# Chapter 2
# *Enterococcus*

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## 2.1 Introduction

The enterococci are a diverse and versatile group of bacteria with several intrinsic characteristics that allow them to survive and grow under a variety of conditions and a remarkable metabolic adaptability in order to fulfill diverse roles as commensals and as opportunistic pathogens. These microorganisms are widely distributed in nature, mainly on the mucosal surfaces of humans and animals, but they are also found in soil, water, dairy products and other foodstuffs, and on plants. Under certain circumstances, they are able to cause a variety of infections in humans and are now recognized among the major etiological agents of nosocomial infections associated with limited therapeutic options, due to their ability to acquire resistance to most of the clinically relevant antimicrobial agents [1–3].

In years past, enterococcal infections were traditionally considered to be acquired endogenously from the patient's own normal flora, and the epidemiology of enterococcal infection attracted little attention. This perspective has dramatically changed and a major interest has focused on the epidemiology of enterococcal infections, because of the increasing documentation of *Enterococcus* as a leading nosocomial pathogen. Furthermore, the emergence and dissemination of multiple antimicrobial resistance traits among enterococcal strains and the evidence supporting the concept of exogenous acquisition of enterococcal infections have generated an

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additional need for typing the isolates as a means of assisting infection control and epidemiological studies both within and among various medical institutions. Therefore, the investigation of epidemiological aspects of nosocomial outbreaks as well as the dissemination of enterococcal strains harboring antimicrobial resistance markers is of major interest, particularly in the light of the increasing occurrence of vancomycin-resistant enterococci (VRE). Ideally, besides outbreak analysis, the methods used for epidemiological investigation of enterococcal isolates must be able to track enterococcal dissemination in different environments and hosts, and the evolution of multiresistant strains.

## 2.2 Characteristics and Current Classification of the Genus

The genus *Enterococcus* is composed of Gram-positive cocci that occur singly, in pairs or as short chains. They are non-sporing, facultatively anaerobic, catalase-negative bacteria, with a fermentative metabolism resulting in L(+) lactic acid as the major product of glucose fermentation.

Characteristics such as growth in broth containing 6.5% NaCl and hydrolysis of esculin in the presence of bile salts (bile–esculin [BE] test) are useful to identify enterococcal strains. Other characteristics presented by most enterococci include hydrolysis of leucine-β-naphthylamide (LAP) and L-pyrrolidonyl-β-naphthylamide (PYR) [3, 4].

The enterococci were earlier considered as a major branch within the genus *Streptococcus,* distinguished by their higher resistance to chemical and physical agents and accommodating most of the serological group D streptococci. After the introduction of molecular methods for studying these microorganisms they have undergone considerable changes in taxonomy, which started with the recognition of *Enterococcus* as a separate genus [5]. *Streptococcus faecalis* and *Streptococcus faecium* were the first species to be transferred to the new genus as *Enterococcus faecalis* and *Enterococcus faecium*, respectively. The continuous use of molecular approaches has allowed major developments in the classification of the enterococci, resulting in the recognition of about 35 enterococcal species to date [3, 4, 6]. The current criteria for inclusion in the genus *Enterococcus* and for the description of new enterococcal species are based on a combination of phenotypic tests and different molecular techniques, including DNA–DNA reassociation experiments, 16S rRNA gene sequencing, and whole-cell protein profiling analysis. Partial or nearly entire sequencing of the 16S rDNA is considered a practical and powerful tool in aiding the identification of enterococcal species: it has been performed for all currently recognized species of *Enterococcus*, and sequences are available from the GenBank database (www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide).

In diagnostic laboratory settings, identification of enterococcal species is generally accomplished by using a series of conventional physiological tests (see references 3, 4, and www.cdc.gov/ncidod/biotech/strep/strep-doc/index.htm for details). Several miniaturized, manual, semiautomated, and automated identification systems are commercially available and may be an alternative for the phenotypic

identification of enterococcal species in routine diagnostic laboratories. The application of molecular techniques for the rapid identification of *Enterococcus* species has also been expanded for use in clinical microbiology laboratories. A variety of molecular procedures have been proposed for the identification of enterococcal species, and with future improvements may also become widely available for the rapid and precise detection of enterococci directly in clinical samples [4, 7].

## 2.3 Clinical Significance and Epidemiology

The enterococci can act as opportunistic agents of infections, particularly in elderly patients with serious underlying diseases and other immunocompromised patients who have been hospitalized for prolonged periods, treated with invasive devices and/or have received broad-spectrum antimicrobial therapy. The spectrum of infections caused by the enterococci includes urinary tract infections (UTIs), wound infections (mostly surgical, decubitus ulcers, and burn wounds), and bacteremia [2]. They are also frequently associated with endocarditis, intra-abdominal, and pelvic infections. Enterococcal infections of the respiratory tract or the central nervous system, as well as otitis, sinusitis, septic arthritis, endophthalmitis, may occur, but are rare. Although the enterococci can cause human infections in the community and in the hospital, these microorganisms began to be recognized with increasing frequency as common causes of hospital-acquired infections in the late 1970s, paralleling the increasing resistance to most currently used antimicrobial agents. As a result, enterococci have emerged as one of the leading therapeutic challenges when associated with serious or life-threatening infections. *E. faecalis* is usually the most frequent enterococcal species isolated from human clinical specimens, representing 80–90% of the isolates, followed by *E. faecium* that is found in 5–10% of enterococcal infections [2, 3]. However, the ratio of isolation of the different enterococcal species can vary according to each setting and can be affected by a number of aspects, including the increasing dissemination of outbreak-related strains such as vancomycin-resistant *E. faecium*.

The pathogenesis of enterococcal infections is still poorly understood. Several potential virulence factors have been identified, although none has been established as having a major contribution to enterococcal virulence. Nevertheless, epidemiological studies show the existence of clonal relationships among outbreak isolates and support the notion that a subset of virulent lineages are often responsible for infections of epidemic proportions [1, 8–10].

## 2.4 Resistance to Antimicrobial Agents

Resistance to several commonly used antimicrobial agents is a remarkable characteristic of most enterococcal species, and can either be intrinsic or acquired. The occurrence of acquired traits leading to high-level resistance to aminoglycosides

(HLR-A), and resistance to glycopeptides, especially to vancomycin, is of particular clinical significance due to the impact in the treatment of enterococcal infections.

The emergence of VRE was first documented in Western Europe and in the United States. Thereafter the isolation of VRE has been continuously reported, indicating epidemic proportions in diverse geographic locations. VRE strains have been classified according to phenotypic and genotypic features [11, 12], and by molecular methods for rapid detection and precise classification which have been developed, mostly based on PCR tests [13]. Nine types of glycopeptide resistance have already been described among enterococci. Each type is associated with different genetic elements, some of which, in turn, can be divided into subtypes. The *vanA* and *vanB* are considered the most clinically relevant genotypes and are usually associated with *E. faecium* and *E. faecalis* isolates, while the VanC resistance is an intrinsic characteristic of *E. gallinarum* (*vanC1* genotype) and *E. casseliflavus* (*vanC2*–-*vanC4* genotypes). The additional types of glycopeptide resistance, encoded by the *vanD*, *vanE*, *vanG*, and *vanL-vanN* genes seem to occur rarely among enterococci. Considering the high frequency and diversity of antimicrobial traits among enterococcal isolates, determination of the genetic profile of genes associated with resistance to a variety of antimicrobials may be used as additional valuable tool for epidemiology and typing purposes.

## 2.5 Typing Methods

### *2.5.1 Early Typing Methods*

Early epidemiological investigations of enterococcal infections were based on classic phenotypic typing methods used to investigate the diversity among enterococcal isolates, including biotyping and antibiotyping, serotyping, bacteriocin typing, and bacteriophage typing (see ref. [4] for additional reading). Although these approaches have occasionally yielded useful information, they frequently fail to adequately discriminate among strains, and therefore, they are of limited value for comprehensive epidemiological studies. On the other hand, the use of phenotypic typing methods in conjunction with molecular typing approaches can contribute valuable information.

### *2.5.2 Molecular Typing Methods*

The introduction of molecular techniques has substantially improved the ability to discriminate enterococcal isolates and has provided critical insights into the epidemiology of the enterococci. By using molecular typing approaches it was possible to demonstrate the exogenous acquisition of enterococcal strains by direct and indirect

contact among patients, breaking the traditional conception that enterococcal infections were endogenous in nature. Intrahospital transmission and interhospital spread have been extensively documented for antimicrobial resistant enterococci, especially VRE [4, 14, 15]. In addition to epidemiological investigations, some of the molecular typing techniques are now used to trace the dissemination of enterococci in different environments and hosts, phylogenetic relationship, and the evolution of multidrug-resistant strains, greatly expanding our understanding of enterococcal epidemiology, population structure, antimicrobial resistance, and virulence. Emergence and global dispersion of certain epidemic enterococcal clonal complexes has been identified [8–10, 16, 17].

Several molecular methods have been proposed to type enterococcal isolates as previously reviewed [4, 18]. The first molecular techniques developed for typing of enterococci were the analysis of plasmids profiles (including both plasmid composition and restriction endonuclease analysis of specific plasmids) and the restriction enzyme analysis (REA) of genomic DNA by conventional electrophoresis. These techniques may be helpful in some instances, but problems related to inconsistencies in plasmid yield and to difficulties in accurate interpretation of the electrophoretic profiles have been encountered with the use of these methods. Multilocus enzyme electrophoresis (MLEE), ribotyping, and the polymerase chain reaction (PCR)-based typing methods, such as the random amplified polymorphic DNA (RAPD-PCR) assay, and the repetitive element sequence (REP)-PCR have also been used to investigate the genetic relationship among enterococcal strains. These methods also have limitations, such as poor reproducibility and/or high technical complexity. DNA sequencing of PCR products and restriction fragment length polymorphism (RFLP) analysis of PCR products have been used to trace and to determine differences among specific resistance genes in enterococci, and therefore representing additional tools for typing resistant strains.

A remarkable contribution to the ability to discriminating among enterococcal strains was noted with the use of techniques involving the analysis of chromosomal DNA restriction endonuclease profiles by pulsed-field gel electrophoresis (PFGE) by either field inversion gel electrophoresis (FIGE) or, ideally, by counter-clamped homogeneous electric field electrophoresis (CHEF), which is the basis for most of the recent PFGE studies. Analysis of chromosomal DNA restriction profiles by pulsed-field gel electrophoresis (PFGE) has been extensively evaluated for epidemiological characterization of enterococcal outbreaks, showing improved strain discrimination and allowing the identification of clonal complexes that predominate among multidrug-resistant enterococci, mainly strains with HLR-A and VRE [4, 14, 19–21]. *Sma*I is the restriction enzyme more frequently used to digest enterococcal DNA, and the usefulness of other enzymes, such as *Apa*I and *Sfi*I, has also been documented [4].

PFGE is possibly the typing method most commonly used in clinical microbiology settings, and it is considered by many investigators as the gold standard for the epidemiological analysis of enterococcal outbreaks. Several protocols for performing PFGE typing of enterococcal strains have been published. However, the development

of standardized protocols for execution, interpretation and nomenclature, as a result of collaborative studies is still needed in order to allow for inter-laboratory data exchange and comparisons. On the other hand, although PFGE is quite discriminatory, epidemiological interpretation of PFGE profiles is not always clear-cut. The occurrence of genetic events can be associated with substantial changes in the PFGE profiles, leading to problems in clonality assessment [22]. Due to the possibility of such inconsistencies in DNA banding patterns of enterococci, PFGE is recommended mostly for the purpose of evaluating the genetic relatedness and tracing transmission of strains that are associated in time and location, as usefulness for long-term epidemiological studies may be limited. The use of PFGE in conjunction with at least one additional typing technique, or independent PFGE analysis using different restriction enzymes, is highly recommended to help clarify epidemiological interpretation. General principles proposed for the interpretation of molecular typing data based on fragment differences are usually applied to interpret PFGE profiles obtained for enterococcal strain. Well-characterized control strains should be evaluated along with unknown isolates. For that purpose, two reference strains, *E. faecalis* OG1RF (ATCC® 47077™) and *E. faecium* GE1 (ATCC® 51558™) have been proposed [23].

Two other robust molecular techniques have become available more recently for typing of enterococcal isolates: multilocus sequence typing (MLST) and multiple-locus variable-number tandem repeat analysis (MLVA). These techniques circumvent the difficulties in data exchange between different laboratories by generating information that is suitable for the development of Web-based databases. MLST is based on identifying alleles after sequencing of internal fragments of a number of selected housekeeping genes, resulting in a numeric allelic profile. Each profile is assigned a sequence type (ST). Internet sites with the possibility for data exchange have been developed (www.mlst.net, and www.pubMLST.org), which contain MLST protocols for *E. faecium* (see ref. [24] and http://efaecium.mlst.net/misc/info.asp) and *E. faecalis* (see ref. [25] and http://efaecalis.mlst.net/misc/info.asp). MLST schemes for these two species are based on sequence analysis of seven loci, each one corresponding to a separate set of different genes. Application of MLST has revealed the occurrence of host-specific genogroups of *E. faecium*, and allowed the recognition of a hospital-adapted *E. faecium* subpopulation (initially named as C1 lineage), that seems to predominate in several geographic areas [8, 9, 15–17]. This hospital-adapted lineage was later renamed as clonal complex-17 (CC17), and classified as an example of the so called high-risk enterococcal complexes (HiRECC). Figure 2.1 shows the eBURST diagram representing clusters of *E. faecium* (as of April 2010) available at the MLST database**.** Major clonal complexes have also been identified among *E. faecalis* isolates [14, 17, 25] by using MLST.

Two simultaneously published studies described the development of MLVA typing schemes for *E. faecalis* [26] and *E. faecium* [27]. MLVA is based on differences in variable-number of tandem repeats (VNTR) in multiple loci dispersed over the enterococcal genome. For each VNTR locus, the number of repeats is determined by PCR using primers based on the conserved flanking regions of the tandem

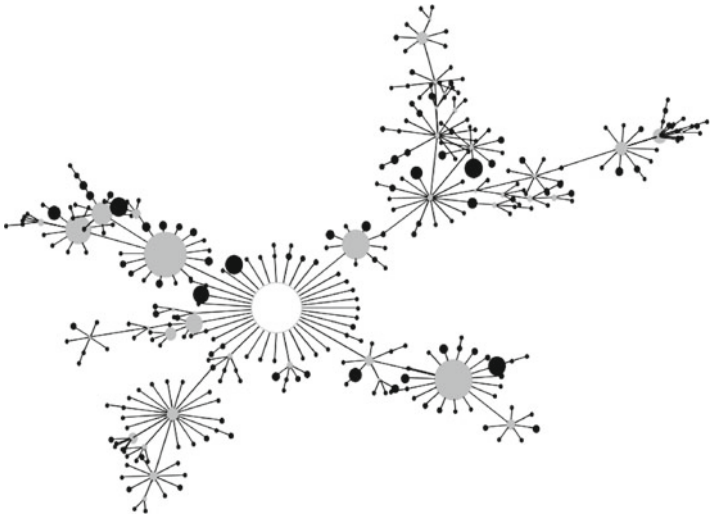

**Fig. 2.1** eBURST diagram showing the clusters of *Enterococcus faecium* presently available at the MLST database (http://www.mlst.net). Each ST is represented as a *node* and the relative size of the *circles* indicates their prevalence in the database. *Lines* connect single locus variants: STs that differ in only one of the seven housekeeping genes. ST17, the presumed founder of the CC17, the major subpopulation representing hospital outbreaks and clinical infections, is represented as the *white circle*

repeats. PCR products are separated on agarose gels and the band size determines the number of repeats. These numbers together result in a MLVA profile and each profile is assigned an MLVA type (MT). The MLVA scheme for *E. faecium* is based on six VNTR loci present in noncoding regions. On the other hand, the MLVA typing scheme for *E. faecalis* is based on seven targets obtained from known genes. Figure 2.2 depicts the MLVA scheme for *E. faecium* showing typical results observed among VRE isolates from Rio de Janeiro, Brazil, belonging to a highly prevalent MT, named MT12. An Internet site has been developed (www.umcutrecht.nl/subsite/MLVA/) to serve as a database and also for the submission of MLVA profiles to assign MTs.

Comparative studies indicate that both MLST and MLVA techniques can achieve high degrees of discrimination between isolates and have comparable discriminatory power [21] that appears to be similar to that of PFGE- based typing [14, 25, 27]. In contrast to the overt advantages of being reproducible, portable, highly discriminatory and unambiguous, MLST is comparatively more expensive, and still limited to laboratories that have facilities for both PCR and sequencing, while MLVA requires PCR and basic electrophoresis facilities. Thus, MLVA may be used as an initial screening and typing method for a more rapid and less expensive alternative to MLST for clinical laboratory settings.

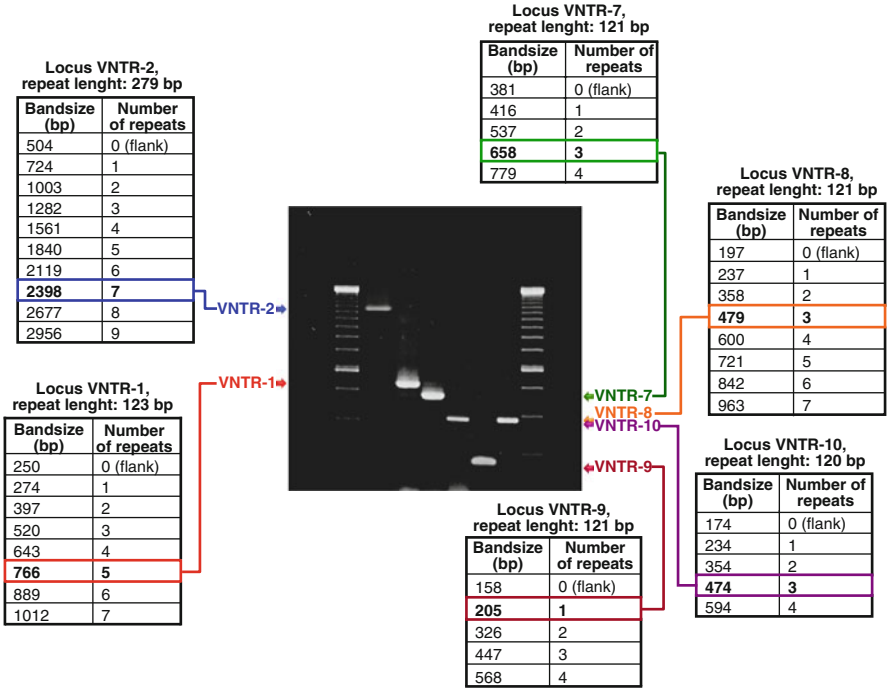


**Fig. 2.2** Schematic representation of the MLVA assay for *Enterococcus faecium* isolates. Six loci are amplified by PCR, so that the size of each locus is measured and the number of repeats can be deduced. The resulting information is a code which can be submitted to the specific database (http://www.umcutrecht.nl/subsite/MLVA/). Typical results observed among VRE isolates from Rio de Janeiro, Brazil, belonging to highly prevalent MT, named MT12 (5 7 3 3 1 3), are shown in the gel

In addition to differences in complexity and costs, molecular typing methods may vary in their reproducibility and discriminatory power. Overall, there is no single definitive method to type the enterococci, so a strong match among the results of different typing techniques, particularly those based on different genomic polymorphisms, should be used as indicative of high relatedness.

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