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Molecular Typing of Enterococci/VRE

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Abstract

With their increased frequency of occurrence as a nosocomial pathogen and thus their elevated overall medical importance, the demand to characterize and differentiate strains of *Enterococcus faecalis* and *E. faecium* has risen. Available techniques vary in ease of use, demands in costs, manpower and time, inter- and intra-laboratory comparability and reproducibility of results, portability of data and discriminatory power. Analysing outbreaks by sophisticated molecular techniques requires methods with a different discrimination than methods to detect and follow transmission of epidemic strains over longer time periods. The latter is especially critical for bacteria showing a rather flexible genome such as *Enterococcus*. The value and application for commonly used techniques for typing (vancomycin-resistant) enterococci is discussed including ribotyping, PCR-based typing, macrorestriction analysis in Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP), Multiple Locus Variable Number of Tandem Repeat Analyses (MLVA), Multi-Locus Sequence Typing (MLST) and some specialist approaches (resistance cluster typing, plasmid typing, next generation sequencing).

Introduction

Enterococci are known as a bacterial entity since the turn of the 20th century but until the mid 1980s they belonged to the genus *Streptococcus*. With the introduction of molecular techniques the intestinal Lancefield group D streptococci were separated into an individual genus termed *Enterococcus* [1]. Nowadays almost 40 different species belong to this genus [2]. For decades majority of enterococcal infections (about 85%) were caused by *Streptococcus/Enterococcus faecalis* and the residual, up to 15% by *Streptococcus/Enterococcus faecium* [3]. Insofar early characterization and typing approaches targeted mainly *E. faecalis* and its known virulence determinants in relation to the technical possibilities of that time. The so-called Maekawa collection contained 21 serotype *E. faecalis* strains differentiated via the polysaccharide capsule and other surface determinants of *E. faecalis* [4,5]. Isolates of *E. faecium* do not contain a capsule locus and cannot be typed by serological assays. In the last two decades two aspects increased the medical importance of *E. faecium*. First, the selection and spread of various types of vancomycin resistance among enterococci is mainly associated with *E. faecium* and less with *E. faecalis*. Second, the increased medical importance of enterococci as a nosocomial pathogen is mainly due to increasing numbers of (hospital-associated) *E. faecium* and less to *E. faecalis*. Both aspects increased the typing requirements and interest especially for *E. faecium*. To the best of our knowledge today, population biology of *E. faecium* differs from that of *E. faecalis* to a certain extent [6,7]. This requires to be considered when assessing available techniques for strain typing and characterization. Since typing is mainly applied for VRE isolates, typing of *E. faecium* is within the focus of this short overview.

Typing parameters and outcomes are always in relation to the quality of the available strain sample to be analyzed and the corresponding epidemiological context to be addressed. Different questions may require different typing techniques providing different levels of discriminatory power; which means that the highest level of discrimination may not be suitable for more general aspects and broader epidemiological correlations. Additional aspects to be considered concern ease of use/applicability, time of analysis and personal input (number, qualification), costs, reproducibility of data and results (intra and interlab) and routes of data storage, exchange and comparison. Elucidating a local VRE outbreak requires different techniques than following the dissemination of epidemic strain types across the country or across borders and for investigating population biological aspects over longer time periods.

Serotyping

Serotyping is a classical typing method assessing differences in immunologic responses to typable bacteria infecting model hosts and resembling differences in surface structures of investigated micro-organisms. A serotyping scheme on a historical collection of *E. faecalis* strains was proposed by Maekawa and co-workers [4]. Rabbit antisera against a large number of *E. faecalis* strains were raised capable to classify 21 distinct serotype strains and to prepare 21 monospecific typing antisera. Diversity of Maekawa type strains T1-T21 was later on confirmed by MLST and capsule locus analyses [6]. Due to a number of reasons (unknown target; less easy to use; limited discrimination) serotyping has never reached a broader acceptance and is from nowadays perspectives somehow outdated.

Ribotyping

Ribotyping is a classical typing method targeting the ribosomal DNA by hybridization or amplification techniques. It is still widely used for typing of pathogens such as *Clostridium difficile* where it is considered as a reference method (see Table 1 for background information on the corresponding techniques and for references). For enterococcal typing it has its limited use for distinct scenarios [8]. However, the depths of analysis, particularly its discriminatory power is rather limited and thus its application and usefulness for typing enterococci is minor [9,10].

PCR-based typing/rep-PCR typing

The zenith of PCR-based typing goes back to the 1990s when PCR became prominent for many applications including typing of bacteria

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[11]. From a today's perspective amplification-based techniques appear outdated due a number of insuperable limitations and disadvantages coming along with the technique itself, which appear as such in relation to sequence-based techniques (see the following). Major limitations in pattern stability (reproducibility) and resolutionary/discriminatory power are partly overcome by a highly standardized approach via a commercial kit in combination with a highly sensitive and precise capillary-based fragment pattern analysis (DiversiLab®, bioMérieux, Marcy-l'Étoile, France). However, it is recommended by the manufacturer for typing of various bacteria including enterococci in the context of outbreak investigations which has been published recently [12,13]. Nevertheless, its discriminatory power has not been shown unambiguously on a reference strain sample set and its application will still be limited due to specific equipment requirements and comparably high costs.

Macrorestriction Analysis in Pulsed-Field Gel Electrophoresis (PFGE)

Macrorestriction analysis in pulsed-field gel electrophoresis casually called "PFGE typing" was introduced in the early 1990s for bacterial strain typing. It involves a very time-consuming procedure, requires a high technical standard and demands qualified and experienced personal on one hand for the lab work and on the other hand for data evaluation [14,15]. Despite these high technical demands on experience and standardization and its obvious limitations, PFGE typing still is considered as the "gold standard" for enterococcal strain typing, especially for elucidating supposed outbreak scenarios [16-18]. As described recently, rates of recombination, to be comparably high in *Enterococcus* in general, may be less pronounced in hospital-associated strain types, mostly prevalent among the nosocomial setting and responsible for the majority of outbreaks [7]. This is in line with recognition of certain VRE strain types prevalent for longer periods and in many hospitals, even country-wide (Figure 1) [19,20].

Nevertheless, the method is purely DNA fragment-based (without any further information) and less portable challenging the intra-laboratory comparability of images and fragment patterns. A harmonised protocol as established for MRSA strain typing [21,22] does not exist for VRE and thus inter-laboratory comparison of data is even more challenging. When using PFGE for VRE typing international agreements to standardize data interpretation and analysis as suggested by some experts, a number of them especially evaluated for VRE, should be considered [15,23].

Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length' polymorphism (AFLP, also fluorescent fAFLP) is a restriction and amplification-based typing technique which has been introduced about 10 years ago for bacterial strain typing [24]. It was the first method to address and open our current understanding of the population biology of *E. faecium*. According to AFLP data and subsequent fragment pattern analysis, hospital strains of *E. faecium* constitute an individual subgroup (at this time called C1) which could be differentiated from strains of commensal (human/animal) and environmental as well as probiotic/food origin [25]. AFLP has already been replaced quite shortly after its introduction by alternative DNA-based techniques with similar or better performance such as MLVA and MLST (see next chapters). AFLP has lately been used as a frontline tool for bacterial genus and species prediction in a medium throughput dimension. It is obvious that with the broader application of MALDI-TOF MS for routine bacterial species identification, also this application field will decline [26,27].

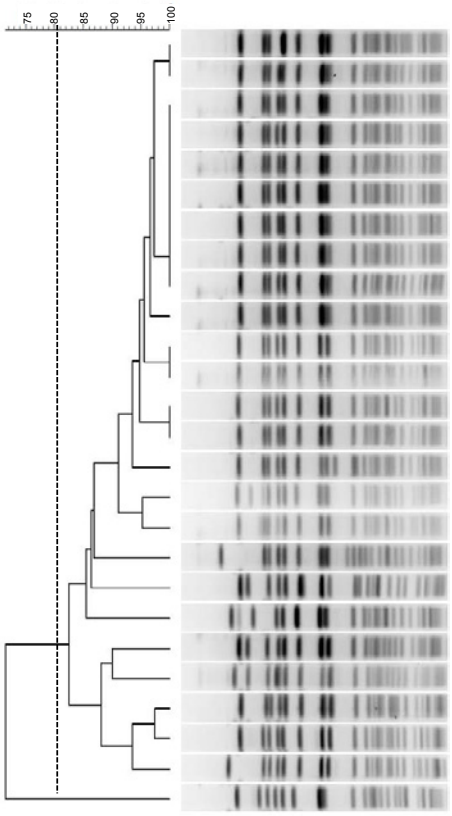
Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA)

MLVA has first been introduced as a typing tool to further differentiate highly clonal bacterial species such as *Bacillus anthracis* and other high threat pathogens about 10 years ago [28,29]. It determines DNA repeat regions and its variations in number and composition. Since majority of these repeat regions (5-8 different per genome) are comparably small (<50 bp), their analyses require some technical demands, e. g. capillary sequencers, which in addition allow multiplexing with differently labeled primers for repeat amplifcation and detection [30]. A MLVA typing scheme for *E. faecium* was introduced in 2004 [31]. The six identified discriminatory Variable-Number Tandem Repeat (VNTR) loci were of comparably long repeat lengths (123–279 bp) allowing an agarose gel based analysis. MLVA discriminated similar to AFLP and MLST allowing to define (confirm) the clade of hospital-associated strain types called C1 or CC17 [32]. MLVA for *E. faecium* is less discriminatory than PFGE typing and different major hospital MLST types could be represented by a single MLVA type (and vice versa; [33]). However, in combination with the determination of additional epidemic marker genes (*esp*, hyl_{Efm}) or strain-specific information (antibiotic resistance profiles) combined MLVA typing allows strain characterization and outbreak analysis [32,34]. A user-friendly platform at the University Centre Utrecht, the Netherlands, allows submission of MLVA profiles and comparison of data (http://www.umcutrecht.nl/subsite/MLVA/; managed by: Dr. Janetta Top; last access: 18.01.2013).

A MLVA scheme for *E. faecalis* has been introduced several years ago [35]. It combines VNTR repeat loci of known virulence genes encoding surface-exposed determinants and unknown loci identified by a repeat finder programme. In the initial study this scheme was comparably discriminatory as PFGE typing for the set of investigated *E. faecalis* strains (VRE and VSE). The *E. faecalis* MLVA scheme has only scarcely been used since its introduction and thus its general usefulness for typing *E. faecalis* strains cannot be properly assessed [36,37].

Multi-Locus Sequence Typing (MLST)

MLST is a logical successor of a technique called Multilocus Enzyme Electrophoresis (MLEE). MLEE was introduced more than 25 years ago for bacterial typing. It determines differences in the amino acid sequences of housekeeping genes assayed phenotypically [38]. Mutational changes in housekeeping genes are seldom and are not exposed to any selective pressure such as bacterial cell wall and surface components or determinants associated with antibiotic resistance. A constant correlation between mutation rate and time is expected. MLST uses a similar experimental approach and circumvents limitations of MLEE. MLST determines the exact nucleotide composition and is capable of identifying also synonymous (silent) mutations. A combination of seven loci distributed across the genome was calculated to be associated with a suitable discriminatory power [39]. An MLST scheme for *E. faecium* was introduced in 2002 [40]. UPGMA (Unweighted Pair Group Method of Alignment) clustering of MLST data of *E. faecium* isolates of various origins identified a sub cluster called C1 consisting of hospital-associated strains, thus confirming the population snapshot suggested on the basis of AFLP data and analysis (and later also for MLVA). A software tool called BURST (Based upon Related Sequence Types; later "extended" (e) BURST) was especially developed for MLST data analysis [41]. The BURST algorithm identifies



Isolate	Origin	MLST	MLVA	Year	*van* type	Sourcel/nfection
UW2383	Berlin Hosp 1	n.d.	n.d.	1999	vanA	Venous catheter/bacteraemia
UW2390	Berlin, Hosp 2	n.d.	n.d.	1999	vanA	urine/UTI
UW2222	Berlin, Hosp 3	ST117	n.d.	1999	vanA	bacteraemia
UW2306	Berlin, Hosp 4	n.d.	n.d.	1999	vanA	BAL
UW2321	Berlin, Hosp 3	n.d.	n.d.	1999	vanA	(multiple abscesses)
UW2322	Berlin, Hosp 5	ST117	MT12	1999	vanA	urine
UW2384	MV, Hosp 1	ST117	MT12	1999	vanA	bacteraemia
UW2413	Berlin, Hosp 6	ST117	n.d.	1999	vanA	swab sample
UW2442	Berlin, Hosp 7	n.d.	n.d.	1999	vanA	peritonitis
UW2320	Berlin, Hosp 8	n.d.	n.d.	1999	vanA	wound
UW931	L. Saxony; Hosp 1	ST117	MT12	1996	vanA	unknown
UW1806	Berlin, Hosp 9	ST117	MT12	1998	vanA	BAL
UW1827	L.Saxony , Hosp 2	ST117	MT12	1998	vanA	tracheal secretion
UW1983	L.Saxony , Hosp 3	ST117	MT12	1998	vanA	bacteraemia
UW1822	MV, Hosp 1	ST117	MT12	1998	vanA	anal swab
UW901	Hesse, Hosp 1	ST117	n.d.	1996	vanA	unknown
UW1505	NRW, Hosp 1	ST117	MT12	1997	vanA	unknown
UW2434	Berlin, Hosp 9	ST117	MT12	1999	vanA	bacteraemia
UW2444	Saxony, Hosp 1	ST117	MT12	1999	vanA	swab sample
UW1823	Berlin, Hosp 10	ST117	MT12	1998	-[1)]	wound swab
UW2319	Hesse, Hosp 2	ST117	MT12	1999	vanA	peritonitis
UW2453	Hesse, Hosp 2	ST117	MT12	1999	vanA	bacteraemia
UW2467	Hamburg, Hosp 1	ST117	MT12	1999	vanA	bacteraemia
UW2862	Saarland, Hosp 1	ST117	n.d.	2000	vanA	bacteraemia
UW2987	NRW, Hosp 2	ST117	n.d.	2000	vanA	bacteraemia
UW3182	Brandenburg, Hosp 1	ST117	MT12	2001	vanA	bacteraemia

Figure 1: *Sma*I-macrorestriction analysis in PFGE of 26 VRE isolates representing individual clusters of infections and colonisations in patients from 22 hospitals collected between 1996 and 2000 in Germany. The origin of the hospital isolates is given as a hospital within a federal state. The dotted line represents the 82% similarity score suggested by Morrison et al. for determining clonal relatedness [23]. Legend: BAL, Broncho Alveolar Lavage; Hosp, hospital; L. Saxony; Lower Saxony; MV, Mecklenburg-West Pomerania; n.d., not determined; NRW, North-Rhine Westphalia; year, year of isolation. Isolate UW1823 may have lost vanA resistance since it was negative in repeated PCR reactions.

Method	Principle	Discrimination	Reproducibility	Data exchange	Applications
Ribotyping	Hybridization of labelled rDNA with digested genomic DNA	medium	good	possible[2]	Too low discrimination for outbreak analysis (short term epidemiology)
RAPID/repPCR	PCR with random primers or primers binding to repetitive target sequences	medium	insufficient - good[1]	possible[2]	Partly suitable for „in house“ outbreak analyses; provided with commercial kits (DiversiLab™)
AFLP	Length polymorphisms in genomic PCR products	good	good–very good[1]	possible	Exchanged by MLST due to better data portability and discriminatory power
PFGE	Genome-based macrorestriction analysis	excellent	good–very good[1]	possible[2]	Still „Gold-Standard“ for outbreak analyses; not suitable for long term epidemiology / population-based analyses (“over-discrimination“)
MLST	DNA sequence comparisons of housekeeping genes	good – very good	excellent	excellent	„Gold Standard“ for population-based analyses; comparably expensive and laborious, too less discriminatory for outbreak analyses
MLVA	Fragment length polymorphisms in genomic repeat regions	good – very good	very good	excellent	Suitable for population-based analyses; too less discriminatory for outbreak analyses
vanA cluster typing	Different schemes exist based on amplification, digestion, sequencing[3]	good – very good	very good	good	Only suitable for specialist analyses and in combination with basic techniques (PFGE, MLST, MLVA)
Plasmid typing	Analysis of the plasmid content and cmposition[3]	limited	very good	possible	Dependent on the corresponding question; suitable for analysis of “plasmid hospitalism” and for enhancing MLST/MLVA analysis’ results
NGS	Analysis of the genome content	highest possible	excellent	excellent	its potential for various epidemiological questions has to be analysed in studies in the near future

Legend: AFLP, Amplified-Fragment Length Polymorphisms; MLST, Multi-locus Sequence Typing; MLVA, Multiple Locus Variable Number of Tandem Repeat Analysis; NGS, Next Generation Sequencing (synonymous for various techniques such as 454, illumina, ion torrent); PFGE, Genomic macrorestriction analysis in Pulsed-field Gel electrophoresis. [1] first specification for Interlab-, second for Intralab reproducability; [2] in relation to a standardized protocol / Kit / machine; [3] no standardized/harmonized typing scheme exists.

Table 1: Comparison of typing methods used for VRE/*Enterococcus*.

mutually exclusive groups of related genotypes in a population and attempts to identify the founding genotype (sequence type or ST) of each group. It then predicts the descent from the predicted founding genotype to the other genotypes in the group, displaying the output as a radial diagramme, centred on the predicted founding genotype (http://eburst.mlst.net/). eBURST phylogenetic analyses are excellent for rather clonal populations/species such as *Streptococcus pneumoniae* and *Staphylococcus aureus*; but reliability is low for species with a high recombination to mutation ratio as predicted for *Enterococcus*[42]. eBURST analysis of MLST datasets of highly recombinogenic species results in a single large straggly eBURST group, which results from the incorrect linking of unrelated groups of strains [42]. Global optimal eBURST (goeBURST) software implemented additional tools and, for instance, allows the introduction of tie break rules reached in deciding and visually evaluating the reliability of the hypothetical links and patterns of descent [43]. However, since it is mainly also based on the BURST algorithm major limitations of the method remain (http://goeburst.phyloviz.net/). Bayesian modelling methods introduced in the BAPS software (Bayesian Analysis of genetic Population Structure; http://www.helsinki.fi/bsg/software/) [44] were applied recently for analysing the population structure of *E. faecium* [45]. Subsequent (nested) BAPS analyses on the entire MLST dataset for *E. faecium* identified several BAPS groups that could be further subdivided. BAPS analyses disproved the concept of a single clonal complex CC17 combining all hospital-associated strains in one major group of relatedness (descent) since major MLST types of hospital-associated strains such as ST17, ST18 and ST78 were placed at different BAPS subgroups and branches at BAPS trees, respectively [45]. Independent from its differences in the core genome and pattern of descent, all hospital strains contain a specific accessory genome content supposedly associated with a specialization to the hospital setting [46-48].

Two schemes exist for MLST typing of *E. faecalis*. Both have some alleles in common, but differ in selecting (a) explicitly only housekeeping genes [49] or (b) mix housekeeping with virulence genes for allele pattern analysis [50]. The latter approach was suitable to identify a clonal lineage highly prevalent in the US and characterized by beta-lactamase production (still rare in *E. faecalis*) and presence of the pathogenicity island. The scheme of Ruiz-Garbajosa et al. [49] follows common rules for MLST allele selection (housekeeping genes only) and has been implemented into the central MLST database (http://efaecalis.mlst.net/; managed by: Rob Willems and Janetta Top (UMC Utrecht, NL, hosted by the Imperial College London and funded by the Wellcome Trust, UK). Consequently, most of the recent papers using MLST for *E. faecalis* rely on the scheme of Ruiz-Garbajosa et al. [37,45,49,51,52]. Population structure of *E. faecalis* appears somehow different from that of *E. faecium*. MLST analysis does not strictly differentiate hospital-associated lineages from colonizing variants. Nevertheless, certain sequence types and clonal complexes appear enriched in the nosocomial setting and which are often found to be multi-resistant. This concerns STs assembled in clonal complexes CC2 and CC9, mainly ST16 which is a classical hospital-associated and "outbreak strain type" [6,49]. However, recent publications also revealed a supposed animal reservoir of this clonal type ST16 [51,53]. Other sequence types appear to be generally distributed among many ecosystems and do not seem to be associated with any form of host specificity. This applies to ST40 which is a quite common ST variant prevalent as a colonizer in humans, animals and the environment but also associated with a various types of infections in animals and humans [6,52-54].

Vancomycin Resistance Gene Cluster Typing

Vancomycin resistance in animal, human and environmental sources is mostly encoded by *vanA*-type resistance clusters of the Tn*1546*-type and its reservoir is in isolates of *E. faecium*. Exchange of resistant strains among different ecosystems is less probable due to the supposed association of distinct *E. faecium* strain types with specific backgrounds, although dissemination of vancomycin- and multi-resistant *E. faecium* across host barriers was described anecdotally [53,55-60]. Vancomycin resistance among enterococci spreads via clonal dispersion and lateral gene transfer, the latter via dissemination of mobile genetic elements of variants of the *vanA*-type element Tn*1546* mostly located on mobilizable or conjugative plasmids [60-66].

Molecular studies revealed a tremendous number of deletions, insertions, and modifications of the original Tn*1546*-like structure in different not epidemiologically linked VRE leading to a wide diversity of various Tn*1546* subtypes [67-72]. Despite its high diversity, identical cluster types were found among clinical human and animal commencal and environmental strains suggesting a common reservoir and exchange of its mobile elements via conjugative plasmids or as part of larger mobile genomic islands in European, Asian and Australian *E. faecium* strain collections [67,68,70,73,74]. Also nowadays, *vanA* cluster typing is used as a typing method for diverse strain collections where clonal spread is less expected [75-79].

In contrast, *vanB*-type elements preferably integrate into the chromosome, but are mobile as part of integrative and conjugative elements ICE [80,81]. Occasionally *vanB* resides on (transferable) plasmids [82-84]; as noticed recently associated with larger *VanB*-type VRE outbreaks [85,86]. In general, the supposed low expression of vancomycin resistance among *vanB* strains may have led to an underestimation of its general prevalence, since in many screening studies comparably high vancomycin concentrations to select VRE were used [87-90]. Rates of clinical *vanB*-type VRE are increasing, at least in some European countries during last years [18,91-93] and a link to a supposed reservoir outside the clinical setting, for instance, among mammal intestinal bacterial colonizers is discussed also in areas where *vanB*-type vancomycin resistance is more prevalent [91,94]. Although several cluster variants exist, a *vanB* cluster typing scheme has not been established yet.

In general, vancomycin resistance gene cluster typing may reveal additional resolution to existing strain typing schemes by assessing a number of structural modifications that do not seem essential to resistance expression and regulation of the element. However, results should be interpreted with caution since the evolutionary clock speed of these changes is unpredictable and IS element and transposon derived modifications could occur quite frequently at hotspots for genomic rearrangements [81].

Plasmid Typing

Plasmid typing may be suitable for demonstrating horizontal *vanA* (and *vanB*) cluster dissemination across different *E. faecium* strain types ("Plasmid hospitalism"). *In vitro* transfer of *vanA* plasmids has been determined in a number of studies [67,95,96] as well as transfer in digestive tracts of animals and human volunteers [97-99] with transfer rates being significantly higher under natural conditions [100]. However, plasmid typing is less well established for resistance plasmids of VRE than it is for multi-resistance plasmids in *Enterobacteriaceae* where for a number of Inc group plasmids, plasmid MLST schemes were established and corresponding databases are managed. A few plasmids

of VRE were fully sequenced [61,62,65,101] and based on these data and further information different plasmid typing schemes based on replicase genes were suggested [102,103]. Results of Jensen et al. [102] were widely used for subsequent plasmid typing studies in *Enterococcus* spp. mainly VRE [60,78,104-107]. Garcia-Migura and co-workers also identified a hot spot for integration of Tn*1546*-like elements and it could be speculated if this integration site is more prevalent among certain plasmid types and the reason for the preferred prevalence of *vanA* clusters with specific plasmids [104,108,109]. Results of a recent study about horizontal transferability of *vanA* plasmids among enterococci, other lactic acid bacteria and bifidobacteria revealed a preferred transfer into and a possible host restriction among *E. faecium* [66]. Only a few studies so far described spread of highly similar plasmids as a means of disseminating *vanA*- or *vanB*-type resistance [65,78,85]. Further progress in whole genome sequencing assessing also the plasmid content of these bacteria will elucidate the role of distinct plasmid types for spreading vancomycin resistance among *Enterococcus*.

Whole Genome Sequencing/Next Generation sequencing (WGS/NGS)

Next generation sequencing techniques provide a tremendous potential to outbreak investigation, elucidating transmission routes of pathogens and tracing emergence and spread of multidrug resistant bacteria [110-112]. A few genomes of enterococcal strains have been elucidated completely [54,113], partly by classical Sanger sequencing [80,114-116], and genomic information to a wide range of additional isolates is available as draft genomes [117,118](check recent data: http://www.ncbi.nlm.nih.gov/genome?term=*Enterococcus%20faecium;* http://www.ncbi.nlm.nih.gov/genome?term=*Enterococcus%20faecalis*). Only a subset of these isolates represents vancomycin-resistant variants [80,113]. It has to be elucidated within the near future if the highest possible discriminatory power of NGS techniques will be capable of assessing relevant information for outbreak analysis and pathogens' transmission as shown very recently for MRSA and multidrug-resistant *Enterobacteriaceae* outbreak analyses [119-121].

Conclusion/outlook

Using molecular typing to elucidate different epidemiological scenarios requires methods with a different discriminatory power. Several techniques have also been established to type VRE which mainly means vancomycin-resistant *E. faecium*. Outbreak analysis still uses macrorestriction analysis in PFGE, although this method is comparably laborious, time-consuming and requires experienced personal for gel preparation and pattern analysis. For VRE outbreak analysis MLST and MLVA may be less suitable since their discriminatory power is limited, but can be compensated by a binary or multimodal typing approach combining MLST with other data (virulence gene patterns, plasmid typing, *vanA* cluster typing, etc.). Some methods are already outdated or do not provide sufficient resolutionary power at all, such as classical ribotyping, AFLP or repPCR-based typing. Plasmid and *vanA* cluster typing may reveal suitable information in addition to basic typing methods (MLST, MLVA, PFGE) but will not provide reliable information as a stand-alone technique. It is expected that next generation sequencing techniques will have their value for VRE outbreak analysis as well, elucidating transmission routes of *vanA* / *vanB* resistance determinants or VRE strains and tracing spread of VRE strain types as shown very recently for a number of other multiresistant, nosocomial pathogens.

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