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REVIEW

Enterococcus: review of its physiology, pathogenesis, diseases and the challenges it poses for clinical microbiology

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Abstract **The genus *Enterococcus* is composed of 38 species, the most important of which are *Enterococcus faecalis* and *Enterococcus faecium*—both human intestinal colonizers. Hospitals within the United States and around the world commonly isolate these bacteria because they are a cause of bacteremia, urinary tract infections (UTIs), endocarditis, wound infections, meningitis, intraabdominal and pelvic infections, and nosocomial and iatrogenic infections. Given the ubiquity of enterococci within the human population, it is important for laboratories to be able to distinguish these agents within hospitalized patients from other bacterial genera and also differentiate different species within the *Enterococcus* genus as well as different strains within each species. Unfortunately, the enterococci are emerging as serious pathogens in both the developed world, where surveillance needs to be improved and speciation procedures are inadequate or cumbersome, and in developing nations, which lack the trained hospital personnel or funding to sufficiently identify enterococci to the genus or species level. This review explores the *Enterococcus* genus and highlights some of the concerns for national and international clinical microbiology laboratories.**

Keywords enterococci, *Staphylococcus*, antibiotic resistance, bacteriology, microbiology

The genus *Enterococcus*

The human intestine is inhabited by its own normal microbial flora, which consists of a multitude of different bacterial species. Although the majority of these bacteria are harmless, avirulent, and/or beneficial, if some of them, such as the enterococci, spread from the intestinal tract to other mucosal and skin surfaces, they can cause disease (reviewed in Murray et al., 2009). Originally, Thiercelin was the first scholar to coin the term "enterococcus" to describe a Gram-positive diplococcus organism found in the intestine (reviewed in Murray, 1990). This same microbe was subsequently named *Streptococcus faecalis* by Andrewes and Horder when they isolated it from a patient (reviewed in Murray, 1990). *Streptococcus faecium* was a fecal organism later described by Orla-Jensen to have similar characteristics to *Streptococcus faecalis* (reviewed in Murray, 1990). The enterococci were essentially classified as Group D streptococci because they possess the Group D cell wall antigen (a glycerol teichoic acid that is associated with the cytoplasmic membrane) according to the serological scheme developed by Rebecca Lancefield (Facklam, 2002; Teixeira et al., 2011). Sherman later divided the streptococci into four distinct groups: pyogenic, viridans, lactic, and enterococcus, with *S. faecalis* and *S. faecium* forming the enterococci (reviewed in Cetinkaya et al., 2000). The enterococci were classified among the genus *Streptococcus* for years until Kalina proposed that they should be placed into their own genus (reviewed in Cetinkaya et al., 2000). Finally, in 1984, the enterococci were indeed reclassified into their own new genus, *Enterococcus*, when DNA-DNA and DNA-rRNA hybridization confirmed significant differences between the enterococci and the other streptococci (reviewed in Murray, 1990; Teixeira et al., 2011).

There are now 38 species within the *Enterococcus* genus (Murray et al., 2009), with only those from humans and animals having been studied in detail. The most important species are the potential human pathogens *Enterococcus faecalis* and *Enterococcus faecium*, though *Enterococcus gallinarum* and *Enterococcus casseliflavus* have also been studied because they are inherently vancomycin-resistant and

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colonize the intestinal tract (Reid et al., 2001; reviewed in Murray et al., 2009).

Enterococcus physiology

Enterococci are robust non-spore-forming bacteria that can proliferate under a variety of hostile conditions. For example, they are facultative anaerobic organisms that have the ability to flourish in both oxygen and reduced oxygen environments, switching from respiration to fermentation readily (Huycke et al., 1998). Similarly, they are able to survive and grow at high salt concentrations (6.5% NaCl) where the Gram-positive streptococci would normally be killed (Devriese et al., 2006; Fisher and Phillips, 2009; Teixeira et al., 2011). In terms of temperature, they can withstand extremes—growing at as low as 5°C, as high as 50°C, and even surviving at 60°C for as long as 30 min (reviewed in Fisher and Phillips, 2009; Teixeira et al., 2011). They proliferate optimally at 37–42.7°C depending on the growth media (reviewed in Fisher and Phillips, 2009; Teixeira et al., 2011). The enterococci are also able to flourish in a broad pH range—growing optimally at pH 7.5, but surviving pH extremes as low as 4.8 and as high as 9.6 (with some strains withstanding pH 10.0) (reviewed in Fisher and Phillips, 2009; Teixeira et al., 2011). Finally, they can resist 40% (w/v) bile salts that would destroy other bacteria like *Streptococcus pneumoniae* (Devriese et al., 2006; Murray et al., 2009; Teixeira et al., 2011). The ability of enterococci to withstand broad pH ranges is likely due to their membrane durability and impermeability to acid and alkali, while their resistance to temperature is attributed to membrane lipids and fatty acids (reviewed in Fisher and Phillips, 2009). The extreme conditions by which enterococci can survive allow them to colonize a wide range of niches, which could have relevance for their clinical importance.

Pathogenesis

Virulence factors are traits or molecules that are produced by pathogens that help these pathogens with colonization, immunoevasion, and immunosuppression of their hosts. Consequently, these virulence factors are often responsible for causing disease. Though the enterococci do not produce potent toxins like some other bacteria, they do possess virulence factors in the form of aggregation substance, enterococcal surface protein (Esp), cytolysin, gelatinase, and antibiotic resistance genes (reviewed in Sava et al., 2010) (Table 1). In addition, they can release superoxide to the extracellular environment (Huycke et al., 1996; Huycke and Gilmore, 1997; Wang and Huycke, 2007) (Table 1). Many of the genes for the enterococcal virulence factors are found on conjugative plasmids or encoded within transposons and are easily transferable (reviewed in Palmer et al., 2010). Indeed, the enterococci have the ability to exchange these genetic determinants not only among themselves but also with bacteria of a different genus (Ray et al., 2003; Sung and Lindsay, 2007).

Three conjugative systems exist among the enterococci, which could lead to the exchange of genetic determinants (reviewed in Jett et al., 1994). The first conjugative system involves the sex pheromone plasmids that are unique to the enterococci, have a narrow host range, and can be transferred at high frequencies in laboratory and *in vivo* environments (reviewed in Dunny, 1990 and Clewell, 1993). The second conjugative system involves plasmids that have a broad host range and are readily transferrable among the enterococci, staphylococci, streptococci, and other bacterial species (reviewed in Clewell, 1990). Finally, the last conjugative system involves transposons found in Gram-negative and Gram-positive bacteria (reviewed in Scott, 1992 and Palmer et al., 2010). Apart from these conjugative systems, infection with bacteriophages is another method of genetic material exchange used by enterococci to allow for the spread of virulence factors or antibiotic resistance (Mazaheri Nezhad Fard et al., 2011; Vinodkumar et al., 2011).

In order for the enterococci to cause disease, they must first adhere to host tissues (reviewed in Sava et al., 2010). Toward this end, they possess surface adhesion proteins, such as aggregation substance, that allow them to bind to the cells of the human intestine (reviewed in Sava et al., 2010) (Table 1). When "sex pheromones" are produced and secreted by enterococci, certain strains of enterococci respond by producing the aggregation substance (reviewed in Sava et al., 2010). The aggregation substance is a hair-like protein that is embedded in the cytoplasmic membrane and it enables cell-to-cell contact between donor and recipient strains for conjugation (Kreft et al., 1992; reviewed in Fisher and Phillips, 2009). When the structural gene for the aggregation substance was sequenced, the presence of a conserved amino acid motif was revealed. This motif is found in fibronectin and other molecules capable of binding to eukaryotic cells via a class of receptors known as integrins (Kreft et al., 1992). As a result, the aggregation substance may also contribute to the ability of the enterococci to colonize sites outside the intestinal tract and to cause infection (Kreft et al., 1992; reviewed in Fisher and Phillips, 2009).

In addition to aggregation substance, the enterococcus surface protein (Esp) is another virulence factor—in this case a cell wall-associated protein that was originally only identified in *E. faecalis* strains (Table 1). However, new evidence has shown that *E. faecium* also possesses this protein (Shankar et al., 1999; Archimbaud et al., 2002). Esp appears to be associated with *E. faecium* biofilm production (Toledo-Arana et al., 2001). This is likely responsible for nosocomial-related strains of *E. faecium* adhering to medical devices, a common source of hospital acquired infections (Heikens et al., 2007). It is also believed that the Esp could be responsible for enterococcal adherence within the urinary bladder (Heikens et al., 2007), an important feature given that enterococci can be the cause of urinary tract infections.

Table 1 Enterococcal virulence factors

Virulence factor	Biological effects
Surface adhesins Aggregation substance	Hair-like protein embedded in cytoplasmic membrane; facilitates binding to host cells; enables cell-to-cell contact between donor and recipient strains for conjugation
Enterococcal surface protein (Esp)	Discovered in *E. faecalis* for adherence in urinary bladder; associated with biofilm production in *E. faecium* (nosocomial infections related to medical devices)
Secreted factors Cytolysin	Hemolytic protein associated with bacteremic strains; bacteriocin that inhibits growth of Gram-positive bacteria (facilitates colonization); can lyse macrophages and neutrophils (gain additional nutrients and escape immune clearance)
Gelatinase	Hydrolyzes gelatin, collagen, casein, hemoglobin; associated with enhanced virulence for endocarditis
Extracellular superoxide	Purpose unknown but may have role in lysis of red blood cells
Antibiotic resistance Multiple plasmid and chromosome genes	Transferred via conjugation, transposons or bacteriophages; resistant to aminoglycosides, β-lactams, vancomycin

Finally, biofilm production is thought to play a role in antibiotic resistance (Foulquié Moreno et al., 2006) and in endodontic disease (Kayaoglu and Ørstavik, 2004; Fisher and Phillips, 2009).

Cytolysin is another virulence factor—a hemolytic protein—that can be produced by the enterococci (Table 1). The gene encoding this protein is found in an operon that is either carried on a plasmid or integrated into the bacterial chromosome (Gilmore et al., 1990; Segarra et al., 1991; Hallgren et al., 2009). Enterococci possessing cytolysin have bacteriocin activity against Gram-positive, but not Gram-negative, bacteria and it is believed that this gives them a selective advantage for growth and survival (Clewell, 2007). The presence of cytolysin containing strains can be determined in clinical laboratories when a β-hemolytic reaction (clearing around bacterial colonies that are indicative of red blood cell lysis) is observed on blood agar plates. The cytolysin is able to lyse red blood cells from either human or horse blood, but sheep blood is somewhat less susceptible to lysis (Haas and Gilmore, 1999). Many bacteremic strains of enterococci possess cytolysin, and the protein can also lyse macrophages and neutrophils, which likely enables enterococci to gain additional nutrients and escape immune clearance (Miyazaki et al., 1993).

Finally, virtually all bacteremic strains of *E. faecalis* produce extracellular superoxide, but the precise purpose for this chemical is still unknown, though there is some suggestion that it may have a role in the lysis of red blood cells (reviewed in Jett et al., 1994) (Table 1). As for gelatinase, *E. faecalis* strains have the gene for gelatinase production (*gel* E) (Su et al., 1991). Gelatinase is a protease that hydrolyzes gelatin, collagen, casein, and hemoglobin (Su et al., 1991), and appears to be responsible for enhanced virulence for endocarditis caused by *E. faecalis* in animal models (Vergis et al., 2002).

Clinical disease

The enterococci are known to be responsible for numerous clinical diseases within communities and hospitals (Table 2). These diseases normally affect the elderly, the immunocompromised, patients with serious underlying illness, patients treated with broad spectrum antibiotics, and patients with additional bacterial infections. Enterococci are the cause of urinary tract infections (UTIs), bacteremia, endocarditis, meningitis, wound infections and intraabdominal and pelvic infections (Moellering, 1992; Teixeira et al., 2011) (Table 2). In addition, the enterococci are becoming one of the most common sources of infections acquired in hospitals (nosocomial infections) and infections due to physician error (iatrogenic infections) (reviewed in Emori and Gaynes, 1993; Teixeira et al., 2011). These infections are especially a problem in developing world countries (Budavari et al., 1997; Cohen, 1997; Khudaier et al., 2007; Singh 2009; Panesso et al., 2010), but are also prevalent in wealthier nations (reviewed in Emori and Gaynes, 1993). According to the National Nosocomial Infections Surveillance (NNIS) system conducted by the Centers for Disease Control and Prevention (CDC), the enterococci are the fourth leading cause of hospital-acquired infections and the third leading cause of bacteremia (reviewed in Emori and Gaynes, 1993). Normally, the routes for enterococcal infections are (1) spread of the patient's normal microbial flora into other body sites as the result of over antibiotic usage or improper patient usage of antibiotics (opportunistic infections), (2) spread of antibiotic resistant bacterial strains in a hospital setting (nosocomial and iatrogenic infections), and (3) wound infections (largely attributed to surgery, decubitus ulcers and burn wounds). Death associated with enterococci is normally due to endocarditis (Megran, 1992). Of all the species, *E. faecalis* and *E. faecium* are the two most important pathogens, with *E. faecalis* accounting for 80%–90% of infection cases and *E. faecium* constituting the majority of the remaining cases (reviewed in Jett et al., 1994). A few clinical isolates involve *E. casseliflavus* and *E. gallinarum* (Reid et al., 2001).

The most common enterococcal infections are UTIs, which are frequently of nosocomial origin and are largely derived from patients using urinary catheters (Moellering, 1992; Teixeira et al., 2011). Worldwide, *Escherichia coli* is considered to be the number one cause of UTIs (Forbes et al., 2007), but *E. faecalis* has overtaken *E. coli* as the number one cause of UTIs in many hospitals including Cedar Sinai in Los Angeles, California, USA. The second most common enterococcal infection, a polymicrobial infection, arises from

Table 2 Clinical diseases caused by enterococci

Disease	Clinical summary
Urinary tract infections	Dysuria and pyuria; frequently nosocomial and derived from patients using urinary catheters
Bacteremia	Caused by organism entering blood stream from sources such as UTI or intraabdominal abscess; can increase likelihood of endocarditis
Endocarditis	Infection of heart endothelium or valves; can lead to significant morbidity and mortality; occurs from sources of infection involving genitourinary and gastrointestinal tracts, wounds, bacteremia
Meningitis	Rare; primarily diagnosed in neonates
Wound infections	Largely attributed to surgery, decubitus ulcers, and burns
Peritonitis	Intraabdominal or pelvic infections; abdominal swelling; polymicrobial; patients typically acutely ill

intraabdominal and pelvic infections in which the enterococci are usually associated with other organisms (Moellering, 1992). Many times these infections are from wounds (Moellering, 1992). Bacteremia is the third most common infection (Caballero-Granado et al., 2001; Song et al., 2003; Teixeira et al., 2011). It is caused by the organism entering the bloodstream from sources such as a UTI or an intraabdominal abscess (Caballero-Granado et al., 2001; Song et al., 2003; Teixeira et al., 2011). Patients undergoing enterococcal bacteremia have an increased likelihood of developing endocarditis (Caballero-Granado et al., 2001; Song et al., 2003; Teixeira et al., 2011). It is rare to see meningitis as a consequence of enterococcal bacteremia but when it does occur it is normally diagnosed primarily in neonates (Moellering, 1992). Finally, there has been some evidence that enterococci are associated with dental infections (Sundqvist et al., 1998; Kayaoglu and Ørstravik, 2004). If the dental infection progresses to a bacteremia, like it can with *Streptococcus mutans* and *Eikenella corrodens*, then it could cause endocarditis.

Again, enterococcal endocarditis is an infection that can lead to significant morbidity and mortality (Caballero-Granado et al., 2001; Song et al., 2003) and can occur from sources of infection involving the genitourinary tract, the gastrointestinal tract, and wound infections and, as stated above, bacteremia (reviewed in Megran, 1992). The mortality associated with endocarditis is 20%–40% with appropriate antibiotic treatment (reviewed in Megran, 1992) and 17%–100% without appropriate antibiotic treatment (reviewed in Megran, 1992). Unfortunately, a relapse rate of 0–14% can occur for those patients who were originally treated efficaciously (reviewed in Megran, 1992). Given the emergence of antibiotic resistant enterococci, endocarditis is likely to remain a common cause of morbidity and mortality. In addition, developing world countries that lack an adequate healthcare system and the funding needed for medicines to treat patients or monitor patient treatment are likely to see enterococcal endocarditis as a continual problem.

Antibiotic resistance to antimicrobial agents

Traditionally, treatment for enterococcal infections consisted of the synergistic effects of an aminoglycoside and a cell wall-active antibiotic such as ampicillin or vancomycin. In addition, careful usage of antibiotics and implementation of infection control practices is believed to reduce the risk of these infections. Unfortunately, the enterococci are becoming significant pathogens worldwide, especially with respect to nosocomial and iatrogenic infections, largely due to their ability to resist antibiotics (reviewed in Moellering, 1992; Teixeira et al., 2011). This can be attributed to the fact that these organisms have been exposed to multiple antibiotics in hospital settings, thus providing them with an evolutionary pressure for selective advantage (reviewed in Moellering, 1992). As already stated, the enterococci have evolved mechanisms for the transfer of resistance genes through exchanging plasmids via conjugation, as well as exchanging resistance genes on transposons or via bacteriophages (Clewell, 1990; Dunny, 1990; Scott, 1992; Clewell, 1993; reviewed in Jett et al., 1994; Mazaheri Nezhad Fard et al., 2011; Vinodkumar et al., 2011). Mutations can also lead to high-level resistance to various antibiotics. Of greatest concern is that the enterococci reside in the gastrointestinal tract where they come into contact with both Gram-negative and Gram-positive organisms and are capable of exchanging resistance genes with other bacterial genera (i.e. staphylococci, streptococci) (Clewell, 1990; Sung and Lindsay, 2007).

Strains of enterococci have now been discovered that are resistant to a plethora of antimicrobial agents: some have acquired resistance to aminoglycosides via aminoglycoside-modifying enzymes, tetracyclines via genes such as *tetM* and *tetN*, chloramphenicol via chloramphenicol acetyltransferase, and the glycopeptides via enzymes that modify the vancomycin target or remove the susceptible target (reviewed in Courvalin, 2006). There are also strains of enterococci that produce β-lactamases that are capable of inactivating penicillin, ampicillin, and related drugs from transferable plasmids (Herman and Gerding, 1991; Teixeira et al., 2011).

In terms of vancomycin resistance, six gene clusters have been discovered that appear to be associated with this phenotype (VanA to VanG) (Fisher and Phillips, 2009). Depending on the cluster, the genes can be either plasmid borne or chromosomal, constitutive or inducible (Fisher and Phillips, 2009). Within the VanA cluster, genes *VanS* and *VanR* are involved in a two-component system that can alter

the enterococcal cell wall composition from the peptidoglycan precursor (D-Ala-D-Ala) to D-Ala-D-lactate (D-Lac). Vancomycin targets the precursor but has reduced affinity for D-Lac, which is the basis for the vancomycin resistance (Stephenson and Hoch, 2002; Fisher and Phillips, 2009).

Some enterococcus strains have the potential to be reservoirs of glycopeptide resistance genes and transfer them to more virulent pathogens such as methicillin-resistant *Staphylococcus aureus* (Noble et al., 1992; Morrison et al., 1997; Sung and Lindsay, 2007). The VanA (with high-level resistance to vancomycin and teicoplanin) and VanB (with variable resistance to vancomycin only) phenotypes are the most common forms of acquired glycopeptide resistance, and these phenotypes are transferable via plasmids or transposons (Woodford et al., 1995). The VanA phenotype has been found in approximately 60% of vancomycin-resistant enterococci (VRE) and VanB found in approximately 40% (Clark et al., 1993). It has been demonstrated that the genes conferring the VanA phenotype could be transferred from *E. faecium* to MRSA, thus heightening concern (Noble et al., 1992).

The problem of vancomycin resistant strains of enterococci is unlikely to abate in the near future. Indeed, even laboratory stocks appear to have been penetrated by the vancomycin resistant phenotype (Vu and Carvalho, in preparation).

Identification of the genus

In order for the enterococci to be identified as disease causing agents, they must be isolated from hospital patients and identified to the genus and species level in clinical laboratories. Clinical microbiologists presently identify enterococci to the genus level via classical culture and biochemical techniques. Enterococci, being fastidious organisms, grow readily on nonselective media such as blood agar and chocolate agar, but "enriched sheep blood agar" enhances their growth (Murray et al., 2009; Teixeira et al., 2011). After 24 h of incubation on blood agar, bacterial colonies may appear as non-hemolytic, or may present as α-hemolytic, or, rarely, β-hemolytic depending on their ability to partially or fully destroy red blood cells (Murray et al., 2009). During microscopy, the enterococci appear as Gram-positive in the Gram stain, ovoid in shape, and arranged in pairs and short chains (Murray et al., 2009; Teixeira et al., 2011). Unfortunately, microscopic examination cannot differentiate *Enterococcus* from *Streptococcus pneumonia* because they both have similar morphologies (Murray et al., 2009). Like the streptococci, enterococci are catalase-negative (Devriese et al., 2006). However, they differ from the streptococci by their ability to survive and grow in high salt concentrations (6.5% NaCl), proliferate within a broad temperature range (5–50°C), withstand pH extremes (4.8 to 9.6), and resist bile salts (Devriese et al., 2006; Fisher and Phillips, 2009; Murray et al., 2009; Teixeira et al., 2011). The enterococci contain the Group D antigen in their cell walls that reacts with Lancefield group D antiserum, but several streptococcal species are also known to react positively (Devriese et al., 1993).

Aside from presumptive identifications based on growth and colony characteristics, the enterococci can be determined to the genus level on the basis of biochemical reactions. They are resistant to the antibiotic optochin, do not dissolve when exposed to bile, and produce L-pyrrolidonyl arylamidase (PYR) (Devriese et al., 2006; Murray et al., 2009; Teixeira et al., 2011). The Voges-Proskauer (VP) test for the presence of acetoin production and the fermentation of the carbohydrate ribose are other useful differential biochemical tests since most enterococcus species give positive results (Devriese et al., 1993). The enterococci also reveal positive reactions in the β-glucosidase and leucinearylamidase tests (Devriese et al., 1993). Biotyping can help with differentiating the enterococci by revealing their carbohydrate utilization and enzymatic patterns, and is typically performed using a series of tubes containing different carbohydrate sources and indicator dyes (Domig et al., 2003). To reduce time and media usage, miniaturized tests were developed with biochemical reagents incorporated all into one strip (Domig et al., 2003). The strips allow an indicator-based determination of carbohydrate utilization and show reactions based on the presence or absence of microbial enzymes (Domig et al., 2003).

Identification of the species

Although phenotypic and biochemical tests may help in the presumptive identification of the *Enterococcus* genus, nucleic acid sequencing and molecular methods are required to differentiate one *Enterococcus* species from another—a costly, time consuming, and technically challenging set of procedures for laboratory technicians that, in themselves, do not necessarily lead to speedy identification of bactericidal antibiotics. Generally, the advantages of molecular techniques are their sensitivity, specificity, safety, speed, and simple interpretation providing that the laboratory employing them has the funding for skilled personnel and purchasing of the materials required. Examples of the molecular methods that can be used to identify and differentiate the enterococci are: polymerase chain reaction (PCR), 16S rDNA (rDNA) nucleic acid sequencing, protein fingerprinting by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), multilocus enzyme electrophoresis (MLEE), pulse field gel electrophoresis (PFGE), rRNA gene restriction analysis (ribotyping), or nucleic acid hybridization (Domig et al., 2003). Of all of these molecular methods, the ones most likely to be used in clinical diagnostic laboratories are PCR and nucleic acid sequencing. However, by far, commercial kits based on physiological tests are the dominant method of choice for hospital laboratories, but these kits are more effective with identifying enterococci to the genus, as opposed to species and strain, level.

The polymerase chain reaction (PCR) has become a powerful molecular tool for bacterial identification, and scientists have developed a rapid PCR assay for the detection of the enterococci to the genus level by targeting the *tuf* gene (elongation factor EF-Tu) (Ke et al., 1999). A multiplex PCR for the identification of the enterococci to the species level was developed by targeting the D-alanyl-D-alanine ligase (*ddl*) gene enabling the identification to the species level of *E. faecium* and *E. faecalis* (Dutka-Malen et al., 1995). The targeting of the D-alanyl-D-serine ligase gene also enabled the identification to the species level of *E. gallinarum* and *E. casseliflavus* (Dutka-Malen et al., 1995). Unfortunately, the multiplex PCR approach fails to identify some *Enterococcus* species if they have nucleic acid targets that reside outside the reach of the primers. As a result, 16S rDNA nucleic acid amplification and sequencing is also required (Williams et al., 1991; Patel et al., 1998; Monstein et al., 2000). Unfortunately, both multiplex PCR and nucleic acid sequencing technologies are not commonly used in developing nations for a variety of different reasons (see below).

PCR has largely overtaken both rRNA gene restriction analysis (ribotyping) and multiplex nucleic acid hybridization as the method of choice for bacterial species identification for most bacteria. Ribotyping, by contrast, has been used in research laboratories, whereby the bacterial chromosomal DNA is isolated, digested into small fragments, transferred to nitrocellulose or nylon membranes, and then probed with sequences from rRNA (Farber, 1996). So far, ribotyping can discriminate species, but is unable to distinguish strains (Domig et al., 2003). The commercially available Accuprobe system for nucleic acid hybridization (Gen-Probe, San Diego, USA) was used to identify 23S rRNA of various *Enterococcus* species (Betzl et al., 1990; Daly et al., 1991) and this kind of technology has been applied to microtiter plates (Behr et al., 2000).

SDS-PAGE of *Enterococcus* proteins, though employed less commonly in clinical (as opposed to research) laboratories, can be used to compare protein profiles among the different *Enterococcus* species to aid in identification since each *Enterococcus* species will have its own bacterial fingerprint (Domig et al., 2003). Multilocus enzyme electrophoresis (MLEE) and pulsed-field gel electrophoresis (PFGE) have also been used to identify enterococci, but more so in research laboratories as opposed to hospital sentinel laboratories (Domig et al., 2003), and MLEE appears to be more effective at recognizing relationships among large numbers of clinical isolates (Tomayko and Murray, 1995). Unlike general gel electrophoresis of DNA, PFGE separates large fragments of DNA through the use of a pulsed electrical field that flows through the gel and provides specific, bacterial DNA fingerprints (Domig et al., 2003). PFGE combined with digestion by rare-cutting endonucleases allows for the differentiation of *Enterococcus* species and estimation of the sizes of their chromosomes (Domig et al., 2003). Again, like with PCR, all of these molecular techniques are not commonly employed in developing world laboratories (see below), and even in the United States the gel electrophoresis approach is not used as widely as PCR or the commercial kits that are based on physiological tests.

The challenge for clinical microbiology

The enterococci are ubiquitous organisms worldwide that colonize both animals and plants. They grow in the intestinal tract of humans and other animals, are flourishing in hospitals and communities, and are even present in the waters and sands of the world's beaches, such as the coastline of Southern California, where sewage contamination and river runoff have increased their numbers (Yamahara et al., 2007). In light of their importance for nosocomial infections, especially within developing countries that lack a quality healthcare infrastructure, it will be paramount for diagnostic laboratories around the world to be able to distinguish these agents within hospitalized patients from other bacterial genera. In addition, the diagnostic laboratories must be able to differentiate species within the *Enterococcus* genus as well as different strains within each species. Similarly, it will be critical for laboratories to perform surveillance of the antibiotic susceptibility profile of the enterococci in order to better identify the most efficacious bactericidal medicines to be administered to infected patients and to monitor patient treatment with such medicines. With regards to the first point, as stated above, enterococci can be discriminated from other bacterial genera on the basis of classical culture and basic biochemical methods (Devriese et al., 1993; Ke et al., 1999; Domig et al., 2003; Devriese et al., 2006; Lehman et al., 2007; Murray, 1990; Murray et al., 2009). However, nucleic acid or protein molecular techniques are presently required to differentiate species within the genus—a costly, time consuming, and technically challenging set of procedures for laboratory technicians (Betzl et al., 1990; Daly et al., 1991; Williams et al., 1991; Dutka-Malen et al., 1995; Tomayko and Murray, 1995; Farber, 1996; Patel et al., 1998; Behr et al., 2000; Monstein et al., 2000; Domig et al., 2003; Lehman et al., 2007; Murray et al., 2009). This is especially the problem for diagnostic laboratories in developing world countries, where hospital acquired infections have become a leading cause of death. For many of these developing countries, molecular technology has been largely absent, except for HIV/AIDS testing (where serologic tests are usually available but other molecular technology, such as the western blot, are lacking) (Archibald and Reller, 2001). The cost of molecular tests for developing nations is cited as the primary reason for their absence (Stetler et al., 1997; Wilkinson et al., 1997; Archibald and Reller, 2001; Pang and Peeling, 2007; Usdin et al., 2010). As it is, molecular testing for HIV/AIDS and tuberculosis has overwhelmed many resources within developing world hospitals, and most of the published literature addresses cost-benefit analysis for

molecular tests related to these two infectious agents, while little is published in the clinical bacteriology area for other pathogens such as the enterococci. When one observes the situation for bacterial induced diseases, one finds many alarming trends: (1) antimicrobial resistant bacteria, such as the enterococci, are on the rise in both hospitals and communities within the developing world, (2) developing world hospitals have less than basic clinical microbiology facilities, (3) developing world hospitals lack the trained staff to perform molecular testing and even basic classical culture and analysis (such as the Gram stain procedure), and (4) developing world hospitals lack adequate infection control programs and surveillance (Budavari et al., 1997; Cohen, 1997; Morrison et al., 1997; Cetinkaya et al., 2000; Archibald and Reller, 2001; Elzinga et al., 2004; Rice, 2006; Khudaier et al., 2007; Chan and Iseman, 2008; Singh 2009; Panesso et al., 2010). In light of the present trend, it is important for clinical microbiology laboratories in more underprivileged nations to invest in classical culture techniques that are rapid, cost effective, feasible within the confinements of developing world hospitals, and that can lead to effective identification of enterococci to the genus and species level. In addition, the results from these classical culture tests should aid the clinical diagnostic scientist to suggest possible treatments for enterococcal infections and help the clinical microbiologist to monitor such treatments in an inexpensive, high throughput fashion. The elaborate molecular technology employed in some of the most advanced hospitals within the United States is unlikely to be widely used in nations that are suffering from a funding crisis and personnel shortage. The biomedical research community must be realistic in its expectations of what technologies and procedures for identification and speciation of the enterococci can feasibly be adopted in developing world hospitals, especially given that most of the budget in these hospitals is earmarked for HIV/AIDS, malaria, and tuberculosis (Archibald and Reller, 2001). With regards to the second point, identification of *Enterococcus* species does not automatically yield information about what antibiotics are needed to relieve patient symptoms or to cure patients of infection. Full antibiogram diagnostic testing is normally performed to acquire such data. Consequently, it will also be important for developing world nations to establish quality clinical laboratories to perform surveillance. Unfortunately, wealthy donor nations and world health governing bodies have not recognized basic clinical microbiology as a priority. Similarly, even developing world governments have not viewed the establishment of more robust clinical microbiology laboratories as a priority, largely due to the emphasis on trying to control HIV, malaria and tuberculosis. Furthermore, there is a lack of quality clinical microbiologists practicing in these developing nations (reviewed in Carvalho, in press). One would expect that these nations might acquire assistance from wealthy donor nations in training their clinical staff, but, unfortunately, the clinical microbiology workforce in the United States and other developed nations is also dwindling due to a variety of factors (reviewed in Carvalho, in press). Consequently, basic surveillance of the enterococci and other disease causing bacteria is presently inadequate and could lead to the inability of hospitals to properly diagnose their patients and treat them effectively with the correct antibiotics in such a way as to prevent the further emergence of antibiotic-resistant pathogens (reviewed in Carvalho, in press; Vu and Carvalho, forthcoming).

In conclusion, the enterococci are likely to continue to acquire antibiotic resistance and pose a world health hazard. It is understandable why governments in the developing world are focusing their efforts and resources on curbing HIV, malaria and tuberculosis given that these diseases are harming so many more people. Nevertheless, the enterococci and other bacteria must also be addressed. Governments around the globe will need to establish quality clinical microbiology laboratories that are cost effective and train the skilled personnel needed to staff them. They will also need to fund the infection control programs necessary to thwart further hospital infections caused by the enterococci. Researchers will need to develop rapid, cost effective, high-throughput, and technically feasible diagnostic methods that can be used to identify and speciate the enterococci. These methods must be successful, but simple and cost effective enough to be adopted by developing world hospitals, which presently do not have access to the more expensive and complicated molecular technologies with respect to bacteria. Finally, extensive surveillance needs to be continually carried out with the enterococci, and, indeed, all bacterial genera to monitor the emergence of antibiotic resistance and the prevalence of the enterococci in hospitals and communities, and so curb the future threat of the enterococci in nations everywhere.

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