the number of hospital admissions and the number of blood-cultured patients (Table 1). Twenty-six patients were included with more than one isolate, and their isolates were equally distributed in time.

Molecular characterization

Of the 630 VSEfm isolates, 28 were identified with rMLST as *E. lactis* (Fig. 1). Dividing the isolates into CC groups, 591 (94%) of the isolates belonged to CC17, 14 (2%) to CC94, and for 25 isolates a CC was not identified. All 14 isolates belonging to CC94 and 14 of the isolates without a CC were *E. lactis*. Thirty-three (5.2%) of the VSEfm isolates were susceptible to ampicillin, leaving 94.8% resistant. Four of the *E. lactis* isolates were resistant to ampicillin.

Of the 26 patients with more than one VSEfm isolate, 14 patients had the same ST-CT combination, 3 patients had VSEfm isolates belonging to the same ST but different CT, 1 patient had isolates with a different ST but the same CT, and the 8 remaining patients had isolates with different ST and CT combinations. Forty-two different STs were found with the most frequent being ST80 and ST117, accounting for 76% of the isolates. The isolates were subdivided into 131 CTs of which 70 were singletons (Table S1).

Application of CT cluster groups consisting of five or more isolates resulted in 20 groups, of which 8 consisted of more than one CT, and included a total of 45 CTs (Table 2). Two of the large CT cluster groups (ST117-CT24 and ST117-CT1180) should have been combined according to the method, but we chose to separate them, because the ST-CTs only were connected with a single isolate and a distance of 19 alleles. A diversity with a mean of 14 different STs, 38 CTs and 33 cluster groups each year was found. The mean of new types each year was 7.75 for STs, 26 for CTs and 22 for CT cluster groups (Table 1). The most prevalent types during the whole period were ST117-CT24 (*n*=139), ST80-CT1160 (*n*=94) and ST117-CT1180 (*n*=81). All the dominating types were typically replaced by a new type after 2 to 3 years (Table 2, Fig. 2).

Transmission

Isolates belonging to at least 7 of the 42 STs and more than 40 of the 131 CTs were involved in putative transmission and occurred during the entire period. Formerly reported STs involved in outbreaks, such as ST17, ST18 and ST192, were retrieved, and involved putative transmissions consisting of 5 to 29 patients (Fig. 1, Table 2).

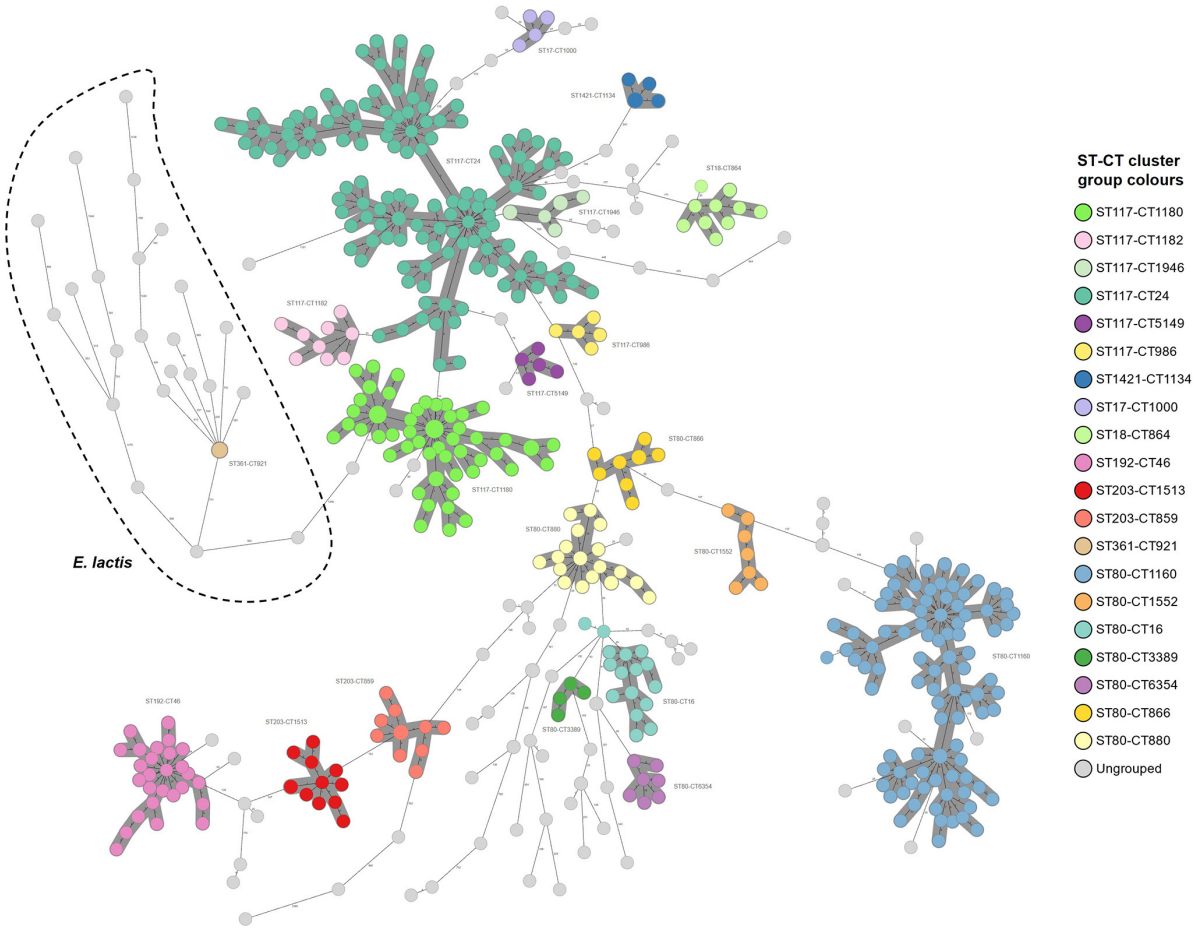


Fig. 1. Minimum-spanning tree of VSEfm blood isolates (*n*=630), detected in the period 2015–2019, at OUH, Denmark. The isolates are coloured by ST-CT cluster groups consisting of five or more isolates each.

Putative transmission was most frequent in the Intensive Care Unit (ICU) and the Department of Haematology, OUH, Denmark. The largest putative transmission episode concerned cluster group ST117-CT24 *E. faecium* and involved 150 patients, of which 59 had been in the ICU and 30 at the Department of Haematology. At the ICU, nine putative transmission episodes were registered according to the definition, with the largest involving 21 patients. At the Department of Haematology, five putative transmission episodes were registered, with the largest involving seven patients.

Investigating the maximum allelic distances between any two isolates in each of the two largest putative transmission episodes, we observed a maximum allelic distance at 11, which was applied in SeqSphere⁺ for determining SLC clusters. This resulted in 44 clusters, of which 14 did not belong to a ST-CT cluster. Seven ST-CT clusters contained more than one SLC cluster, and included 17 SLC clusters all together (Table 2).

In 10 of the 44 SLC clusters, two or less departments were involved, and the ICU was represented in 34 of the 44 SLC clusters. In seven cases, the SLC clusters provided a more specific epidemiologic information than by use of the ST-CT cluster information.

VSEfm and VREfm relatedness

A total of 27 VREfm blood isolates from 27 patients was identified in the period 2015 to 2019. The number of VREfm isolates was distributed with 1 to 5 isolates each year in the period 2015–2018, while 15 isolates were identified in 2019 (Table 3).

All 27 isolates were ampicillin resistant and belonged to CC17. Five different STs and eight CTs were found, with the most frequent being the VVEfm clone ST1421-CT1134 (*n*=15) and 12 of these isolates were detected in 2019.

There was no correlation between the total prevalence, the diversity in STs, CTs or cluster groups of VSEfm during the years with the introduction of VREfm (Table 3). The first VSEfm ST1421-CT1134 blood isolate was detected more than 4 months after

Table 2. Distribution of VSEfm-bacteraemia CT cluster groups consisting of five or more isolates, and stratified by STs, CTs and departments during the period 2015–2019, at OUH, Denmark (*n*=630)

Departments: A, ICU1; B, Haematology; C, Gastroenterology; D, Abdominal surgery 1; E, ICU2; F, Infectious diseases; G, Urinary tract diseases; H, Nephrology; I, Abdominal surgery 2; J, Oncology; K, Geriatric diseases.

CT cluster group	MLST	cgMLST	Single linkage clusters	Year, total no., departments ≥2 isolates (exact no.)					
	ST	CT (*n*)	Count	2015	2016	2017	2018	2019	Total (*n*)
ST117-CT24	ST117	CT24 (139), CT1487 (1), CT1834 (1), CT2056 (1), CT6351 (2), CT6364 (1), CT6380 (1), CT6382 (3), CT6408 (1)	–	78	43	16	8	5	150
			3	A (35) B (12) C (5) D (5) E (3) F (5)	A (18) B (12) C (5) D (3)	A (4) B (4) D (2–)	B (2)	–	A (59) B (30) C (11) D (10) E (4) F (5)
ST192-CT46	ST192+ST2146	CT46 (21), CT6389 (1), CT6394 (1), CT1838 (4) ST2146-CT1838 (2)	–	15	11	3	0	0	29
			1	A (8) G (2)	A (8) B (2)	–	–	–	A (16) B (3) C (2) G (2)
ST18-CT864	ST18	CT864 (9), CT1835 (2), CT6373 (1)	–	9	3	0	0	0	12
			2	A (7)	H (2)	–	–	–	A (7) H (2)
ST80-CT866	ST80	CT866 (10), CT6369 (2)	–	6	5	1	0	0	12
			3	A (5)	A (3)	–	–	–	A (9)
ST361-CT921	ST361	CT921 (5)	–	3	2	0	0	0	5
			1	–	A (2)	–	–	–	A (2)
ST80-CT16	ST80	CT16 (6), CT1840 (11)	–	2	4	7	1	3	17
			2	–	A (2)	A (2) B (2)	–	–	A (5) B (3)
ST80-CT880	ST80	CT880 (15), CT5907 (4), CT6350 (2), CT6376 (1), CT6384 (1)	–	5	10	4	3	1	23
			2	A (2)	A (2) C (2)	–	A (3)	–	A (7) C (5) I (3)
ST17-CT1000	ST17	CT1000 (5)	–	4	1	0	0	0	5
			1	B (4)	–	–	–	–	B (4)
ST203-CT859	ST203	CT859 (15)	–	1	1	2	6	5	15
			1	–	–	A (2)	A (3)	A (3)	A (8) C (2)
ST80-CT1160	ST80	CT1160 (94), CT2516 (1), CT6342 (1), CT6345 (1) CT6392 (1), CT6415 (1),	–	2	25	44	18	10	99
			3	A (2)	A (13) B (2) G (2)	A (18) B (7) C (5) D (3) E (3) G (2)	A (6) B (4) C (3) J (2)	A (4)	A (43) B (13) C (9) D (5) E (4) G (4) J (2)
ST80-CT1552	ST80+ST2149	CT1552 (6+1)	–	0	1	3	2	1	7
			1	–	–	–	–	–	K (2)
ST117-CT1180*	ST117	CT1180 (81), CT6398 (2)	–	0	1	17	34	31	83
			2	–	–	A (8) B (5)	A (15) B (11) E (2)	A (15) B (3) C (6) J (3)	A (39) B (19) C (6) E (4) J (4)

Continued

Table 2. Continued

CT cluster group	MLST	cgMLST	Single linkage clusters	Year, total no., departments ≥2 isolates (exact no.)					
	ST	CT (*n*)	Count	2015	2016	2017	2018	2019	Total (*n*)
ST117-CT1182	ST117	CT1182 (9)	–	0	1	2	2	4	9
			1	–	–	A (2)	A (2)	–	A (6)
ST80-CT6354	ST80	CT6354 (7)	–	0	0	5	1	1	7
			1	–	–	B (4)	–	–	A (2) B (5)
ST117-CT1946	ST117	CT1946 (11)	–	0	0	1	7	3	11
			1	–	–	–	A (6)	A (3)	A (10)
ST117-CT986	ST117	CT986 (5)	–	0	0	1	1	3	5
			1	–	–	–	–	J (2)	A (2) J (2)
ST203-CT1513	ST203	CT1513 (14)	–	0	0	0	7	7	14
			1	–	–	–	A (5)	A (3) B (2)	A (8) B (3) I (2)
ST1421-CT1134	ST1421	CT1134 (9)	–	0	0	0	3	6	9
			1	–	–	–	B (2)	B (6)	B (8)
ST80-CT3389	ST80	CT3389 (5)	–	0	0	0	2	3	5
			1	–	–	–	A (2)	B (2)	A (3) B (2)
ST117-CT5149	ST117	CT5149 (5)	–	0	0	0	1	4	5
			1	–	–	–	–	–	A (2)

*ST117-CT1180 is separated from ST117-CT24 by only 19 alleles.

the first VREfm bacteraemia of the same type. There were too few VREfm blood isolates to investigate for correlation between exchanges of VREfm main types and the exchanges of VSEfm blood isolate types.

Clinical impact

The included VSEfm bacteraemia patients were distributed equally in number and age each year. The youngest was <1 year and the oldest 99 years of age. The men/women ratio was 1.6, with a mean age of 67.7 years for women and 66.5 years for men, and a median of 69 years for women and 70 years for men. Of the 599 patients included in the study, 95% had one VSEfm bacteraemia episode in the investigation period, 4% had two episodes and 1% three or more. The number of departments with VSEfm bacteraemia patients was 25 out of 37 possible. The yearly affected number of departments was stable and ranged from 17 to 19 each year.

Of the 630 bacteraemia isolates, 297 (47%) were obtained from patients at the ICU, 105 (17%) from the Department of Haematology, 43 (7%) from the Department of Gastroenterology and 30 (5%) from the Department of Abdominal Surgery. The rest of the isolates were found in a variety of departments with less than 20 isolates for each place. The medical departments without the ICU accounted for around 36% of the findings. Of the strains susceptible to ampicillin, 39% were obtained from patients at the ICU, and the rest from a variety of departments.

Almost all STs, CTs or CT cluster groups were represented in patients hospitalized in the ICU or the Department of Haematology. Patients having an *E. lactis* isolate were in half of the cases admitted to the ICU, while the other half of the patients were from seven different wards.

Of the 599 patients, 438 (73%) had one or more arterial or central venous catheters, 160 patients (27%) did not have a catheter, and for 1 patient it was unknown whether a catheter was present or not. Presence of a catheter or not was equally distributed within the ampicillin-susceptible group, and there was no correlation to specific CCs. Of the 438 patients with a catheter, 93% had a blood culture drawn from the catheter, and in 91% of these cases, VSEfm was found in the catheter blood. No significant relation was found between specific CT cluster groups and the presence of a positive catheter blood culture (P>0.05).

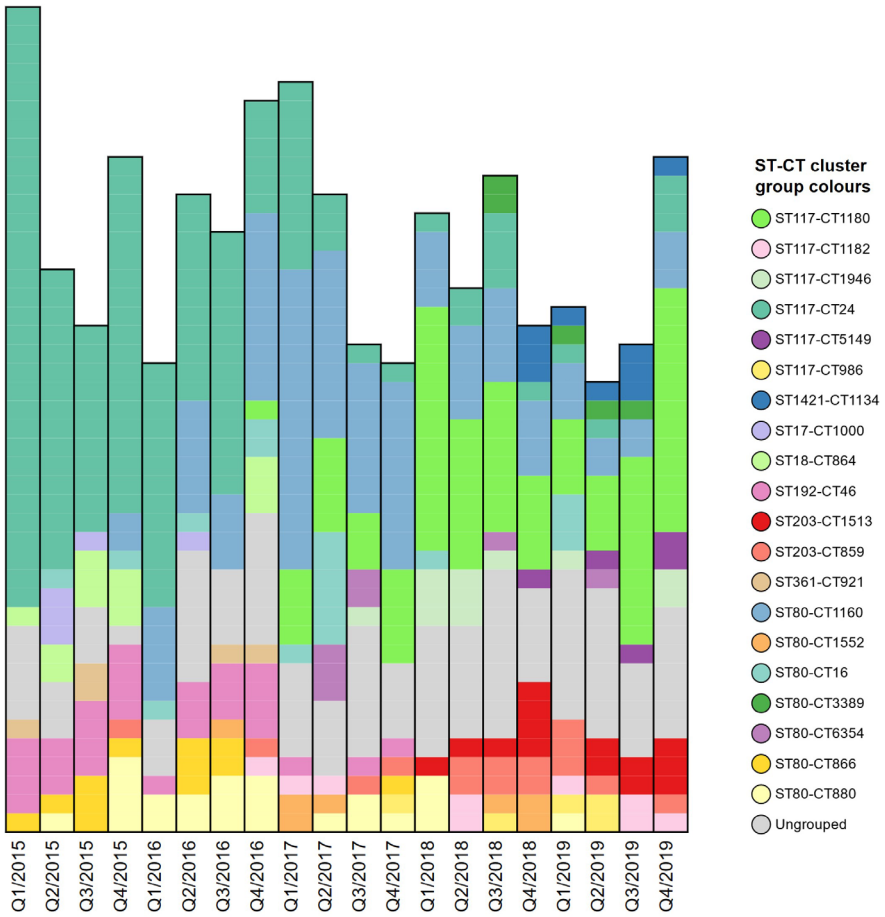


Fig. 2. Timeline distribution of VSEfm blood isolates (*n*=630), detected in the period 2015–2019, at OUH, Denmark. The isolates are stratified by ST-CT cluster groups consisting of five or more isolates each, and year of the collection.

Of the 160 patients without a catheter, 33 (21%) did not get any antibiotic treatment for the VSEfm bacteraemia. For patients with a catheter, this amounted to 15%, and a further 11% had the catheter removed or changed without any VSEfm active antibiotics (Table 4). Patients with a catheter, who did not receive antibiotics as a part of the treatment, had a significant (P<0.001) lower 30 day mortality if the catheter was changed or removed compared to not removing or changing it. Patients with a catheter who received antibiotics did not have a significant reduction in 30 day mortality if the catheter was removed or changed (P>0.5).

Table 3. Distribution of blood isolates of VREfm (*n*=27) and the corresponding VSEfm by CT cluster groups, during the period 2015–2019, at OUH, Denmark

ST-CT cluster	Pathotype	Year					Total
		2015	2016	2017	2018	2019	
ST80-CT880	VSEfm/VREfm	5/1	10/0	4/0	3/0	1/0	23/1
ST203-CT859	VSEfm/VSEfm	1/0	1/1	2/0	6/1	5/1	15/3
ST80-CT993	VSEfm/VREfm	0/0	0/2	0/0	0/1	0/0	0/3
ST80-CT1545	VSEfm/VREfm	0/0	0/0	0/1	0/0	0/0	0/1
ST1421-CT1134	VSEfm/VREfm	0/0	0/0	0/0	3/3	6/12	9/15
ST80-CT1512	VSEfm/VREfm	0/0	0/1	0/0	0/0	0/0	0/1
ST117-CT991	VSEfm (CT1182)/VREfm (CT991)	0/0	1/0	2/0	2/0	4/2	9/2
ST18-CT1584	VSEfm/VREfm	0/0	0/0	0/1	0/0	0/0	0/1

Table 4. Treatment, catheter intervention and death of VSEfm-bacteraemia patients in the period 2015–2019 at OUH, Denmark (*n*=599)

Initial antibiotic treatment	Duration (days)	No. of patients (*n*=599)	Amp^S (*n*=33)	Patients with ACVC (*n*=438)	ACVC changed or removed (*n*=318)	Enterococcal active secondary treatment (*n*=23)	Not dead in the investigation period (*n*=144)	No. of deaths >30 days after the latest VSE bacteraemia episode (*n*=216)	Cause of death for patients dead within 30 days after the latest VSE bacteraemia episode (*n*=238)			
									Total	Likely	Possible	Unlikely
No antibiotic	–	149*	8	116	50	0	31	48	69	2	3	64
Unknown	–	7	1	5	3	0	1	4	2	0	0	2
Penicillins†	SD	2	2	1	0	0	0	0	2	0	0	2
	≤1	0	0	0	0	0	0	0	0	0	0	0
	2–7	3	3	1	1	2	0	2	1	0	0	1
	>7	4	4	0	0	1	1	3	0	0	0	0
	Total	9	9	2	1	3	1	5	3	0	0	3
Vancomycin	SD	115	5	111	108	0	32	38	45	1	0	44
	≤1	11	3	7	6	4‡	3	3	5	1	0	4
	2–7	129	5	87	68	8§	21	45	63	7	8	48
	>7	178	2	109	82	8‖	55	73	50¶	4	7	38
	Unknown#	1	0	1	–	–	0	0	1	0	0	1
	Total	434	15	315	264	20	111	159	164¶	13	15	135
Total	–	599	33	438	318	23	144	216	238¶	15	18	204

Amp^S, Ampicillin susceptible; SD, single dose.
*Unknown whether one patient is dead or not.
†Penicillins=ampicillin and/or piperacillin/tazobactam.
‡Three patients were treated with piperacillin/tazobactam (all three isolates Amp^S) and one with linezolid.
§All eight was treated with linezolid.
‖Seven were treated with linezolid and one with daptomycin.
¶Unknown cause of death for one patient.
#Patients moved to another hospital.

Neither did we find a significant reduction for patients who had their catheter changed or removed if an antibiotic treatment was added (P>0.25).

The distribution of treatment and catheter intervention can be found in Table 4. The overall 30 day mortality was 40% and unrelated to the presence of a catheter, specific STs, CTs and cluster groups. Dividing the patients into age groups of each 10 years, the 30 day mortality rose from age of 40 with the highest mortality (85%) for patients in the group 90–99 years.

Only 15 (6.3%) of the patients died from the VSEfm bacteraemia within 30 days, i.e. VSEfm was the likely cause of death. In 18 (7.6%) of the cases, VSEfm bacteraemia was a possible cause of death, and in 86% cases, the VSEfm bacteraemia was unlikely to have caused death. All the patients with an *E. lactis* bacteraemia belonged to the unlikely group. Eight of the 15 patients with VSEfm as a likely cause of death, died with an isolate belonging to ST80, of which three were in CT cluster group ST80-CT880 and three in ST117-CT1180.

The 15 patients with VSEfm as a likely cause of death were distributed with seven patients in the ICU and the rest in each different department. Of the 18 cases with VSEfm as a possible cause of death, 50% of the patients were admitted in the ICU and 17% in the Department of Haematology. The distribution of 30 day mortality and cause of death can be found in Table 4.

DISCUSSION

Molecular characterization

Our molecular investigation found that most of the VSEfm isolates related to the hospital have remained ampicillin resistant and are designated as CC17. All the isolates designated as CC94 were identified as *E. lactis*, which supports the recent findings and explanation of the phylogenetic split into clades found in earlier studies [11, 12, 33]. However, not all the *E. lactis* isolates were found to be susceptible to ampicillin. Due to new classification, *E. lactis* has recently been found included in older studies as *E. faecium*, but clinical practices have been the same for these two species; therefore, the *E. lactis* isolates remained included in our study.

The most frequent STs of VSEfm were ST80 and ST117. Some of the formerly worldwide spread STs, e.g. ST17 and ST78, were also detected, but only in a few patients [2, 9, 11]. We found that a substitution of the dominating VSEfm STs seems to happen every second to third year, which almost applies to the changes in Danish VREfm isolates [22]. Using cgMLST as a typing tool, the isolates were found to be diverse, and with a high rate of CT exchange each year, which might be an indication of the rate of recombination in this species.

Transmission

Many patients with several VSEfm CT clusters were found to have been involved in putative transmissions during the 5 year investigation period without our knowledge. By using the criteria for molecular relatedness of 20 alleles or less in difference, we found that some CTs were inadvertently grouped together [17]. Combining isolates in CT cluster groups may blur the number of mutations between the different CTs inside the group, raising the question whether all isolates can be assumed connected. For example, we found that two large cluster groups (ST117-CT24 and ST117-CT1180) had been combined according to the method. By using local SLC with a threshold at 11 alleles, we found an increased number of clusters than by using cgMLST, but only in a few putative transmission episodes this implied a more specific epidemiological information. There is a wide discrepancy in the chosen cluster thresholds between studies, and it has been suggested as tight as three alleles for hospital-outbreaks [34]. Our SLC threshold was based on investigating the two largest outbreaks according to our definition of putative transmission, but the allele threshold might have changed if the timespan in our definition was reduced. Reducing the allele threshold may increase the number of sub-clusters, but if the threshold gets too small there is a risk of missing linked patients.

Combining the WGS-based strain typing and analyses of clonal clusters with epidemiological data is necessary to enhance the probability of detecting true transmission, since cluster thresholds or SNP borders cannot be set by a reliable, single number – especially not with a highly recombinant micro-organism such as *E. faecium* [35]. The combination of the molecular and epidemiological results can be used to identify where the transmission might have taken place, saving time and costs in achieving infection control. However, no such system can be complete since ward-move data can be difficult to obtain, transmission might happen outside the ward, and links between patients might be missing [14].

The large number of putative transmissions in our study may be due to the use of the official allele distance threshold for cgMLST clusters, and by using a smaller allele threshold on our data set, the number of patients involved in possible transmissions may be reduced. But still, putative transmissions of VSEfm are found during the entire period. This might be used as an indicator of the presence of risk factors, e.g. sub-optimal hand hygiene and cleaning procedures of utensils [36]. These risk factors could support transmission of VREfm and other micro-organisms as well, since many micro-organisms use the same transmission pathways. Achieving infection control in a hospital is not only a matter of preventing transmission of the most resistant micro-organisms. It should also prevent transmission of bacteria in general, regardless of the resistance profiles. If we prevent transmission of

antibiotic-susceptible clones, we also prevent transmission of the more resistant strains. In this study, only blood isolates were available, which is a limitation in detecting the extent of transmission, but due to the large amount of blood isolates, it is still an indicator of the enterococci flourishing in the hospital.

VSEfm and VREfm relatedness

We found no correlation between the total prevalence, the prevalence of specific STs, CTs or CT clusters groups of VSEfm and the rise of VREfm or VVEfm in blood isolates (Table 4). The STs and CTs of the included VREfm and VVEfm in this study correspond, in general, to the findings in the rest of Denmark, and an introduction of VREfm and VVEfm into our hospital might be explained by hospital transfers of patients unaware of carrying them [13].

We used cgMLST for investigating relatedness between isolates, but there are a lot of other genetic material in the bacteria that could be relevant to study. Besides plasmids, other known possible transmission links could be horizontal transfer of mobile genetic elements, TN structures or transposons, and it could, therefore, be interesting to investigate for these elements in our isolates, to see whether we can find a connection between the VSEfm and the VREfm detected at OUH [37].

It has previously been discussed whether vancomycin resistance in *E. faecium* arises from an introduction of a resistance mechanism in many different receptive VSEfm types at the same time, or whether the resistance arises in a single clone that afterwards causes a clonal outbreak. A recent study from Ireland has found the *efm* gene to be a possible explanation of introduction of VREfm, supporting the first hypothesis. The study suggests that the spread of VREfm, besides the directly transfer of VREfm isolates between patients, mainly is due to genomic-related vancomycin-sensitive *efm* genes that transfer between *E. faecium* in patients, and afterwards acquire a vancomycin-resistant plasmid [38]. Unfortunately, the study does not describe whether this only concerns isolates found in certain human materials. We did not investigate for the presence of the *efm gene* in our isolates, but we found that ST1421-CT1134 VVEfm – the most dominating clone in Denmark during 2018 –2020 – did appear the same year as the first corresponding VSEfm was found in a blood culture [21]. The VVEfm though appeared months before the corresponding VSEfm ST1421-CT1134, and it was not possible to investigate whether this also applied to isolates from other materials. This could be due to an unknown VVEfm introduction followed by the VVEfm having lost the vancomycin resistance or due to the use of blood isolates only. Therefore, we call for studies investigating clinical isolates from other locations and faecal screening isolates, and the presence of the *efm* gene in those.

Clinical impact

In our study, we found that the distribution of age and sex of the VSEfm bacteraemia patients corresponds to earlier findings from Denmark and Canada [26, 39]. We found that patients with VSEfm hospital-acquired bacteraemia were admitted to the ICU and medical departments in 47 and 36% of the cases, respectively. In a 10-year-old Danish study, patients with enterococcal hospital-acquired bacteraemia were admitted to the ICU and medical departments in 34.8 and 37.7% of the cases, respectively [39]. The difference in the ICU findings can be due to differences in local ward allocation plans and the bacterial environment of the hospital.

We also found that almost all the detected CTs were represented in the ICU or the Department of Haematology. This was not a surprise, because the patients in these departments often are critically ill and have received broad-spectrum antibiotic treatment for long periods, leaving an environment suitable for antibiotic-resistant micro-organisms. Other departments with a high prevalence were the departments taking care of abdominal diseases, which was expected due to the natural habitat of enterococci.

We found that 25% of the VSEfm bacteraemia patients did not receive any comprehensive enterococcal antibiotic treatment. Furthermore, we found a significant impact of catheter removal or change on reducing the 30 day mortality in patients not treated with antibiotics active against enterococci, which corresponds to results in other studies [40]. This may indicate the ability of *E. faecium* to colonize foreign materials, but the presence of a catheter together with the possibility to change or remove it may also indicate the patient's health condition. This is supported by our findings that the catheter removal or change had no significant impact on the 30 day mortality if the patient received antibiotics active against enterococci at the time of change or removal.

We found that the overall 30 day mortality of VSEfm bacteraemia was 40%, a result similar to another Danish–Dutch study, which found the 30 day mortality for VSEfm at 38% and VREfm at 48% [30]. This is a surprisingly high 30 day mortality compared to bacteraemia from *S. aureus* and *Escherichia coli* with levels for meticillin-sensitive *S. aureus* (MSSA) at 18%, and meticillin-resistant *S. aureus* (MRSA) at 25%, while *Escherichia coli* with an hospital-onset was found at 31% [41, 42]. By investigating the cause of death in detail, we revealed that in only 6.3% of the VSEfm bacteraemia cases was the VSEfm likely to have caused death. Most of the patients had underlying severe illness, which constituted a confounder and resulted in VSEfm appearing to have a greater impact on a fatal outcome than is the case. This divergence may also apply to other species and, therefore, it is important to investigate the actual cause of death including other diseases [43].

Conclusion

In this study, we found a changing and diverse molecular pattern of VSEfm bacteraemia isolates during a 5 year period. Putative transmission of VSEfm occurred consistently in our hospital, possibly indicating the presence of risk factors, which could support transmission of other micro-organisms as well. The resistant isolates can be considered the tip of the iceberg, and maybe it is time to also have a look at microbes not having a significant resistance profile. We did not find any molecular patterns of VSEfm to predict the introduction of VREfm, which could be due to the use of blood isolates only. With this study, we also demonstrated that VSEfm bacteraemia rarely causes death, i.e. the 30 day mortality does not reflect the actual cause of death, indicating that the 30 day mortality must be interpreted with care.

Funding information
The authors received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Acknowledgements
Elisa Knudsen is thanked for her excellent technical assistance in the laboratory.

Conflicts of interest
The authors declare that there are no conflicts of interest

Ethical statement
The Danish Patients Safety Authority has approved the collection of data from the hospital records (reference no. 3-3013-2554/1).

References

1. Fiore E, Van Tyne D, Gilmore MS. Pathogenicity of enterococci. *Microbiol Spectr* 2019;7:gpp3-0053-2018.
2. Arias CA, Murray BE. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat Rev Microbiol* 2012;10:266–278.
3. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis* 2008;197:1079–1081.
4. Wagenvoort JHT, De Brauwer E, Penders RJR, Willems RJ, Top J, *et al*. Environmental survival of vancomycin-resistant *Enterococcus faecium*. *J Hosp Infect* 2011;77:282–283.
5. Otter JA, Vickery K, Walker JT, deLancey Pulcini E, Stoodley P, *et al*. Surface-attached cells, biofilms and biocide susceptibility: implications for hospital cleaning and disinfection. *J Hosp Infect* 2015;89:16–27.
6. Coque TM, Willems RJL, Fortún J, Top J, Diz S, *et al*. Population structure of *Enterococcus faecium* causing bacteremia in a Spanish university hospital: setting the scene for a future increase in vancomycin resistance? *Antimicrob Agents Chemother* 2005;49:2693–2700.
7. Leavis HL, Bonten MJM, Willems RJL. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* 2006;9:454–460.
8. Top J, Willems R, van der Velden S, Asbroek M, Bonten M. Emergence of clonal complex 17 *Enterococcus faecium* in the Netherlands. *J Clin Microbiol* 2008;46:214–219.
9. Lester CH, Sandvang D, Olsen SS, Schønheyder HC, Jarløv JO, *et al*. Emergence of ampicillin-resistant *Enterococcus faecium* in Danish hospitals. *J Antimicrob Chemother* 2008;62:1203–1206.
10. Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of *Enterococcus faecium*. *PLoS One* 2012;7:e30187.
11. Willems RJL, Top J, van Schaik W, Leavis H, Bonten M, *et al*. Restricted gene flow among hospital subpopulations of *Enterococcus faecium*. *mBio* 2012;3:e00151-12.
12. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, *et al*. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *mBio* 2013;4:e00534-13.
13. DANMAP. *DANMAP Reports (*https://www.DANMAP.org/reports*)*. Lyngby, Copenhagen: Technical University of Denmark, Statens Serum Institut; 2022.
14. Raven KE, Gouliouris T, Brodrick H, Coll F, Brown NM, *et al*. Complex routes of nosocomial vancomycin-resistant *Enterococcus faecium* transmission revealed by genome sequencing. *Clin Infect Dis* 2017;64:886–893.
15. van Hal SJ, Willems RJL, Gouliouris T, Ballard SA, Coque TM, *et al*. The interplay between community and hospital *Enterococcus faecium* clones within health-care settings: a genomic analysis. *Lancet Microbe* 2022;3:e133–e141.
16. Belloso Daza MV, Almeida-Santos AC, Novais C, Read A, Alves V, *et al*. Distinction between *Enterococcus faecium* and *Enterococcus lactis* by a *gluP* PCR-based assay for accurate identification and diagnostics. *Microbiol Spectr* 2022;10:e0326822.
17. de Been M, Pinholt M, Top J, Bletz S, Mellmann A, *et al*. Core genome multilocus sequence typing scheme for high-resolution typing of *Enterococcus faecium*. *J Clin Microbiol* 2015;53:3788–3797.
18. Mellmann A, Bletz S, Böking T, Kipp F, Becker K, *et al*. Real-time genome sequencing of resistant bacteria provides precision infection control in an institutional setting. *J Clin Microbiol* 2016;54:2874–2881.
19. van Hal SJ, Ip CLC, Ansari MA, Wilson DJ, Espedido BA, *et al*. Evolutionary dynamics of *Enterococcus faecium* reveals complex genomic relationships between isolates with independent emergence of vancomycin resistance. *Microb Genom* 2016;2:e000048.
20. Jensen LB, Garcia-Migura L, Valenzuela AJS, Løhr M, Hasman H, *et al*. A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* 2010;80:25–43.
21. DANMAP. *DANMAP 2018 – Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria from Food Animals, Food and Humans in Denmark (www.DANMAP.org)*. Lyngby, Copenhagen: Technical University of Denmark, Statens Serum Institut; 2019.
22. Hammerum AM, Justesen US, Pinholt M, Roer L, Kaya H, *et al*. Surveillance of vancomycin-resistant enterococci reveals shift in dominating clones and national spread of a vancomycin-variable *vanA Enterococcus faecium* ST1421-CT1134 clone, Denmark, 2015 to March 2019. *Euro Surveill* 2019;24:1900503.
23. Kjær Hansen S, Andersen L, Detlefsen M, Holm A, Roer L, *et al*. Using core genome multilocus sequence typing (cgMLST) for vancomycin-resistant *Enterococcus faecium* isolates to guide infection control interventions and end an outbreak. *J Glob Antimicrob Resist* 2021;24:418–423.
24. Thomsen MCF, Ahrenfeldt J, Cisneros JLB, Jurtz V, Larsen MV, *et al*. A bacterial analysis platform: an integrated system for analysing bacterial whole genome sequencing data for clinical diagnostics and surveillance. *PLoS One* 2016;11:e0157718.

25. Agudelo Higuita NI, Huycke MM. Enterococcal disease, epidemiology, and implications for treatment. In: *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston, MA: Massachusetts Eye and Ear Infirmary, 2014.
26. Bright M, Parfitt EC, Pasquill K, Steele L, Laupland KB. Occurrence and determinants of enterococcal bloodstream infections: a population-based study. *Infect Dis* 2020;52:638–643.
27. Ali GA, Goravey W, Najim MS, Shunnar KM, Ibrahim SI, *et al*. Epidemiology, microbiological and clinical characteristics of *Enterococcus* species bloodstream infections: a 10-year retrospective cohort study from Qatar. *Ann Med Surg* 2022;80:104258.
28. Prematunge C, MacDougall C, Johnstone J, Adomako K, Lam F, *et al*. VRE and VSE bacteremia outcomes in the era of effective VRE therapy: a systematic review and meta-analysis. *Infect Control Hosp Epidemiol* 2016;37:26–35.
29. López-Luis BA, Sifuentes-Osornio J, Lambraño-Castillo D, Ortiz-Brizuela E, Ramírez-Fontes A, *et al*. Risk factors and outcomes associated with vancomycin-resistant *Enterococcus faecium* and ampicillin-resistant *Enterococcus faecalis* bacteraemia: a 10-year study in a tertiary-care centre in Mexico City. *J Glob Antimicrob Resist* 2021;24:198–204.
30. Rottier WC, Pinholt M, van der Bij AK, Arpi M, Blank SN, *et al*. Attributable mortality of vancomycin resistance in ampicillin-resistant *Enterococcus faecium* bacteremia in Denmark and the Netherlands: a matched cohort study. *Infect Control Hosp Epidemiol* 2022;43:719–727.
31. Higgs C, Sherry NL, Seemann T, Horan K, Walpola H, *et al*. Optimising genomic approaches for identifying vancomycin-resistant *Enterococcus faecium* transmission in healthcare settings. *Nat Commun* 2022;13:509.
32. Kirkwood BR. *Essentials of Medical Statistics*. Hoboken, NJ: Wiley-Blackwell; 1988.
33. Belloso Daza MV, Cortimiglia C, Bassi D, Cocconcelli PS. Genome-based studies indicate that the *Enterococcus faecium* clade B strains belong to *Enterococcus lactis* species and lack of the hospital infection associated markers. *Int J Syst Evol Microbiol* 2021;71:004948.
34. Kampmeier S, Tönnies H, Correa-Martinez CL, Mellmann A, Schwierzeck V. A nosocomial cluster of vancomycin resistant enterococci among COVID-19 patients in an intensive care unit. *Antimicrob Resist Infect Control* 2020;9:154.
35. Turner KME, Hanage WP, Fraser C, Connor TR, Spratt BG. Assessing the reliability of eBURST using simulated populations with known ancestry. *BMC Microbiol* 2007;7:30.
36. Hughes A, Ballard S, Sullivan S, Marshall C. An outbreak of vanA vancomycin-resistant *Enterococcus faecium* in a hospital with endemic vanB VRE. *Infect Dis Health* 2019;24:82–91.
37. Gorrie C, Higgs C, Carter G, Stinear TP, Howden B. Genomics of vancomycin-resistant *Enterococcus faecium*. *Microb Genom* 2019;5:e000283.
38. Leigh RJ, McKenna C, McWade R, Lynch B, Walsh F. Comparative genomics and pangenomics of vancomycin-resistant and susceptible *Enterococcus faecium* from Irish hospitals. *J Med Microbiol* 2022;71:001590.
39. Pinholt M, Ostergaard C, Arpi M, Bruun NE, Schønheyder HC, *et al*. Incidence, clinical characteristics and 30-day mortality of enterococcal bacteraemia in Denmark 2006-2009: a population-based cohort study. *Clin Microbiol Infect* 2014;20:145–151.
40. Heybati K, Seeger R, Thyagu S, Piticaru J, Ahluwalia N, *et al*. Catheter management across patients with hematologic malignancies and catheter-related blood stream infections: a systematic review. *Ann Hematol* 2022;101:2515–2524.
41. Austin ED, Sullivan SS, Macesic N, Mehta M, Miko BA, *et al*. Reduced mortality of *Staphylococcus aureus* bacteremia in a retrospective cohort study of 2139 patients: 2007–2015. *Clin Infect Dis* 2020;70:1666–1674.
42. Feldman SF, Temkin E, Wullfhart L, Nutman A, Schechner V, *et al*. A nationwide population-based study of *Escherichia coli* bloodstream infections: incidence, antimicrobial resistance and mortality. *Clin Microbiol Infect* 2022;28:879.
43. Nørgaard M, Larsson H, Pedersen G, Schønheyder HC, Sørensen HT. Haematological malignancies – a predictor of a poor outcome in patients with bacteraemia. *J Infect* 2006;53:190–198.

Paper II - Supplementary

Supplementary

Table S1.

Complete distribution list of CT cluster groups for VSEfm blood isolates during the period 2015-2019 at Odense University Hospital, Denmark. The list is ranged after the ST-CT number

E. lactis isolates	CT cluster group	MLST	cgMLST (no. of isolates)	Year					
	ST-CT	ST	CT	2015 (n)	2016 (n)	2017 (n)	2018 (n)	2019 (n)	Total (n)
	ST17-CT1000	ST17	CT1000	4	1	0	0	0	5
	ST17-CT6359		CT6359	1	0	0	0	0	1
	ST17-CT6361		CT6361	1	0	0	0	0	1
	ST17-CT6362		CT6362	1	1	0	0	0	2
	ST17-CT6363		CT6363	0	1	0	0	0	1
	ST17-CT6372		CT6372	0	1	0	0	0	1
	ST18-CT222	ST18	CT222	1	0	0	0	0	1
	ST18-CT388		CT388	0	0	0	1	0	1
	ST18-CT864		**CT864** (9), CT1835 (2), CT6373 (1)	9	3	0	0	0	12
	ST18-CT1898		CT1898	0	0	0	1	1	2
	ST18-CT6385		CT6385	0	0	1	0	0	1
	ST22-CT6071	ST22	CT6071	0	0	0	0	1	1
	ST27-CT6346	ST27	CT6346	0	0	0	0	1	1
	ST52-CT6397	ST52	CT6397	0	0	0	1	0	1
	ST56-CT6400	ST56	CT6400	0	0	0	1	0	1
E. lactis	**ST74-CT6433**	ST74	CT6433	0	0	1	0	0	1
	ST78-CT1438	ST78	CT1438	1	0	0	0	1	2
	ST80-CT16	ST80	**CT16** (6), CT1840 (11)	2	4	7	1	3	17
	ST80-CT866		**CT866** (10), CT6369 (2)	6	5	1	0	0	12
	ST80-CT880		**CT880** (15), CT5907 (4), CT6350 (2), CT6376 (1), CT6384 (1)	5	10	4	3	1	23
	ST80-CT899		CT899	0	2	0	0	0	2
	ST80-CT1053		CT1053	0	0	1	0	3	4
	ST80-CT1160		**CT1160** (94), CT2516 (1), CT6342 (1), CT6345 (1), CT6392 (1), CT6415 (1)	2	25	44	18	10	99
	ST80-CT1179		CT1179	0	1	2	0	0	3
	ST80-CT1530		CT1530	0	0	0	0	1	1
	ST80-CT1552	**ST80** + ST2149	CT1552 (6 + 1)	0	1	3	2	1	7
	ST80-CT1836		CT1836	0	1	0	0	0	1
	ST80-CT1837	**ST80** + ST2148	CT1837 (3 + 1)	0	4	0	0	0	4
	ST80-CT1860		CT1860	0	0	0	1	1	2
	ST80-CT1953		CT1953	0	0	0	1	2	3
	ST80-CT2472		CT2472	0	0	0	0	1	1
	ST80-CT3389		CT3389	0	0	0	2	3	5
	ST80-CT6344		CT6344	0	0	0	0	1	1
	ST80-CT6352		CT6352	0	0	0	0	1	1
	ST80-CT6354		CT6354	0	0	5	1	1	7
	ST80-CT6355		CT6355	0	0	0	0	1	1
	ST80-CT6366		CT6366	0	1	0	0	0	1
	ST80-CT6367		CT6367	0	1	0	0	0	1
	ST80-CT6375		CT6375	0	1	0	0	0	1
	ST80-CT6383		CT6383	0	0	1	0	0	1
	ST80-CT6388		CT6388	0	0	1	0	0	1
	ST80-CT6391		CT6391	0	0	1	2	0	3
	ST80-CT6395		CT6395	0	0	0	1	0	1
	ST80-CT6396		CT6396	0	0	0	1	0	1
	ST80-CT6403		CT6403	0	0	0	1	0	1
	ST80-CT6407		CT6407	0	0	0	1	0	1
	ST80-CT6413		CT6413	0	0	0	1	0	1
	ST80-CT6414		CT6414	0	0	0	1	0	1
E. lactis	**ST94-CT5422**	ST94	CT5422	0	1	0	0	0	1
E. lactis	**ST94-CT6365**		CT6365	0	1	0	0	0	1
E. lactis	**ST94-CT6379**		CT6379	0	0	1	0	0	1
E. lactis	**ST94-CT6387**		CT6387	0	0	1	0	0	1
E. lactis	**ST94-CT6399**		CT6399	0	0	0	1	0	1
E. lactis	**ST94-CT6402**		CT6402	0	0	0	1	0	1
	ST117-CT24	ST117	**CT24** (139), CT1487 (1), CT1834 (1), CT2056 (1), CT6351 (2), CT6364 (1), CT6380 (1), CT6382 (3), CT6408 (1)	78	43	16	8	5	150
	ST117-CT873		CT873	0	2	0	0	1	3

	ST117-CT877		CT877	0	0	0	0	1	1
	ST117-CT986		CT986	0	0	1	1	3	5
	ST117-CT1180		**CT1180** (81), CT6398 (2)	0	1	17	34	31	83
	ST117-CT1182		CT1182	0	1	2	2	4	9
	ST117-CT1946		CT1946	0	0	1	7	3	11
	ST117-CT1997		CT1997	0	0	2	1	0	3
	ST117-CT5149		CT5149	0	0	0	1	4	5
	ST117-CT6347		CT6347	0	0	0	0	1	1
	ST117-CT6358		CT6358	1	0	0	0	0	1
	ST117-CT6393		CT6393	0	0	1	0	0	1
	ST117-CT6406		CT6406	0	0	0	1	0	1
	ST117-CT6412		CT6412	0	0	0	1	0	1
E. lactis	**ST163-CT6386**	ST163	CT6386	0	0	1	0	0	1
	ST168-CT851	ST168	CT851	1	0	0	0	0	1
	ST192-CT46	**ST192** + ST2146	**CT46** (21), CT1838 (4), CT6389 (1), CT6394 (1), ST2146-CT1838 (2)	15	11	3	0	0	29
	ST192-CT6357		CT6357	1	0	0	0	0	1
	ST192-CT6360		CT6360	1	0	0	0	0	1
	ST192-CT6368		CT6368	1	1	0	0	0	2
	ST203-CT859	ST203	CT859	1	1	2	6	5	15
	ST203-CT1513		CT1513	0	0	0	7	7	14
	ST210-CT6348	ST210	CT6348	0	0	0	0	1	1
E. lactis	**ST240-CT6374**	ST240	CT6374	0	1	0	0	0	1
	ST262-CT6441	ST262	CT6441	1	0	0	0	0	1
	ST266-CT6409	ST266	CT6409	0	0	0	1	0	1
E. lactis	**ST329-CT6401**	ST329	CT6401	0	0	0	1	0	1
E. lactis	**ST345-CT6404**	ST345	CT6404	0	0	0	1	0	1
E. lactis	**ST361-CT921**	ST361	CT921	3	2	0	0	0	5
E. lactis	**ST361-CT6343**		CT6343	0	0	0	0	1	1
	ST437-CT2250	ST437	CT2250	0	0	0	2	0	2
	ST502-CT6390	ST502	CT6390	0	0	1	0	0	1
	ST612-CT1026	ST612	CT1026	0	1	0	0	0	1
	ST612-CT2942		CT2942	0	0	0	0	1	1
	ST612-CT6349		CT6349	0	0	0	0	1	1
E. lactis	**ST874-CT5357**	ST874	CT5357	1	0	0	0	0	1
E. lactis	**ST928-CT6378**	ST928	CT6378	0	0	1	0	0	1
E. lactis	**ST1031-CT6405**	ST1031	CT6405	0	0	0	1	0	1
E. lactis	**ST1031-CT6410**		CT6410	0	0	0	1	0	1
E. lactis	**ST1103-CT6353**	ST1103	CT6353	0	0	0	0	1	1
E. lactis	**ST1137-CT6370**	ST1137	CT6370	0	1	0	0	0	1
E. lactis	**ST1202-CT6381**	ST1202	CT6381	0	0	1	0	0	1
E. lactis	**ST1268-CT6377**	ST1268	CT6377	0	0	1	0	0	1
	ST1421-CT1134	ST1421	CT1134	0	0	0	3	6	9
	ST1693-CT2532	ST1693	CT2532	0	0	0	0	2	2
E. lactis	**ST1937-CT5271**	ST1937	CT5271	0	0	0	0	1	1
	ST2089-CT6432	ST2089	CT6432	0	0	0	0	1	1
E. lactis	**ST2142-CT6371**	ST2142	CT6371	0	1	0	0	0	1
E. lactis	**ST2150-CT6411**	ST2150	CT6411	0	0	0	1	0	1
	ST2151-CT6356	ST2151	CT6356	0	0	0	0	1	1

DEFINITIONS AND CRITERIA FOR CAUSE OF DEATH

The cause of death for patients with a vancomycin-sensitive *E. faecium* (VSE) bacteremia was assessed by screening the hospital records of all patients included in the study and grouping the cause of death into likely, unlikely, and possibly caused by VSE bacteremia.

The screening was done in two steps with one clinical microbiologist grouping all patients followed by a second microbiologist further scrutinizing the group "possible".

STEP ONE – CRUDE DIVISION
The assessment was done by using:

- clinical informations.
- Vital signs and paraclinical information such as temperature, infection parameters (CRP, leukocytes), and x-rays.
- Microbiological findings.
- The duration from onset of bacteremia until death.
- Antibiotic treatment.
    - if the antibiotic treatment was active or not against VSE.
    - if there was improvement or not on the given antibiotic treatment.

Death due to* VSE bacteremia = *Likely
No other cause of death was recognized, and other diseases were stable.

Death with* VSE bacteremia = *Unlikely
There was another evident cause of death.

a) Death of a non-infectious cause

- All treatment was discontinued due to another reason than infection.
- Cause of death was due to a terminal disease such as cancer, injury, or surgical complications.

b) Death of an infectious disease different from VSE bacteremia
If one or both of the following criteria were present:

- The patient was treated for an infection, which was not compatible with a usual VSE infection - e.g., pneumonia.
- Another pathogenic microorganism with a corresponding clinical picture was found – e.g., *Legionella pneumophila* and pneumonia, *Streptococcus spp.* and necrotizing fasciitis.

Death possible due to* VSE bacteremia = *Possible
Patients who died of an infection and where VSE cannot be eliminated as a cause of death, because there is no other evident cause of death and,

- Antibiotic treatment was not given even though general treatment of the patient continued.
- The patient improved on the antibiotic treatment against VSE, but all other treatment of the patient was stopped before the VSE treatment had finished, and the patient died with an infection.
- There was no improvement on antibiotic active against VSE, and no other pathogenic microorganism was found as possible cause.

Unknown
It is not evident from the hospital record what the cause of death was (in most cases due to death outside the hospital without any contact to the hospital, or because the patient had moved abroad).

Alive
The patient was alive on the date of the hospital record investigation (June 2022)

STEP TWO – METICULOUS DIVISION OF THE GROUP "POSSIBLE"

Table 1: Criteria and score used for evaluating the implication of *E. faecium* bacteraemia in causing death.

Question	Answer (points)	
	Yes	No
Did the patient have bacteraemia with VSE detected in >1 blood culture	1	0
Were there any acute non-infectious causes of death? (Evidently non-infectious cause, e.g. brain stem stroke)	0	1
Were there any bacteria in the blood cultures more pathogenic than VSE? (Other infectious causes of death)	0	1
Is it your medical estimation based on treatment, clinical and para-clinical information, that the patient would have survived more than 14 days if there had not been *E. faecium* bacteraemia?	2	0

Interpretation:

0-2 points	Unlikely cause of death
3 points	Possible cause of death
4-5 Points	Likely cause of death

DEFINITIONS

30-days mortality

Death within or 30 days after the latest VSE find in a blood culture

Antibiotics used against VSE

Requires the isolate to be deemed susceptible according to EUCAST susceptibility testing.

Drugs:

Ampicillin
Tazobactam/Piperacillin
Vancomycin
Linezolid
Daptomycin
Teicoplanin

Paper III

Journal of Hospital Infection 146 (2024) 82–92

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevier.com/locate/jhin

Vancomycin-resistant *Enterococcus faecium*: impact of ending screening and isolation in a Danish University hospital

S.G.K. Hansen [a,b,*], K. Klein [a,b], A. Nymark [a,b], L. Andersen [a], K.O. Gradel [c,d], J. Lis-Toender [e], C. Oestergaard [e], M. Chen [f], R. Datcu [g], M.N. Skov [a,b], A. Holm [a,b], F.S. Rosenvinge [a,b]

[a] *Department of Clinical Microbiology and Infection Control, Odense University Hospital, Odense, Denmark*
[b] *Research Unit of Clinical Microbiology, Department of Clinical Research, University of Southern Denmark, Odense, Denmark*
[c] *Center for Clinical Epidemiology, Odense University Hospital, Odense, Denmark*
[d] *Research Unit of Clinical Epidemiology, Department of Clinical Research, University of Southern Denmark, Odense, Denmark*
[e] *Department of Clinical Microbiology, Lillebaelt Hospital, University Hospital of Southern Denmark, Vejle, Denmark*
[f] *Department of Clinical Microbiology, University Hospital of Southern Denmark, Aabenraa, Denmark*
[g] *Department of Clinical Microbiology, Esbjerg and Grindsted Hospital, University Hospital of Southern Denmark, Esbjerg, Denmark*

ARTICLE INFO

Article history:
Received 13 November 2023
Accepted 27 January 2024
Available online 13 February 2024

Keywords:
Enterococcus faecium
Vancomycin
Mortality
Morbidity
Treatment
Isolation

SUMMARY

Background: Substantial resources are used in hospitals worldwide to counteract the ever-increasing incidence of vancomycin-resistant and vancomycin-variable *Enterococcus faecium* (VREfm and VVEfm), but it is important to balance patient safety, infection prevention, and hospital costs.

Aim: To investigate the impact of ending VREfm/VVEfm screening and isolation at Odense University Hospital (OUH), Denmark, on patient and clinical characteristics, risk of bacteraemia, and mortality of VREfm/VVEfm disease at OUH. The burden of VREfm/VVEfm bacteraemia at OUH and the three collaborative hospitals in the Region of Southern Denmark (RSD) was also investigated.

Methods: A retrospective cohort study was conducted including first-time VREfm/VVEfm clinical isolates (index isolates) detected at OUH and collaborative hospitals in the period 2015–2022. The intervention period with screening and isolation was from 2015 to 2021, and the post-intervention period was 2022. Information about clinical isolates was retrieved from microbiological databases. Patient data were obtained from hospital records.

Findings: At OUH, 436 patients were included in the study, with 285 in the intervention period and 151 in the post-intervention period. Ending screening and isolation was followed by an increased number of index isolates. Besides a change in *van* genes, only minor non-significant changes were detected in all the other investigated parameters. Mortality within 30 days did not reflect the VREfm/VVEfm-attributable deaths, and in only four cases was VREfm/VVEfm infection the likely cause of death.

Conclusion: Despite an increasing number of index isolates, nothing in the short follow-up period supported a reintroduction of screening and isolation.

* Corresponding author. Address: Department of Clinical Microbiology, J.B. Winsløwsvej 21, 2. floor, Odense, 5000, Denmark. Tel.: +45 65414798.
E-mail address: sanne.groenvall.kjaer.hansen@rsyd.dk (S.G.K. Hansen).

https://doi.org/10.1016/j.jhin.2024.01.019

Introduction

Enterococcus faecium is a part of the intestinal microbiota and associated with nosocomial infections — especially in the urinary tract, abdomen, and bloodstream [1,2].

E. faecium infections have been treatable using glycopeptides such as vancomycin, but resistance appeared in the 1980s [3—5]. Based on phenotypic susceptibility and the presence of different *van*-resistance genes, *E. faecium* can be classified as vancomycin-susceptible *E. faecium* (VSEfm), vancomycin-resistant *E. faecium* (VREfm), and vancomycin-variable *E. faecium* (VVEfm) [5,6].

In Denmark, VREfm were rarely found before 2012, and VVEfm was detected for the first time in 2015. Both have since spread throughout the country and caused several outbreaks. In 2022, VREfm/VVEfm comprised 9% of Danish *E. faecium* bacteraemia isolates, and 0.4% of the Danish population were colonized [5,7]. To counteract the development, a variety of different screening and isolation strategies are used in Danish hospitals. However, screening and isolation increase hospital workload and costs, and studies indicate that isolation increases the patient's overall risk of complications and death, due to fewer tests of vital parameters, delayed examinations, and fewer contacts with hospital staff. In addition, patients report social stigmatization and reduced physical contact with family members [8—12].

When screening and isolation are ended, studies from countries with high incidences of VREfm have reported that the number of patients with VREfm rises to a steady level within a year or two [13—17]. The consequence of ending screening and isolation has not been studied in a low-prevalence setting, but knowledge on the topic is important to balance patient safety, infection prevention, and hospital costs.

During 2019—2021, the yearly mean number of patients detected with VREfm/VVEfm at Odense University Hospital (OUH) was 260 [5]. In this period, we observed only few infections caused by VREfm/VVEfm, despite frequent colonization and empiric antibiotic regimes not active against VREfm/VVEfm. Studies report a 24—66% 30-day mortality after *E. faecium* bacteraemia despite adequate antibiotic treatment. Mortality is correlated to severe underlying illness, but no study has investigated whether *E. faecium* was the actual cause of death [18—22]. In a recent study we found that only 6% of the 30-day mortality in patients with VSEfm bacteraemia was attributable to infection *per se* [23]. Therefore, OUH ended screening and isolation against VREfm/VVEfm at the end of 2021.

The aim of this study was to examine the impact of ending VREfm/VVEfm screening and isolation at OUH by investigating changes in the VREfm/VVEfm patients: age, gender, treatment departments, site of infection, treatment, bacteraemia within 30 days of primary infection, 30-day mortality, VREfm/VVEfm-attributable death, and burden of bacteraemia at OUH. To investigate a possible increased transmission to the collaborative hospitals we investigated the burden of VREfm/VVEfm bacteraemia in all hospitals in the Region of Southern Denmark (RSD).

Methods

This study was conducted as a retrospective cohort study in RSD.

Setting

RSD covers approximately one-fifth (1.2 million) of the Danish population. There are four hospitals in the region with frequent inter-hospital referrals. OUH is the largest (~1000 beds and 90,000 admissions/year) and has a number of highly specialized clinical functions. The three non-OUH hospitals — Lillebaelt Hospital, Esbjerg and Grindsted Hospital, and Hospital Sønderjylland — are regional collaborative hospitals with a total of ~1240 beds and 135,000 admissions per year.

Each hospital has its own Department of Clinical Microbiology (DCM) and Infection Prevention and Control (IPC), and a high proportion of two- and four-bed rooms.

Inclusion criteria

All patients with their first-time clinical VREfm/VVEfm isolate (index isolate) detected by culture at a DCM in the RSD from January 2015 through December 2022 were included. Clinical isolates were defined as all VREfm/VVEfm isolates excluding isolates from rectal swabs. We included both inpatients and outpatients regardless of symptoms and prescribed antibiotics.

The four DCMs did not use the same diagnostic methods to detect vancomycin resistance, nor the same thresholds for including *E. faecium* from different sample categories. However, diagnostic algorithms for blood cultures were identical and the analysis of the non-OUH hospitals was therefore restricted to the number of index isolates from blood culture.

Investigation periods

The study was divided into an intervention period (January 1st, 2015 to December 31st, 2021) with specific precautions and a post-intervention period (January 1st to December 31st, 2022) with standard precautions.

VREfm/VVEfm-specific precautions

Screening was performed as a single rectal swab on patients admitted to the hospital in case of:

- hospitalization outside the Nordic countries within the last six months
- positive VREfm/VVEfm sample (clinical or screening) within the last six months

- detection of VREfm/VVEfm in another inpatient located in the same hospital room
- suspicion of an outbreak in the ward.

Wards with repeated outbreaks performed periodic screening of all patients on admission and submission.

All VREfm/VVEfm-positive patients were isolated (single or cohort) when admitted to hospital, until six months after the last positive finding.

Infection precautions were continuously adjusted to deal with local outbreaks, including enhanced cleaning frequency and hydrogen peroxide decontamination.

Bacterial identification and susceptibility testing

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Microflex LT; Bruker Daltonik Gmb, Bremen, Germany) was used for bacterial identification.

Susceptibility to vancomycin was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST (www.eucast.org). In-house polymerase chain reaction (PCR) was used for detecting the vancomycin-resistance genes *vanA*, *vanB*, and a deletion in the *vanX* gene [24]. An isolate was registered as VREfm if *vanA* and/or *vanB* were detected without a deletion in *vanX*, and as VVEfm if a deletion was found. The combination of the *vanA* gene and the *vanX* deletion was designated *vanAXd*.

Whole-genome sequencing

Clinical VREfm and VVEfm isolates were referred for whole-genome sequencing at Statens Serum Institut (SSI), Denmark, as part of a national surveillance programme. Results of multilocus sequence typing (MLST) and core-genome MLST (cgMLST) were available to the DCMs as sequence types (ST) and complex types (CT).

Data sources

All Danish residents have a unique identification number that holds information on age and sex and enables unambiguous identification in administrative and healthcare systems [25].

Number of admissions was provided by the Departments of Data and Automation.

At OUH, data on samples containing *E. faecium* were retrieved from the Microbiology Department Database System (MADS, Aarhus, Denmark) and The Danish Microbiology DataBase (MiBa) and included sample date, requesting ward, specimen, anatomical location, presence of arterial and/or central lines, urinary tract catheters, and abdominal drains [26,27].

Information about date of death, clinical parameters, antibiotic treatment, and removal of indwelling catheters was extracted from electronic hospital records (Cambio COSMIC; https://www.cambiogroup.com) and EPJ SYD [28].

Blood culture data (number, number of patients, and results) were extracted from MADS at both OUH and non-OUH.

Samples

The first sample containing VREfm/VVEfm was defined as the index sample. If more than one sample with VREfm/VVEfm were collected at the same date from different locations, the index sample was categorized as mixed and further subdivided; if the mixed sample-set included a VREfm/VVEfm-positive blood culture, the sample-set was grouped as 'blood', otherwise as 'other'.

If samples collected within two days of the index sample contained both VREfm/VVEfm and other bacterial species, the index sample was categorized as polymicrobial.

Coagulase-negative staphylococci in a single blood culture were regarded as contamination and were not included.

Treatment

Linezolid, daptomycin, and tigecyclin were regarded as active against VREfm/VVEfm. Teicoplanin and quinopristin–dalfopristin were not available in our hospital.

Antibiotic treatment was registered if started within seven days after the index sample was obtained. Duration was counted as number of days where at least one dose of antibiotic was administered.

Catheter removal or replacement was registered within seven days after the index sample.

Mortality

Mortality within 30 days after the index sample date was registered. For patients who died within 30 days, death attributable to VREfm/VVEfm was categorized as 'likely', 'possible', 'unlikely', and 'unknown', based on data from hospital records and a previously described algorithm [23].

Statistical analyses

The two periods were compared using χ^2-statistics for categorical and Student's *t*-test for continuous variables in the univariate analyses, and logistic regression with odds ratios (ORs) and 95% confidence intervals (CIs) in the multivariate analyses. The multivariate analyses were adjusted for *van* genes and requesting ward. We reiterated all the analyses by including only 2021 in the intervention period. Stata/SE, vs 17 (StataCorp., College Station, TX, USA) was used for statistical analyses. *P*-Values were two-sided and $P < 0.05$ was considered statistically significant.

Ethics approval

The Danish Patients Safety Authority has approved the collection of data from the hospital records (ref. no.: 3-3013-2554/1).

Results

A total of 436 patients with a VREfm/VVEfm index isolate detected at OUH were included; 285 (65.4%) were detected in the intervention period and 151 (34.6%) in the post-intervention period (Table I).

A total of 471,975 blood cultures were obtained at OUH in the periods; 38,881 (8.2%) with bacterial growth, 2135 with *E. faecium* (929 patients), and 105 with VREfm/VVEfm (47 patients; 35 with an index isolate at OUH).

Table I
Clinical and microbiological characteristics for VREfm/VVEfm index isolate patients at Odense University Hospital in the intervention period (2015–2021) vs post-intervention period (2022) ($N = 436$)

Variable	Total	Intervention 2015–21	Post-intervention 2022
No. of patients	436	285	151
Van gene			
vanA	59 (13.5%)	54 (18.9%)	5 (3.3%)
vanB	169 (38.8%)	43 (15.0%)	126 (83.4%)
vanAXd	206 (47.2%)	186 (65.2%)	20 (13.2%)
vanA + *vanB*	2 (0.5%)	2 (0.7%)	0
Sex			
Men	203 (46.6%)	134 (47%)	69 (45.7%)
Age (years)			
<18	3 (0.7%)	1 (0.3%)	2 (1.3%)
≥18	433 (99.3%)	284 (99.7%)	149 (98.7%)
Mean	72.75	73.1	72.1
Median (interval)	75 (0; 99)	75 (0; 99)	75 (11; 96)
Place of detection			
General practitioner	46 (10.6%)	28 (9.8%)	18 (11.9%)
Hospital	390 (89.4%)	257 (90.2%)	133 (88.1%)
Intensive care units	56 (14.4%)[a]	46 (17.9%)	10 (7.5%)
Internal medicine: total	178 (45.6%)	115 (44.7%)	63 (47.4%)
Abdominal	16 (4.1%)	9 (3.5%)	7 (5.2%)
Nephrology	24 (6.1%)	15 (5.8%)	9 (6.8%)
Haematology/oncology	49 (12.5%)	33 (12.8%)	16 (12.0%)
Other	89 (22.8%)	58 (22.5%)	31 (23.3%)
Surgery: total	89 (22.8%)	57 (22.2%)	32 (24.0%)
Abdominal	25 (6.4%)	16 (6.2%)	9 (6.8%)
Urology	26 (6.7%)	18 (7.0%)	8 (6.0%)
Orthopaedic/plastic/wound	38 (9.7%)	23 (8.9%)	15 (11.3%)
Paediatric	1 (0.2%)	1 (0.4%)	0
Other	66 (16.9%)	38 (14.8%)	28 (21.1%)
Specimen			
Blood culture	35 (8.0%)	27 (9.5%)	8 (5.3%)
Urine	335 (76.8%)	212 (74.4%)	123 (81.5%)
Abdominal fluid	29 (6.7%)	21 (7.4%)	8 (5.3%)
Skin/soft tissue/bone/visceral	24 (5.5%)	14 (4.9%)	10 (6.6%)
Other, e.g. sputum	13 (3.0%)	11 (3.9%)	2 (1.3%)
Patients with positive VREfm/VVEfm blood culture within 30 days, excluding index blood VREfm/VVEfm isolates	12 (2.8%)	12 (4.2%)	0
Microbiological culture results			
VREfm/VVEfm mono-microbial	256 (58.7%)	167 (58.6%)	89 (58.9%)
Polymicrobial total	180 (41.3%)	118 (41.4%)	62 (41.1%)
Enterobacterales	66 (36.7%)[b]	40 (33.9%)	26 (41.9%)
Non-fermentative Gram-negative rods	31 (17.2%)	21 (17.8%)	10 (16.1%)
Gram-positive, catalase-negative cocci	16 (8.9%)	14 (11.9%)	2 (3.2%)
Staphylococcus aureus	4 (2.2%)	3 (2.5%)	1 (1.6%)
Coagulase-negative staphylococci	15 (8.3%)	10 (8.5%)	5 (8.1%)
Yeast	55 (30.6%)	36 (30.5%)	19 (30.6%)
Anaerobe	9 (5.0%)	6 (5.1%)	3 (4.8%)
Other	9 (5.0%)	5 (4.2%)	4 (6.5%)
VREfm/VVEfm active antibiotic treatment initiated ≤7 days from the index sample			
No	378 (86.7%)	248 (87%)	130 (86.1%)
Yes	33 (7.6%)	28 (9.8%)	5 (3.3%)
Unknown	25 (5.7%)	9 (3.2%)	16 (10.6%)
VREfm/VVEfm active antibiotic treatment length (days), median (range)			
Total	4 (1; 28)	3.5 (1; 28)	10 (4; 14)
	$N = 33$	$N = 28$	$N = 5$

(*continued on next page*)

Table I (*continued*)

Variable	Total	Intervention 2015–21	Post-intervention 2022
Blood cultures	6 (1; 17) $N = 18$	3 (1; 17) $N = 13$	10 (4; 14) $N = 5$
Urine samples	2.5 (1; 17) $N = 8$	2.5 (1; 17) $N = 8$	–
Abdominal samples	11 (4; 17) $N = 3$	11 (4; 17) $N = 3$	–
Catheter present at the anatomical location of the positive VREfm/VVEfm sample, and removal/change ≤7 days after the index sample			
Yes: total	194 (44.5%)	131 (46.0%)	63 (41.7%)
Removal/change ≤7 days	100 (51.5%)	62 (47.0%)	38 (60.3%)
Arterial and/or intravenous	20 (57.1%)[c]	15 (55.6%)	5 (62.5%)
Removal/change ≤7 days	17 (85%)[d]	12 (80%)	5 (100%)
Urinary tract	150 (44.8%)[c]	98 (46.2%)	52 (42.3)
Removal/change ≤7 days	76 (50.7%)[d]	47 (48%)	29 (55.8%)
Abdominal	24 (82.8%)[c]	18 (85.7%)	6 (75.0%)
Removal/change ≤7 days	7 (29.2%)[d]	3 (16.7%)	4 (66.7%)
30-day mortality and cause of death ≤30 days from the index sample			
Dead ≤30 days	97 (22.2%)	65 (22.8%)	32 (21.2%)
Likely dead due to VREfm/VVEFm	4 (4.1%)	4 (6.2%)	0
Possibly dead due to VREfm/VVEFm	7 (7.2%)	1 (1.5%)	6 (18.8%)
Unlikely dead due to VREfm/VVEFm	82 (84.5%)	57 (87.7%)	25 (78.1%)
Unknown dead due to VREfm/VVEFm	4 (4.1%)	3 (4.6%)	1 (3.1%)

VREfm/VVEfm vancomycin-resistant/vancomycin-variable *Enterococcus faecium*.
[a] Percent of hospital isolates.
[b] Percent of the number of polymicrobial samples.
[c] Percent of the number of the equivalent VREfm/VVEfm specimens.
[d] Percent of the total number of patients with a catheter present at the anatomical location for the positive VREfm/VVEfm sample.

Differences in patient, clinical, and microbiological characteristics between the two periods were, with few exceptions, minor (Table I). Men:women ratios were identical: 0.87 for all cases, and 1.9 for bacteraemia cases only. Distribution of departments did not differ between the two periods, except for intensive care units (ICUs) with 46 (17.9%) patients in the intervention period vs 10 (7.5%) in the post-intervention period ($P < 0.01$).

Of the 35 patients with a blood index sample, 12 (34.3%) were treated at the ICU, and eight (22.9%) at the Departments of Haematology/Oncology, while the rest were treated in a variety of other departments.

Overall 30-day mortality was 22.2% ($N = 97$) and there was no significant difference ($P = 0.70$) between the periods (Table I and Figure 1). VREfm/VVEfm was the 'likely' cause of death in 4.1% of the patients ($N = 4$). Of the 35 bacteraemia patients, 16 (45.7%) died within 30 days, and in two patients (12.5%), VREfm/VVEfm was the 'likely' cause of death.

VRE/VVEfm active antibiotic treatment was associated with increased 30-day mortality ($P < 0.01$). Thirteen (39%) of the 33 treated patients died within 30 days, whereas 83 (22%) of the 378 patients not treated died. Nine of the 18 treated bacteraemia patients died within 30 days.

Thirty-day mortality was not related to the presence, removal, or replacement of intra-abdominal or intra-vascular catheters. Patients with VREfm/VVEfm in the urine had a higher 30-day mortality ($P < 0.01$) if a urinary tract catheter was present. The mortality was not related to change or removal of the urinary tract catheter.

There was no difference in the number of polymicrobial samples in the two periods. Yeast was detected in 18.6% and Enterobacterales spp. in 13.4% of patients who died within 30 days.

The distributions of *van* genes in the two periods were significantly different ($P < 0.01$). *vanA* (18.9%) and *vanAXd* (65.2%) dominated the intervention period, whereas *vanB* (83.4%) dominated the post-intervention period. In addition, there was a shift from *vanA* to *vanAXd* and *vanB* within the intervention period (Table I and Figure 2). The distribution of characteristics was therefore assessed in relation to the two periods for each *van* gene separately (Table II). Most of the numbers in these groups were too small for meaningful statistical assessment, but there were no conspicuous differences between the periods or *van* genes when focusing on percentages for the larger numbers. The multivariate analyses corroborated these results as only the *van* genes differed between the two periods (OR: 0.04, 95% CI: 0.01–0.10 for *vanA*; 0.03, 0.02–0.06 for *vanAXd*) whereas there were no differences between any of the wards, including ICUs.

Whole-genome sequencing was performed on 74.1% ($N = 323$) of the isolates. Types and distribution during the years can be found in Supplementary Table A1.

To account for possible heterogeneity in the seven-year intervention period, the last year (2021) of the intervention was compared with the post-intervention period (2022) (Supplementary Table A2). No significant difference was detected in any parameter between the two periods except for *van*-gene types ($P < 0.01$).

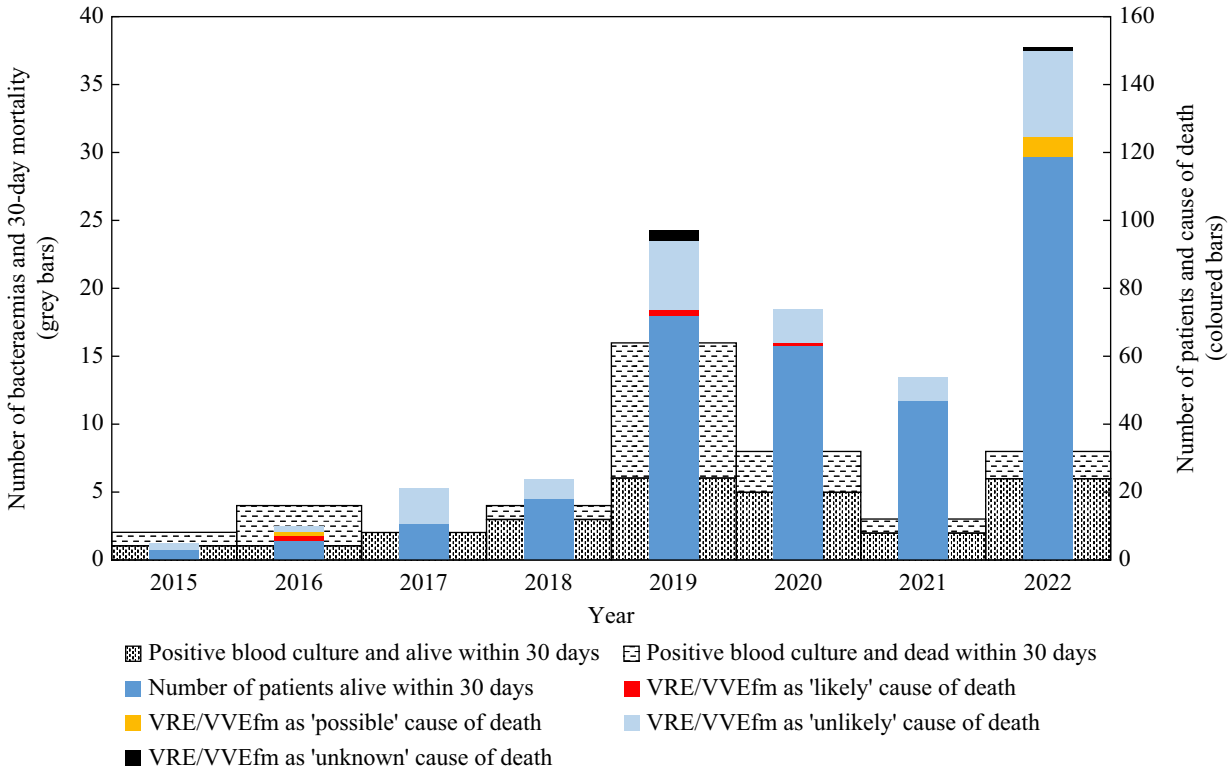


Figure 1. Distribution of 30-day mortality and vancomycin-resistant/vancomycin-variable *Enterococcus faecium* (VREfm/VVEfm) as the cause of death for VREfm/VVEfm index isolate patients, at Odense University Hospital in the period 2015–22 ($N = 436$).

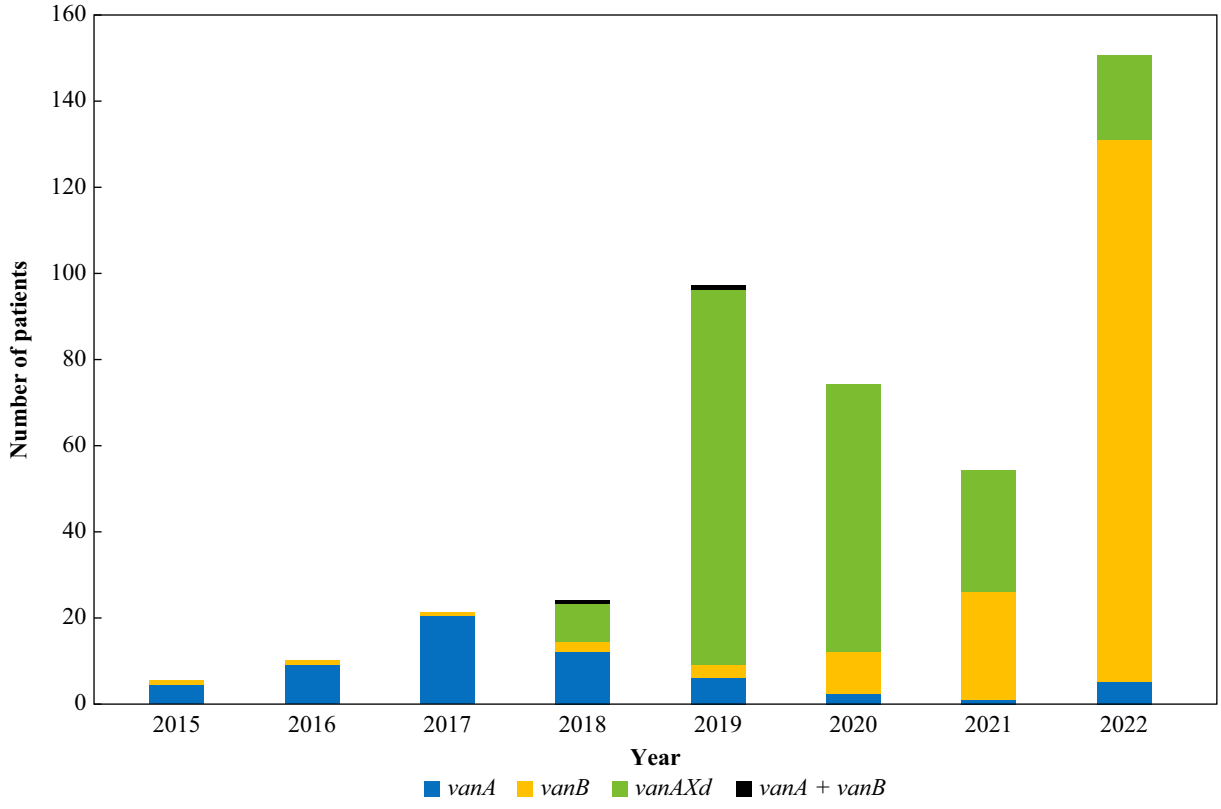


Figure 2. Distribution of *van* genes in vancomycin-resistant/vancomycin-variable *Enterococcus faecium* (VREfm/VVEfm) index isolates detected at Odense University Hospital in the period 2015–22 ($N = 436$).

Table II
Association between clinical, microbiological information, and *van* genes detected in the VREfm/VVEfm index isolates at Odense University Hospital in the period 2015–21 vs 2022 ($N = 436$)

Variable	*vanA*		*vanB*		*vanAXd*	
	2015–21	2022	2015–21	2022	2015–21	2022
Total no.[a]	54	5	43	126	186	20
Specimen						
Blood culture	5 (9.3%)	0	4 (9.3%)	7 (5.6%)	18 (9.7%)	1 (5%)
Urine	35 (64.8%)	4 (80%)	29 (67.4%)	101 (80.2%)	147 (79%)	18 (90%)
Abdominal fluid	9 (16.7%)	1 (20%)	5 (11.6%)	7 (5.6%)	6 (3.2%)	0
Skin/soft tissue/bone/visceral	2 (3.7%)	0	4 (9.3%)	9 (7.1%)	8 (4.3%)	1 (5%)
Other, e.g. sputum	3 (5.6%)	0	1 (2.3%)	2 (1.6%)	7 (3.8%)	0
Microbiological culture results						
VREfm/VVEfm monomicrobial	30 (55.6%)	4 (80%)	23 (53.5%)	71 (56.3%)	114 (61.3%)	14 (70%)
Polymicrobial total	24 (44.4%)	1 (20%)	20 (46.5%)	55 (43.7%)	72 (38.7%)	6 (30%)
Enterobacterales	6 (25%)[b]	0	7 (35%)	24 (43.6%)	27 (37.5%)	2 (33.3%)
Non-fermentative Gram-negative rods	3 (12.5%)	1 (100%)	4 (20%)	8 (14.5%)	14 (19.4%)	1 (16.7%)
Gram-positive, catalase-negative cocci	1 (4.2%)	0	2 (10%)	2 (3.6%)	11 (15.3%)	0
Staphylococcus aureus	0	0	2 (10%)	1 (1.8%)	1 (1.4%)	0
Coagulase-negative staphylococci	5 (20.8%)	0	1 (5%)	5 (9.1%)	4 (5.6%)	0
Yeast	9 (37.5%)	0	4 (20%)	15 (27.3%)	23 (31.9%)	4 (66.7%)
Anaerobe	1 (4.2%)	0	1 (5%)	3 (5.5%)	4 (5.6%)	0
Other	0	0	3 (15%)	4 (7.3%)	2 (2.8%)	0
VREfm/VVEfm active antibiotic treatment initiated ≤7 days from the index sample						
No	45 (83.3%)	5 (100%)	39 (90.7%)	107 (84.9%)	162 (87.1%)	18 (90%)
Yes	9 (16.7%)	0	4 (9.3%)	5 (4%)	15 (8.1%)	0
Unknown	0	0	0	14 (11.1%)	9 (4.8%)	2 (10%)
Median (range) VREfm/VVEfm active antibiotic treatment length in days						
Total	3 (1; 18) $N = 9$	–	6.5 (1; 11) $N = 4$	10 (4; 14) $N = 5$	2 (1; 28) $N = 15$	–
Blood cultures	8 (3; 10) $N = 3$	–	5.5 (1; 10) $N = 2$	10 (4; 14) $N = 5$	1.5 (1; 17) $N = 8$	–
Urine samples	2 (1; 4) $N = 4$	–	3 $N = 1$	–	2 (1; 17) $N = 3$	–
Abdominal samples	4 $N = 1$	–	11 $N = 1$	–	17 $N = 1$	–
Catheter present at the anatomical location of the positive VREfm/VVEfm sample, and removal/change ≤7 days after the index sample						
Yes: total	31 (57.4%)	1 (20%)	18 (41.9%)	51 (40.5%)	81 (43.5%)	11 (55%)
Removal/change ≤7 days	15 (48.4%)	0	10 (55.6%)	31 (60.8%)	37 (45.7%)	7 (63.6%)
Arterial/intravenous	3 (60%)[c]	0	2 (50%)	4 (57.1%)	10 (55.6%)	1 (100%)
Removal/change ≤7 days	1 (33.3%)[d]	0	2 (100%)	4 (100%)	9 (90%)	1 (100%)
Urinary tract	20 (57.1%)[c]	1 (25%)	11 (37.9%)	41 (40.6%)	67 (45.6%)	10 (55.6%)
Removal/change ≤7 days	14 (70%)[d]	0	5 (45.5%)	23 (56.1%)	28 (41.8%)	6 (60%)
Abdominal	8 (88.9%)[c]	0	5 (100%)	6 (85.7%)	4 (66.7%)	0
Removal/change ≤7 days	0	0	3 (60%)	4 (66.7%)	0	0
30-day mortality and cause of death ≤30 days from the index sample						
Alive ≤30 days	31 (57.4%)	1 (20%)	38 (88.4%)	102 (81%)	149 (80.1%)	16 (80%)
Dead ≤30 days	23 (42.6%)	4 (80%)	5 (11.6%)	24 (19%)	37 (19.9%)	4 (20%)
Likely dead due to VREfm/VVEfm	1 (4.3%)	0	0	0	3 (8.1%)	0
Possibly dead due to VREfm/VVEfm	1 (4.3%)	1 (25%)	0	3 (12.5%)	0	2 (50%)
Unlikely dead due to VREfm/VVEfm	20 (87%)	3 (75%)	5 (100%)	20 (83.3%)	32 (86.5%)	2 (50%)
Unknown dead due to VREfm/VVEfm	1 (4.3%)	0	0	1 (4.2%)	2 (5.4%)	0

VREfm/VVEfm vancomycin-resistant/vancomycin-variable *Enterococcus faecium*.
[a] The two isolates containing a *vanA* and a *vanB* gene are not included in the table.
[b] Percent of the number of polymicrobial samples.
[c] Percent of the number of the equivalent VREfm/VVEfm specimen.
[d] Percent of the total number of patients with a catheter present at the anatomical location for the positive VREfm/VVEfm sample.

At all four hospitals, from 2015 to 2022, the number of admissions decreased, whereas both the total number of obtained blood cultures and the number of patients who had at least one blood culture taken increased.

A total of 20 blood index isolates was included from non-OUH. During the entire period, at OUH, there was an overall increase in the number of index isolates per 10,000 blood-cultured patients (Figure 3). The numbers were small, but the number of blood index isolates per 10,000 blood-cultured patients seemed to be stable since 2019 – both at OUH and non-OUH.

Discussion

There was an increased number of index isolates after ending screening and isolation precautions against VREfm/VVEfm. No differences in age, gender, site of infection, number of bacteraemia cases within 30 days of primary infection, 30-day mortality, death attributable to VREfm/VVEfm, and burden of bacteraemia at hospitals in RSD were detected between the two periods.

There were significant changes in the *van* gene distribution in the investigation period, but no obvious differences in the patient characteristics in relation to each *van* gene separately between the two periods.

The increased number of index isolates in the post-intervention period is in agreement with findings from high-incidence countries ending screening and isolation regimes [13,16,29]. In most studies from high-incidence countries the incidence stabilized within 34 months, but due to our short post-intervention period it is unknown whether this will happen in our low-incidence setting [13]. Measures to contain the COVID-19 pandemic may have reduced the VREfm/VVEfm transmission in the intervention period. The COVID-19 restrictions were partially lifted during the post-intervention period. It is possible that fewer VREfm/VVEfm first-time cases would have been detected in the post-intervention period if only the VREfm/VVEfm precautions had been ended.

As demonstrated in other studies, VREfm/VVEfm bacteraemia was mostly found in men – a finding for which there is still no definitive explanation [30]. However, more women had an index isolate, especially from the urinary tract system. This might be explained by Danish women living longer than men, and by bacteriuria being more common in women and older patients [31,32].

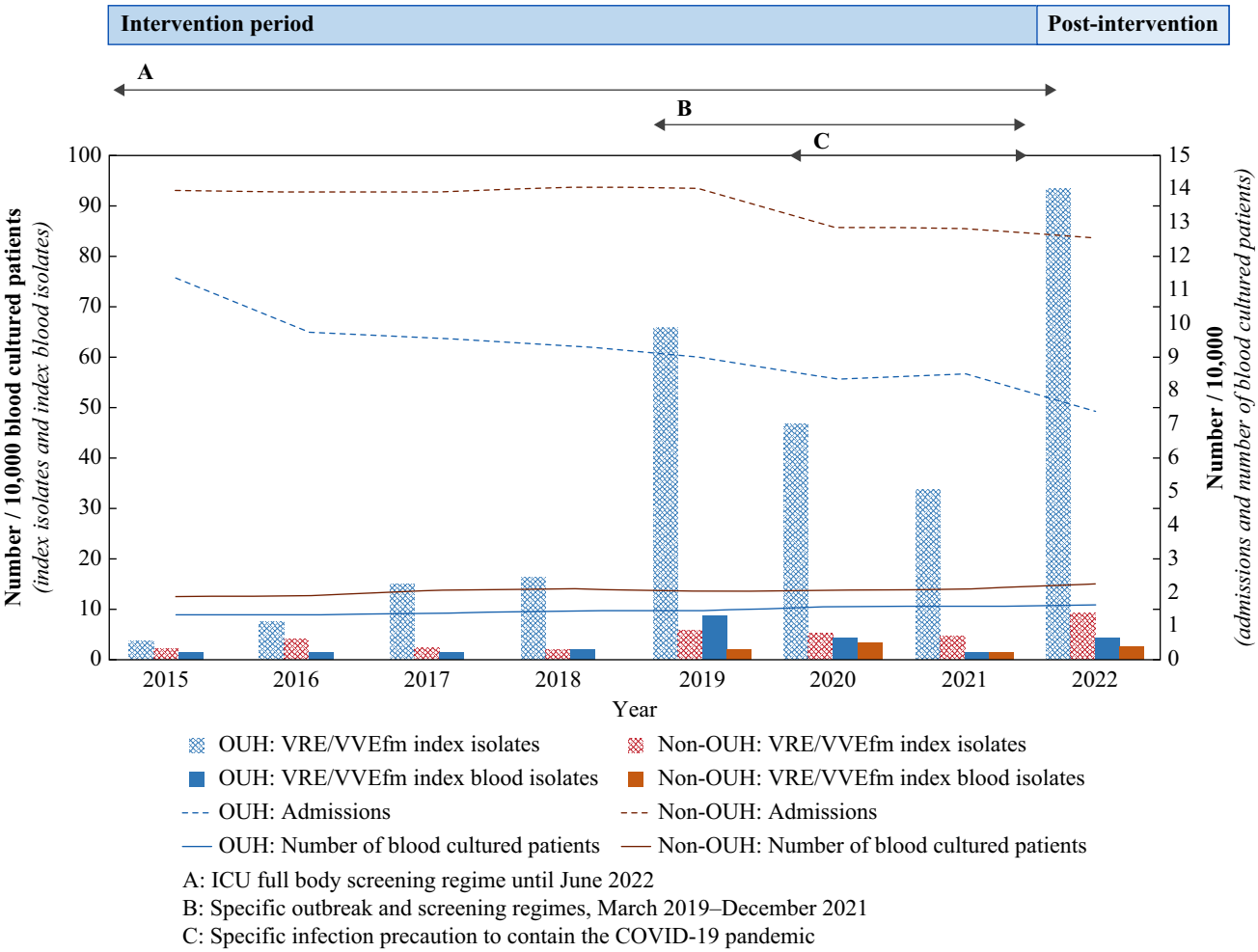


Figure 3. Number of vancomycin-resistant/vancomycin-variable *Enterococcus faecium* (VREfm/VVEfm) index isolates at Odense University Hospital (OUH) (all-case and bacteraemia) and at non-OUH (only bacteraemia) in the period 2015–22, related to number of blood cultured patients and infection control interventions.

The high number of ICU and haematology/oncology inpatients matches earlier findings, and is probably linked to various risk factors, e.g. high age, severe disease, immunosuppression, use of catheters and drains, long duration of hospitalization, and prolonged use of broad-spectrum antibiotics [4,18,22,33,34].

There was a significant reduction in index isolates detected at the ICU in the post-intervention period, which may have been due to ceasing a regimen of full-body microbiological screening three times a week in mid-2022.

In both periods, most samples were from the urinary tract and may reflect the number of colonized patients. The observed non-significant decrease in blood isolates from the intervention to the post-intervention period could be due to the change in the ICU full-body screening as described above or to other uninvestigated factors.

The low number of patients treated with VREfm/VVEfm active antibiotics was comparable to a recent German study [35]. Fewer patients were treated in the post-intervention period, and treatment was mainly given for VREfm/VVEfm bacteraemia and in longer duration. Although there were no changes in the recommended empiric antibiotic regimen at OUH, more patients had their catheters changed without a supplementary antibiotic treatment in the post-intervention period. This practice is supported by earlier findings of recovery taking place without use of antibiotics, but with removal of the infected foreign devices [36].

The higher 30-day mortality in relation to antibiotic treatment is probably due to a higher likelihood of treating critically ill patients. Treatment of VREfm/VVEfm could therefore indicate severe underlying disease and risk of death.

The number of index isolates fell from 2019 to 2021 and rose in 2022. The changes were non-significant, and may be related to a bundle of infection control interventions and their cessation at OUH, and are coincident with the COVID-19 pandemic.

The risk factors mentioned above for patients at ICU and Departments of Haematology/Oncology are associated with both a poor prognosis and an increased risk of being colonized with antibiotic-resistant micro-organisms, that may or may not contribute to the poor outcome [33,34].

The 30-day mortality was high, but VREfm/VVEfm was only the 'likely' cause of death in a few cases. This discrepancy between 30-day mortality and 'likely' cause of death is in accordance with our recent study on VSEfm bacteraemia [23].

For collaborating hospitals, the prevalence of resistant bacteria is affected by carryover from the hospital with the highest prevalence [37]. In all the hospitals in RSD, the number of admissions decreased during the period while the number of patients having a blood culture increased. This could be due to the changes in the Danish Public Health Services, where more and more patients are treated by the general practitioner or as outpatients, and only patients with relatively severe illness are admitted to hospital. Despite this, the number of VREfm/VVEfm first-time bacteraemia cases per 10,000 blood-cultured patients did not increase at non-OUH hospitals.

The strengths of this study are that all clinical cases regardless of sample material were included.

All cases were investigated and evaluated by examination of the hospital records. The same systems and procedures were used for recording data in the before-and-after period. Data were not affected by the hypothesis of this study, as this was unknown at the time of data registration.

One limitation of the study is that routine PCR for detecting the *van* genes at OUH was not introduced until 2018. We therefore used this method retrospectively on stored VREfm/VVEfm isolates. One consequence may be reduced detection of VREfm/VVEfm before 2018. Major limitations were the short duration of the post-intervention period and the small numbers of bacteraemia cases.

The before-and-after study design without a control group in general makes definitive conclusions about causal relationship difficult. The results may not be generalizable to other healthcare settings or populations.

During the last decade, patients admitted to hospital have become older, more comorbid, and more ill. Technological improvements entail more patients receiving advanced treatments and intensive care. They are often treated with broad-spectrum antibiotics resulting in a changed microbiota [38]. Together with a non-normal functioning immune system, this may cause difficulties in isolating the clinical impact of low-pathogenic, resistant bacteria such as VREfm/VVEfm. Treatment and specific infection control interventions against VREfm/VVEfm should be used with caution. It may be more efficient to use efforts to improve adherence to standard precautions and antibiotic stewardship — to reduce not only VREfm/VVEfm, but also other nosocomial pathogens [3,17,39,40].

In conclusion, this study investigated the impact of ending VREfm/VVEfm screening and isolation in a Danish university hospital. The number of patients with a first-time clinical VREfm/VVEfm isolate increased, but we found no changes that could support the need for reintroducing screening and isolation. The follow-up period was short and the development must be monitored closely in the years to come. Further research on the consequences of and need for continued screening and isolation in low-incidence countries is highly relevant.

Acknowledgements

Thanks to A.M. Hammerum and Louise Roer at the Reference Laboratory, Statens Serum Institut (SSI), Denmark for providing us with the whole-genome sequencing data.

Author contributions

Conceptualization, methodology: S.G.K.H., K.O.G., M.N.S., A.H., F.S.R.; software: S.G.K.H., K.G.O., C.O., M.C., R.D., F.S.R.; data collection: S.G.K.H., K.K., A.N., L.A., K.O.G., J.L.-T., C.O., M.C., R.D., F.S.R.; data curation, formal analysis, validation, interpretation, visualization: S.G.K.H., K.O.G., F.S.R.; investigation: F.S.R.; project administration, funding acquisition, resources, supervision: S.G.K.H., M.N.S., A.H., F.S.R.; writing — original draft: S.G.K.H., K.O.G., F.S.R.; writing — review and editing: S.G.K.H., K.K., L.A., K.O.G., J.L.-T, C.O., M.C., R.D., M.N.S., A.H., F.S.R.

Conflict of interest statement

None declared.

Funding sources

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2024.01.019.

References

[1] Agudelo Higuita NI, Huycke MM. Enterococcal disease, epidemiology, and implications for treatment. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. Enterococci: from commensals to leading causes of drug resistant infection [Internet]. Boston: Massachusetts Eye and Ear Infirmary; 2014.

[2] Fiore E, Van Tyne D, Gilmore MS. Pathogenicity of enterococci. Microbiol Spectr 2019;7.

[3] Zhou X, Willems RJL, Friedrich AW, Rossen JWA, Bathoorn E. *Enterococcus faecium*: from microbiological insights to practical recommendations for infection control and diagnostics. Antimicrob Resist Infect Control 2020;9:130.

[4] Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol 2012;10:266—78.

[5] DANMAP. Reports. n.d. Available at: https://www.danmap.org/reports [last accessed November 2022].

[6] Hammerum AM, Justesen US, Pinholt M, Roer L, Kaya H, Worning P, et al. Surveillance of vancomycin-resistant enterococci reveals shift in dominating clones and national spread of a vancomycin-variable vanA *Enterococcus faecium* ST1421-CT1134 clone, Denmark, 2015 to March 2019. Eurosurveillance 2019;24:1.

[7] Skjøt-Arkil H, Mogensen CB, Lassen AT, Johansen IS, Chen M, Petersen P, et al. Carrier prevalence and risk factors for colonisation of multiresistant bacteria in Danish emergency departments: a cross-sectional survey. BMJ Open 2019;9.

[8] Birgand G, Moore LSP, Bourigault C, Vella V, Lepelletier D, Holmes AH, et al. Measures to eradicate multidrug-resistant organism outbreaks: how much do they cost? Clin Microbiol Infect 2016;22:162.e1. —162.e9.

[9] European Centre for Disease Prevention and Control. Economic evaluations of interventions to prevent healthcare-associated infections. 2017. https://doi.org/10.2900/4617. Literature review.

[10] Evans HL, Shaffer MM, Hughes MG, Smith RL, Chong TW, Raymond DP, et al. Contact isolation in surgical patients: a barrier to care? Surgery 2003;134:180—8.

[11] Martin EM, Bryant B, Grogan TR, Rubin ZA, Russell DL, Elashoff D, et al. Noninfectious hospital adverse events decline after elimination of contact precautions for MRSA and VRE. Infect Control Hosp Epidemiol 2018;39:788.

[12] Stelfox HT, Bates DW, Redelmeier DA. Safety of patients isolated for infection control. JAMA 2003;290:1899—905.

[13] Popiel KY, Miller MA. Evaluation of vancomycin-resistant enterococci (VRE)-associated morbidity following relaxation of VRE screening and isolation precautions in a tertiary care hospital. Infect Control Hosp Epidemiol 2014;35:818—25.

[14] Martin EM, Colaianne B, Bridge C, Bilderback A, Tanner C, Wagester S, et al. Discontinuing MRSA and VRE contact precautions: defining hospital characteristics and infection prevention practices predicting safe de-escalation. Infect Control Hosp Epidemiol 2022;43.

[15] Marra AR, Edmond MB, Schweizer ML, Ryan MPHGW, Diekema DJ. Discontinuing contact precautions for multidrug-resistant organisms: a systematic literature review and meta-analysis. Am J Infect Control 2018;46:333—73.

[16] Lemieux C, Gardam M, Evans G, John M, Suh KN, Vanwalraven C, et al. Longitudinal multicenter analysis of outcomes after cessation of control measures for vancomycin-resistant enterococci. Infect Control Hosp Epidemiol 2017;38:24—30.

[17] Kleyman R, Cupril-Nilson S, Robinson K, Thakore S, Haq F, Chen L, et al. Does the removal of contact precautions for MRSA and VRE infected patients change health care-associated infection rate?: a systematic review and meta-analysis. Am J Infect Control 2021;49:784—91.

[18] Bright M, Parfitt EC, Pasquill K, Steele L, Laupland KB. Occurrence and determinants of enterococcal bloodstream infections: a population-based study. Infect Dis (Auckl) 2020;52:638—43.

[19] Ali GA, Goravey W, Najim MS, Shunnar KM, Ibrahim SI, Daghfal J, et al. Epidemiology, microbiological and clinical characteristics of *Enterococcus* species bloodstream infections: a 10-year retrospective cohort study from Qatar. Ann Med Surg 2022;80:104258.

[20] Prematunge C, MacDougall C, Johnstone J, Adomako K, Lam F, Robertson J, et al. VRE and VSE bacteremia outcomes in the era of effective VRE therapy: a systematic review and meta-analysis. Infect Control Hosp Epidemiol 2016;37:26—35.

[21] Rottier WC, Arpi M, Blank SN, Nabuurs-Franssen MH, Ruijs GJHM, Tersmette M, et al. Attributable mortality of vancomycin resistance in ampicillin-resistant *Enterococcus faecium* bacteremia in Denmark and the Netherlands: a matched cohort study. Infect Control Hosp Epidemiol 2022;43:719—27.

[22] López-Luis BA, Sifuentes-Osornio J, Lambraño-Castillo D, Ortiz-Brizuela E, Ramírez-Fontes A, Tovar-Calderón YE, et al. Risk factors and outcomes associated with vancomycin-resistant *Enterococcus faecium* and ampicillin-resistant *Enterococcus faecalis* bacteraemia: a 10-year study in a tertiary-care centre in Mexico City. J Glob Antimicrob Resist 2021;24:198—204.

[23] Hansen SGK, Roer L, Karstensen KT, Hoegh SV, Hansen F, Klein K, et al. Vancomycin-sensitive *Enterococcus faecium* bacteraemia — hospital transmission and mortality in a Danish University Hospital. J Med Microbiol 2023:72.

[24] Kjær Hansen S, Andersen L, Detlefsen M, Holm A, Roer L, Antoniadis P, et al. Using core genome multilocus sequence typing (cgMLST) for vancomycin-resistant *Enterococcus faecium* isolates to guide infection control interventions and end an outbreak. J Glob Antimicrob Resist 2021;24:418—23.

[25] Schmidt M, Pedersen L, Sørensen HT. The Danish Civil Registration System as a tool in epidemiology. Eur J Epidemiol 2014;29:541—9.

[26] Voldstedlund M, Haarh M, Mølbak K. The Danish microbiology database (MIBA) 2010 to 2013. Eurosurveillance 2014;19:20667.

[27] Møller JK. A microcomputer-assisted bacteriology reporting and information system. Acta Pathol Microbiol Immunol Scand B 1984;92:119—26.

[28] Systematic.com. Systematic Columna CIS. n.d. Available at: https://systematic.com/en-gb/industries/healthcare/hospital-care/columna-cis/#6af214b4-6126-44ff-b38f-6b70094cc97c [last accessed June 2023].

[29] Cho SY, Kim HM, Chung DR, Choi JR, Lee MA, Huh HJ, et al. The impact of vancomycin-resistant *Enterococcus* (VRE) screening policy change on the incidence of healthcare-associated VRE bacteremia. Infect Control Hosp Epidemiol 2022;43:603—8.

[30] Correa-Martínez CL, Schuler F, Kampmeier S. Sex differences in vancomycin-resistant enterococci bloodstream infections — a systematic review and meta-analysis. Biol Sex Differ 2021:12.

[31] Nicolle LE. Asymptomatic bacteriuria and bacterial interference. Microbiol Spectr 2015;3(5).

[32] Statistics Denmark. Life expectancy. n.d. Available at: https://www.dst.dk/en/Statistik/emner/borgere/befolkning/middellevetid [last accessed July 2023].

[33] Nørgaard M, Larsson H, Pedersen G, Schønheyder HC, Rothman KJ, Sørensen HT. Short-term mortality of bacteraemia in elderly patients with haematological malignancies. Br J Haematol 2006;132:25—31.

[34] Nørgaard M, Larsson H, Pedersen G, Schønheyder HC, Sørensen HT. Haematological malignancies — a predictor of a poor outcome in patients with bacteraemia. J Infect 2006;53:190—8.

[35] Bui MT, Rohde AM, Schwab F, Märtin N, Kipnis M, Boldt A-C, et al. Prevalence and risk factors of colonisation with vancomycin-resistant *Enterococci faecium* upon admission to Germany's largest university hospital. GMS Hyg Infect Control 2021;16:Doc06.

[36] Linden PK. Optimizing therapy for vancomycin-resistant enterococci (VRE). Semin Respir Crit Care Med 2007;28:632—45.
[37] Donker T, Henderson KL, Hopkins KL, Dodgson AR, Thomas S, Crook DW, et al. The relative importance of large problems far away versus small problems closer to home: insights into limiting the spread of antimicrobial resistance in England. BMC Med 2017;15:86.
[38] Remschmidt C, Schröder C, Behnke M, Gastmeier P, Geffers C, Kramer TS. Continuous increase of vancomycin resistance in enterococci causing nosocomial infections in Germany — 10 years of surveillance. Antimicrob Resist Infect Control 2018;7:54.
[39] Leigh RJ, McKenna C, McWade R, Lynch B, Walsh F. Comparative genomics and pangenomics of vancomycin resistant and susceptible *Enterococcus faecium* from Irish hospitals across 20 years. J Med Microbiol 2022;71:10.
[40] Wilson J, Prieto J. Re-visiting contact precautions — 25 years on. J Infect Prev 2021;22:242—4.

Paper III - Supplementary

Supplementary Appendix

Whole-genome-sequencing was performed on 323 (74.1%) of the 436 index isolates by the Danish National Reference Center, Statens Serum Institut (SSI), Denmark. The MLST and cgMLST results were distributed to the DCM, OUH.

The distribution can be found in Supplementary Table A1.

Supplementary Table A1

Distribution list of STs, CTs and van-genes for VREfm/VVEfm index isolates in the period 2015-2022 at Odense University Hospital, Denmark (n=323)

CT cluster group	MLST	cgMLST (no. of isolates)	Van-genes	Year								
ST-CT	ST	CT		2015 (n)	2016 (n)	2017 (n)	2018 (n)	2019 (n)	2020 (n)	2021 (n)	2022 (n)	Total (n)
	ST17	CT496	*vanA, vanB*				1				2	3
		CT7023	*vanB*								1	1
		CT7086	*vanA, vanB*								2	2
		CT7140	*vanB*								2	2
		CT7201	*vanB*								1	1
	ST18	CT1584	*vanA*			1						1
	ST78	CT1438	*vanA*	1								1
	ST80	CT14	*vanA*	1	1							2
		CT32	*vanB*			1						1
		CT866	*vanA*	1								1
		CT880	*vanB*	1	1							2
		CT993	*vanA*		7	7	3					17
		CT1064	*vanA + vanB*				1	1				2
		CT1160	*vanA, vanAXd*							1	1	2
		CT1508	*vanA*			1						1
		CT1545	*vanA*			1						1
		CT1830	*vanB*								1	1
		CT2309	*vanA*				1					1
ST80-CT2406		CT2406 (17) CT2946 (5) CT2949 (3) CT3234 (35) CT6254 (13) CT6435 (1) CT6598 (1) CT7030 (2)	*vanB*						3	10	64	77
		CT2414	*vanA*					1				1
		CT2680	*vanB*								1	1
		CT2840	*vanA*					1				1
		CT5180	*vanB*								2	2
		CT5191	*vanAXd*					1				1
		CT7035	*vanB*								1	1
		CT7124	*vanB*								1	1
		CT7141	*vanB*								1	1
		CT7200	*vanB*								1	1
		CT7206	*vanB*								1	1
		CT7497	*vanB*								1	1
	ST117	CT24	*vanA*	1								1
		CT36	*vanB*				1	2	1			4
		CT71	*vanB*				1		2			3
		CT991	*vanB*					1	1			2
		CT1180	*vanA, vanB*			1		2	1	1	1	6
		CT1182	*vanA*			1	1					2
		CT1686	*vanB*								12	12
		CT2456	*vanAXd, vanB*								5	5
		CT2531	*vanB*								1	1
		CT5113	*vanB*						1	2		3
		CT7145	*vanB*								1	1
	ST203	CT550	*vanA*							1		1
		CT859	*vanA*		1	4	6	1				12
		CT1143	*vanA*			1						1
		CT1144	*vanA*			1						1
		CT1800	*vanAXd*								1	1
	ST316	CT6506	*vanA*								2	2
	ST612	CT1026	*vanA*						1			1
	ST1350	CT6999	*vanB*								1	1

	ST1421	CT1134	*vanAXd*				9	66	23	15	13	126
		CT5189	*vanAXd*							1		1
		CT6046	*vanAXd*							1		1
		CT6548	*vanAXd*								3	3
	ST1424	CT6743	*vanB*								1	1

Supplementary Table A2

Clinical and microbiological characteristics for VREfm/VVEfm index isolate patients, in the last year of the intervention period (2021) vs. post-intervention period (2022) (n=205)

	Total[1]	Intervention Year 2021	Post-intervention Year 2022
Number of patients	205	54	151
Van-gene			
vanA	6 (2.9)[1]	1 (1.9)	5 (3.3)
vanB	151 (73.7)	25 (46.3)	126 (83.4)
vanAXd	48 (23.4)	28 (51.9)	20 (13.2)
vanA+ vanB	0	0	0
Sex			
Men, n (%)	94 (45.9)	25 (46.3)	69 (45.7)
Age			
- < 18 years	2 (1)	0	2 (1.3)
- ≥ 18 years	203 (99)	25 (100)	149 (98.7)
- Mean, years	73.2	74.2	72.1
- median [interval], years	75 [11;96]	75 [47;94]	75 [11;96]
Place of detection			
General practitioner	27 (13.2)	9 (16.7)	18 (11.9)
Hospital	178 (86.8)	45 (83.3)	133 (88.1)
- Intensive care units	16 (9)[2]	6 (13.3)	10 (7.5)
- Internal medicine - total	86 (48.3)	23 (51.1)	63 (47.4)
- Abdominal	9 (5.1)	2 (4.4)	7 (5.2)
- Nephrology	17 (9.6)	8 (17.8)	9 (6.8)
- Haematology/oncology	23 (12.9)	7 (15.6)	16 (12)
- Other	37 (20.8)	6 (13.3)	31 (23.3)
- Surgery - total	41 (23)	9 (20)	32 (24)
- Abdominal	10 (5.6)	1 (2.2)	9 (6.8)
- Urology	10 (5.6)	2 (4.4)	8 (6)
- Orthopaedic/Plastic/Wound	21 (11.8)	6 (13.3)	15 (11.3)
- Paediatric	0	0	0
- Other	35 (19.7)	7 (15.6)	28 (21.1)
Specimen			
Blood culture	10 (4.9)	2 (3.7)	8 (5.3)
Urine	167 (81.5)	44 (81.5)	123 (81.5)
Abdominal fluid	12 (5.9)	4 (7.4)	8 (5.3)
Skin/soft tissue/bone/visceral	14 (6.8)	4 (7.4)	10 (6.6)
Other e.g., sputum	2 (1)	0	2 (1.3)
Patients with positive VREfm/VVEfm blood culture within 30 days, excluding index blood VREfm/VVEfm isolates			
	1	1	0
Microbiological culture results			
VREfm/VVEfm mono-microbial	121 (59)	32 (59.3)	89 (58.9)
Polymicrobial total	84 (41)	22 (40.7)	62 (41.1)
- *Enterobacteriales*	37 (44)[3]	11 (50)	26 (41.9)
- Non-fermentative Gram-negative rods	14 (16.7)	4 (18.2)	10 (16.1)
- Gram-positive, catalase-negative cocci	4 (4.8)	2 (9.1)	2 (3.2)
- *Staphylococcus aureus*	2 (2.4)	1 (4.5)	1 (1.6)
- Coagulase-negative staphylococci	4 (4.8)	1 (4.5)	5 (8.1)
- Yeast	24 (28.6)	5 (22.7)	19 (30.6)
- Anaerobe	4 (4.8)	1 (4.5)	3 (4.8)
- Other	6 (7.1)	2 (9.1)	4 (6.5)
VREfm/VVEfm active antibiotic treatment initiated ≤ 7 days from the index sample			
No	177 (86.3)	47 (87)	130 (86.1)
Yes	9 (4.4)	4 (7.4)	5 (3.3)
Unknown	19 (9.2)	3 (5.6)	16 (10.6)
Median [range] VREfm/VVEfm active antibiotic treatment length in days			
Total	9 [1;17], n=9	2 [1;17], n=4	10 [4;14], n=5
Blood cultures[3]	9.5 [1;14], n=6	1, n=1	10 [4;14], n=5

Urine samples[3]	2 [1;3], n=2	2 [1;3], n=2	'-'
Abdominal samples[3]	17, n=1	17, n=1	'-'
Catheter present at the anatomical location of the positive VREfm/VVEfm sample, and removal/change ≤7 days after the index sample			
Yes - total	83 (40.5)	20 (37)	63 (41.7)
- Removal/change ≤7 days	49(59)	11 (55)	38 (60.3)
Arterial and/or intravenous	7 (70)[4]	2 (100)	5 (62.5)
- Removal/change ≤7 days	7 (100)[5]	2 (100)	5 (100)
Urinary tract	66 (39.5)[4]	14 (31.8)	52 (42.3)
- Removal/change ≤7 days	36 (54.5)[5]	7 (50)	29 (55.8)
Abdominal	10 (83.3)[4]	4 (100)	6 (75)
- Removal/change ≤7 days	6 (60)[5]	2 (50)	4 (66.7)
30-day mortality and cause of death ≤ 30 days from the index sample			
Dead ≤ 30 days	39 (19)	7 (13)	32 (21.2)
- Likely dead due to VREfm/VVEfm within 30 days	0	0	0
- Possible dead due to VREfm/VVEfm within 30 days	6 (15.4)[6]	0	6 (18.8)
- Unlikely dead due to VREfm/VVEfm within 30 days	32 (82.1)	7 (100)	25 (78.1)
- Unknown dead due to VREfm/VVEfm within 30 days	1 (2.6)	0	1 (3.1)

[1] Number (%), unless stated otherwise

[2] Percent of hospital isolates

[3] Percent of the number of polymicrobial samples

[4] Percent of the number of the equivalent VREfm/VVEfm specimen

[5] Percent of the total number of patients with a catheter present at the anatomical location for the positive VREfm/VVEfm sample

[6] Percent of VREfm/VVEfm cases dead within 30 days