Chapter

The Genus *Enterococcus* and Its Associated Virulent Factors

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Abstract

Enterococci, the Gram-positive, catalase negative, non-spore forming and aero-tolerant fermentative organisms form the second largest group of bacteria studied with reference to microbial source tracking in view of their ability to survive adverse environmental conditions and adaptable nature to revolutionize from low number commensals to a predominant population of host microbiota thus creating a consequence for pathogenesis. Despite being a member of normal human intestinal flora, they are not regarded anymore as generally recognized as safe (GRAS) organisms and some of its species may turned out to be a major cause of nosocomial infections. Ecological and epidemiological studies showed that these bacteria enter in the environment via feces and colonize because of their high adaptability. The main contributors in pathogenesis of enterococci are the presence of various virulence factors and antibiotic resistance genes. This chapter aims to highlight the infections caused by enterococci and their respective virulent determinants.

Keywords: *enterococcus*, virulence, resistance, hemolysis, lactic acid bacteria, nosocomial infections

1. Introduction

Enterococci (ENT), the Gram-positive (G +ve), catalase negative, benzidine negative, non-spore forming and aero-tolerant fermentative organisms form the second largest group of bacteria studied with reference to microbial source tracking (MST) [1, 2]. It is a non-filamentous microorganism but some species like *E. casseliflavus* and *E. gallinarum* exhibit motility by scanty flagella. They produce lactic acid [L (+)- lactic acid enantiomer in case of glucose fermentation] by homofermentative Embden-Meyerhof-Parnas pathway, hence called Lactic Acid Bacteria (LAB). All the species except *E. faecalis* [(*E. fl*) (which contains lysine alanine 2–3 type)] contains lysine-D-asparagine linkages with D-isoasparagine as cross bridge in peptidoglycan. Their ability to survive in adverse environmental conditions and adaptable nature revolutionize them from low number commensals to a predominant population of host microbiota which ultimately results in creating a consequence for their pathogenesis [3]. Despite being a member of normal human intestinal flora, they are not regarded as GRAS (Generally Recognized As Safe) organisms anymore [4] as some of its species have turned out to be a major cause of nosocomial infections including hepatobiliary sepsis, urinary tract infections (UTI), surgical wound infections, endocarditis, bacteremia and neonatal sepsis [5]. From a medical perspective, ENT have been recognized as an important hospital acquired pathogen due to their ability to transfer or acquire resistance genes via

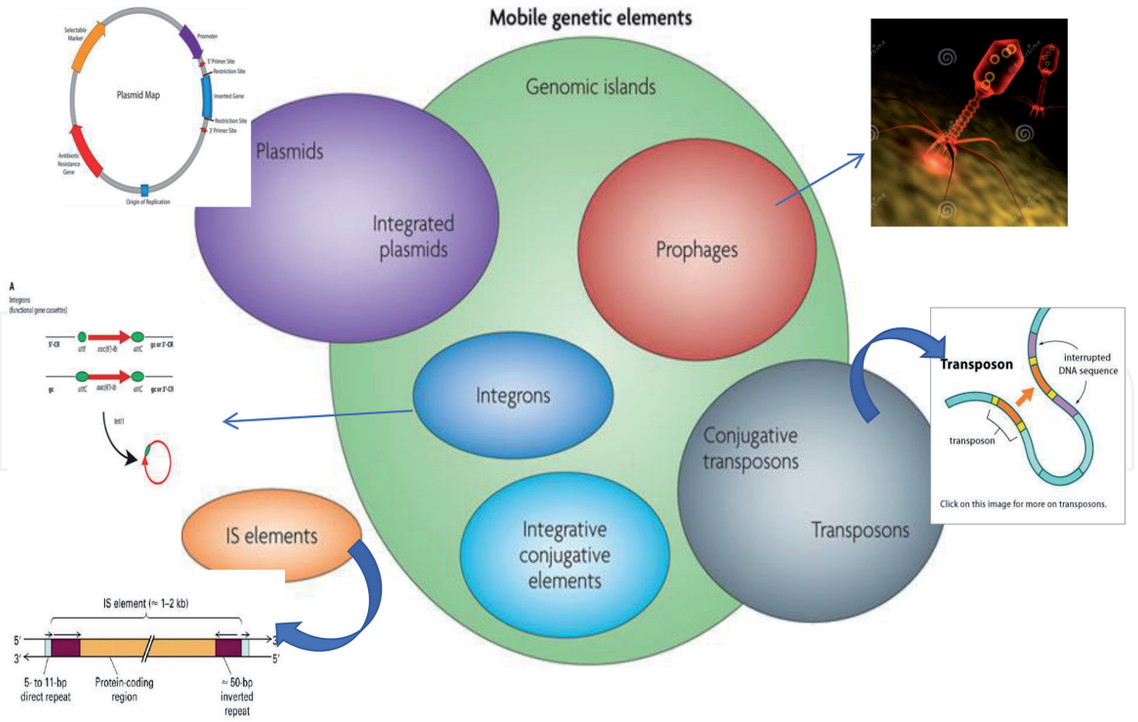


Figure 1.
Bacterial mobile genetic elements.

chromosomal exchange as well as plasmid or transposon (**Figure 1**). This can lead to increment in dangerous nosocomial infections, thus limiting therapeutic options [6]. This is the reason for exploitation of this genus as an important key indicator bacterium for humans and veterinary resistance surveillance system [7].

2. Enterococcal infections and their treatments

Over the past few decades, members of the genus *Enterococcus* have emerged as an important nosocomial pathogen causing different infections. Their transformation from gut commensal to pathogen is attributed by increasing antibiotic resistance especially resistance to vancomycin, high-level aminoglycosides (HLA), and penicillin is of interest. Moreover, resistance to new antimicrobial agents, like linezolid, quinupristin/dalfopristin, and daptomycin has also been emerged (**Figure 2**). Being more resistant than *E. fl*, *E. faecium* (*E. fm*) has come out to be the leading cause of multidrug resistant (MDR) infections in U.S. Because of its resistance to vancomycin, ampicillin and high-level aminoglycosides, infections caused by this species is very difficult to treat. According to National Healthcare Safety Network (NHSN) report, majority of device associated infections (for example, central lines infections, urinary drainage catheters infection and ventilator infections) were caused by 80% vancomycin and 90.4% ampicillin resistant *E. faecium* [8]. Other enterococcal species including *E. avium, E. casseliflavus, E. durans, E. hirae, E. raffinosus, E. gallinarum and E. mundtii* accounts for less human's infection [9]. Enterococci can cause variety of infections directly as sole cause of an infection or indirectly as a contributor in co-infection with other microorganisms [10] (**Figure 3**).

Enterococcal infections particularly those caused by vancomycin resistant enterococci (VRE) are associated with prolonged hospital stay and excess mortality. World Health Organization (WHO), in its report published in February 2017 placed Vancomycin Resistant *E. faecium* in the "HIGH PRIORITY category in global priority pathogens list (global PPL)" of antibiotic resistant bacteria to help in prioritizing

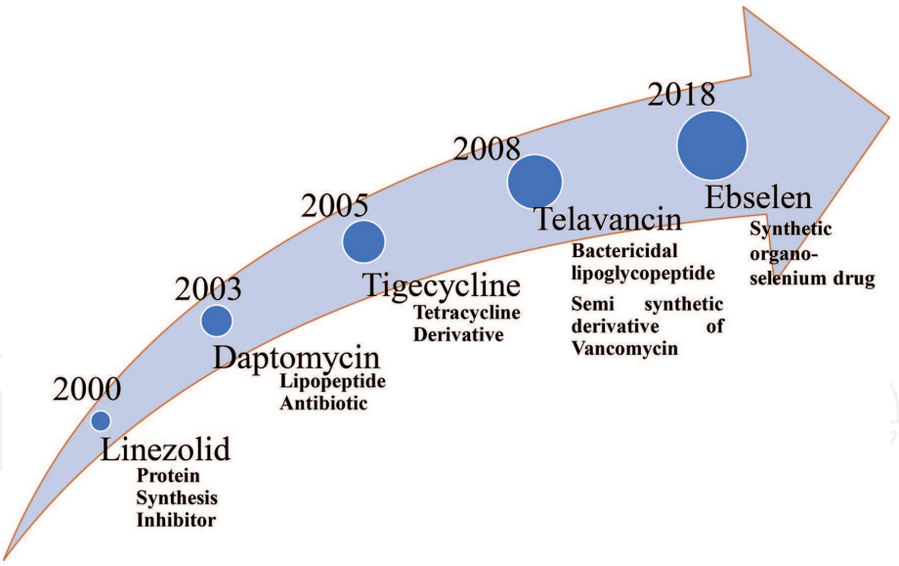


Figure 2.
Examples of recently approved drugs.

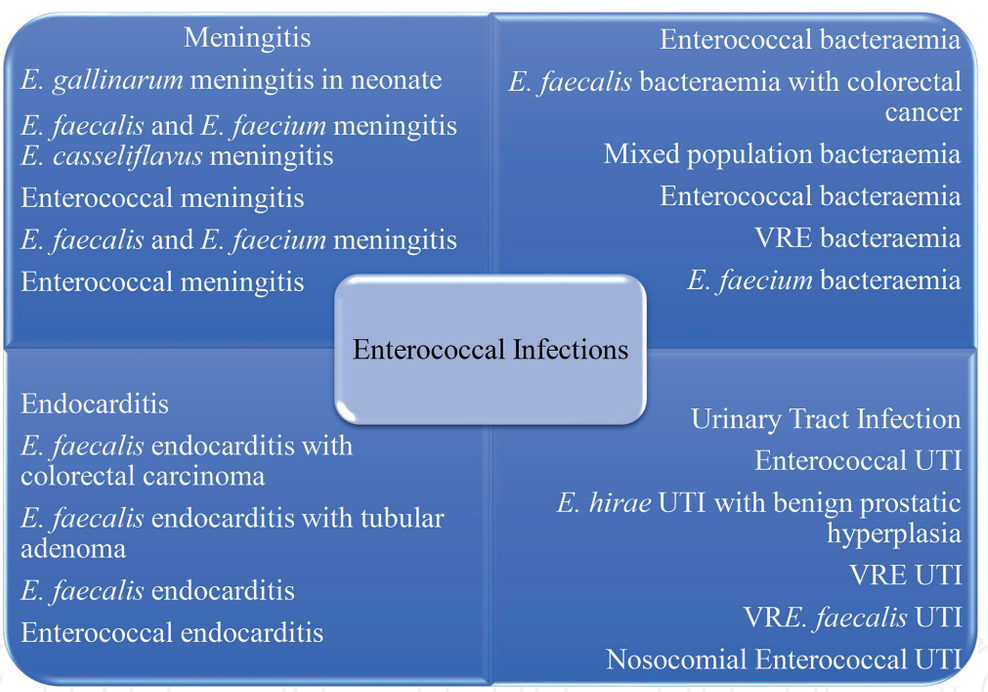


Figure 3.
Different infections caused by genus Enterococcus.

the research and development of new and effective antibiotic treatments [11, 12]. Earlier to this, VRE was also categorized as "microorganisms with a threat level of serious" with estimated 20,000 drugs resistant enterococcal infections, 1300 death tolls and 66,000 *Enterococcus* infections per year in United States [13].

2.1 Urinary tract infections (UTIs)

UTIs including prostatitis, epididymitis and cystitis are the most common types of infections caused by ENT. Majority of the patients includes older men as compared to young women. Upper UTIs which lead to bacteremia also occurred in young men [14]. According to a report presented to NHSN by center of disease control and prevention (CDC), *Enterococcus spp.* account for 14.9% of the total catheter associated UTIs between 2006 and 2007 [8]. Moreover, it is also reported

that 15% of UTIs occur in ICU setting with VRE being the major health care associated pathogen [15].

2.2 Intra-abdominal, pelvic and soft tissue infections

ENT are often recovered as a component of mixed microbial flora from cultures of pelvic, soft tissues and intra-abdominal infections. They rarely cause monomicrobial infections at these sites. Enterococcal bacteremia is accompanied with intra-abdominal and pelvic abscesses and wounds; this is the reason why many clinicians prescribe antibiotic regimens for infections at these sites [14, 16, 17]. Moreover, ENT are frequently found in cultures from foot ulcers, decubiti and in diabetics in association with osteomyelitis [15]. Tigecycline, a semi synthetic, bacteriostatic in nature analogue of TET is active against many Gram negative (G −ve) and G +ve bacteria has been used use for the treatment of skin, intra-abdominal and soft tissue infections [18].

2.3 Bacteremia

Incidence of enterococcal blood stream infections are rising day by day [19]. Starting from 6th position in early 80's, ENT is now the 2nd most common cause of health care associated bacteremia [8]. Bacteremia is designated as a major cause of mortality with *Enterococcus* spp. being the third and fourth most common etiological agent of blood stream infections in U.S and Denmark, respectively [20–22]. Genitourinary tract, intra-abdominal, biliary sources, soft tissues infections and indwelling central lines are the common sources of bacteremia from which ENT are isolated as a polymicrobial component [17]. Although enterococcal bacteremia occurs in patients with underlying immunity and illnesses, it rarely affects distant organs or cause metastatic abscesses. Usage of inappropriate antibiotics or late treatment is associated with excess mortality [19]. However, some studies found no decrease in mortality with appropriate antibiotic treatment [23, 24], while some revealed a better outcome after using appropriate antibiotics both for vancomycin and high-level gentamicin resistant enterococci [25, 26].

2.4 Endocarditis

Endocarditis is one of the major enterococcal infections for which antibiotic treatment is difficult because of enterococci's intrinsic resistance to many antibiotics. First case report of endocarditis with details of clinical and pathological description of a strain called *Micrococcus zymogens* (*Enterococcus faecalis*) was published in 1899 [27]. Since then this species is responsible for 8–17% of all infective endocarditis (IE) cases affecting mainly elderly patients with prosthetic heart valve, degenerative heart valve diseases, urinogenital or GIT infections leading to bacteremia and becoming third most frequent etiologic agent of both native and prosthetic valve IE [28–30]. In certain cases, dual antibiotic therapy including aminoglycoside (preferably gentamicin) and cell-wall synthesis inhibitor (vancomycin or β-lactam) is required for IE therapy.

American Heart Association (AHA) and European Society of Cardiology (ESC) recommends 4 to 6 weeks of combined antibiotic treatment with success rate of 80%. Due to nephrotoxic effects of long-term aminoglycoside usage, Danish guidelines on endocarditis treatment endorsed aminoglycoside usage but for 2 weeks only [30]. In case of VRE and HLGR enterococcal IE, surgery remains the only option to remove the infected valve [15]. Among *Enterococcus* spp., *E. fl* was thought to be the most common causative agent of endocarditis infecting mostly older persons as compared to women [31, 32] but recently a more problematic MDR strain of

E. fm belonging to well characterized hospital-associated clade was also identified as a cause of IE. The strains of *E. fm* has high resistance against first line antibiotics (i.e., MIC >64 mg/L ampicillin and vancomycin) due to which their application in curing IE is obsolete [33, 34]. In response to this, AHA recommends Quinupristin-dalfopristin (Q/D; 30% Streptogramin B and 70% A) and linezolid as alternate to treat MDR *E. fm* IE [35]. In fact, many reports suggest better efficacy of Q/D (24 g/day) when use in combination with imipenem, levofloxacin, doxycycline, rifampicin, high-dose ampicillin [36, 37]. Two main and critical steps in the pathogenesis of IE are attachment to tissues and production of biofilm. Biofilm associated proteins which facilitates occurrence of IE includes aggregation substance protein, i.e., *Asc10* [38], microbial surface components recognizing adhesive matrix molecules (MSCRAMM) proteins *ace* for *E. fl* [39] and *acm* for *E. fm* [40], *esp* and its homolog in *E. fm*, esp_{fm} [41, 42], endocarditis and biofilm associated pili of *E. fl*, i.e., *ebp* [42–44]. The main complication of enterococcal IE is heart failure occurring in half of the patients. Moreover, MDR *E. fm* is also an important factor in increasing epidemiology of enterococcal IE because >90% of *E. fl* are susceptible to ampicillin and vancomycin [45].

3. Pathogenesis and virulence associated with enterococci

Virulence factors are potential traits that define the pathogenesis of most infections which involves a series of events namely, colonization, adhesion to the host's cells, tissue invading and resistance to non-specific defensive mechanisms. Researchers are encouraged to characterize the factors involved in etiology of infections caused by pathogenic ENT in immunocompromised or impaired immunity patients. Two major classes of virulent factors have been well characterized: (1) surface factors that promote colonization in host cells, and (2) protein and peptides secreted by ENT that damage the tissues [46].

3.1 Gelatinase (*gelE*), serine protease (*sprE*) and *fsr* regulator

Gelatinase is a zinc metalloprotease expressed extracellularly and hydrolyze gelatin, collagen and casein [47]. It is proved to be a full virulence factor expressed in mouse model of peritonitis, endocarditis [48, 49], endophthalmitis [50], in nematode [51] and in vitro translocation [52]. It is encoded by *gelE* and *sprE* operon and expressed in regulation by a quorum sensing system encoded by the *fsr* locus [53]. The *fsr* locus (*E. fl* regulator) is a well characterized locus containing *fsrA*, *fsrB*, *fsrC* and *fsrD* genes which is homologs to staphylococcal *agrBCA* loci [54]. A signaling peptide in *fsrB* liberates gelatinase biosynthesis activating pheromone (GBAP) peptide by auto-processing and a quorum sensing system. *gelE* and *sprE* genes are induced when GBAP accumulates from exponential to stationary phase. *Fsr* regulon is present above the *sprE* and *gelE* and encode a serine protease and gelatinase, respectively [55]. Possible molecular mechanism behind the expression of *gelE* and *sprE* is shown in **Figure 4** [56]. Epidemiological data suggests the involvement of *fsr* locus and gelatinase in virulence traits, like adhesion capacity (biofilm) established by processing of C-terminal gelatinase protein [57, 58].

3.2 Catalase (EC 1.11.1.6)

Catalase is a renowned enzyme present in all three domains of life. It catalyzes the decomposition of hydrogen peroxide (HP) to water and oxygen, protecting the cell from oxidative damage of HP. HP is a reactive oxygen species (ROS) in

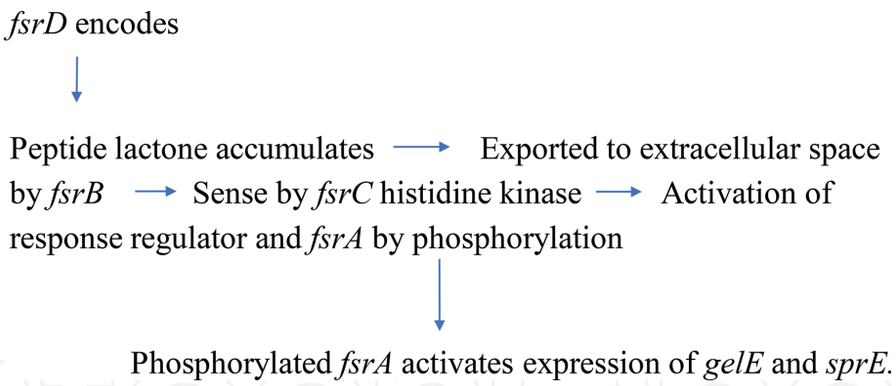


Figure 4.
Flow diagram showing the possible mechanism of gelE *and* sprE *gene expression.*

biosphere. It is produced as a by-product in aerobic metabolism such as in oxygen activation, in photosynthetic and respiratory electron transport chain and as product of oxidases activity. First step of catalase reaction is the reduction of HP to water forming cationic heam radical and an oxoiron [compound 1 (Fe^{IV}=O ion)]. In the second step, dismutation is completed by the reaction of a second HP, resulting in the release of oxygen and water. The enzyme is regenerated in the resting Fe^{III} state. NADPH binding catalases prevent the build-up of an inactive partially oxidized dead-end form of the enzyme called compound II [59].

Catalases are of three types: Prokaryotic Mn-catalases (minor bacterial protein family), bifunctional catalase peroxidases (not found in plants and animals and exhibit both catalytic and peroxidative activities) and haem catalases (most abundant group found in Archaebacteria, Eubacteria, Fungi, Protista, Animalia and Plantae). Despite catalyzing the same reaction ($2H_2O_2 \rightarrow 2H_2O + O_2$), all three families differ in architecture of active site and mode of reaction [60]. Among G +ve lactic acid bacteria (LAB), *E. fl* are unable to make porphyrin compounds, including heam groups. It exhibits catalase activity but only when it is grown in heme containing medium [61]. *E. fl* catalase (*katA*) is a homo-tetrameric protein containing only one heme group (protoheme IX) and belongs to the group of heme containing mono functional catalases [62]. In the absence of heme, *E. fl* produces NADH peroxidase (*Npr*) that degrades HP to water. Factors involve in biogenesis of catalase was not known until Baureder and Hederstedt [62] carried out a research in which they used two different transposon systems to construct libraries of *E. fl* mutants and screened for clone defective in catalase activity by using colony zymogram staining procedure. They identified nine genes (in addition to *katA*, which codes for catalase enzyme protein) distributed over five chromosomal loci which are important for expression of catalase activity in *E. fl*. The proteins encoded by those genes have diverse functions such as NADH oxidation and HP detoxification (*npr*), global regulation of RNA turnover (*rnjA*, *srmB*), membrane transport (*oppBC*) and/or stress response (*etaR*) [62].

3.3 Hyaluronidase (EC 4.2.2.1)

These are the enzymes capable of degrading hyaluronate (Hyaluronic acid, hyaluronan) found in several body parts, like umbilical cord, synovial fluid, cartilage, brain, muscles and extracellular matrix (ECM) in connective tissues. Almost half of the total body hyaluronate is found in the skin. The viscous ground substance release by the connective tissues provides a barrier for the entry of bacteria or toxin into the body. However, ground substance contains hyaluronate as a major component which is degraded by hyaluronidases. Rooster's combs and certain bacteria like

streptococci also produce hyaluronidases [63]. Many pathogenic bacteria release some extracellular products which helps them in damaging the tissue thus acting as a virulent factor and smoothen the progress of bacterial toxin into the tissues and are commonly named as "spreading factors." Bacterial hyaluronidases (BH) are among some of the spreading factors released by certain G +ve and G −ve bacteria. BH belongs to the third type of hyaluronidases commonly called as hyaluronate lyases. They eliminate β 1–4 linkage resulting in the production of unsaturated disaccharides by acting as endo-*N*-acetylhexosaminidases [63]. Different models of *E. fm* trans conjugant's virulence that harbors conjugative mega plasmid have been reported [64, 65] to carried *hyl* gene. According to some previous studies, the *hyl* gene was more prevalent in clinical isolates rather than community base isolates. According to a recent study, *hyl* gene is considered as a passive marker of virulence because deletion of this gene caused no effect on mouse peritonitis model [66, 67].

3.4 Cytolysin (Cyt)

Enterococcal Cyt is a broad range prokaryotic and eukaryotic lysin usually plasmid encoded. It is reported to enhance virulence of *E. fl* in animal models. It was originally described as lanthionine-containing bacteriocins of G +ve bacteria [68]. The Cyt operon is a part of *E. fl* PAI consisting of 6 genes related to toxin biosynthesis and two promoters namely P_L (involve in regulation of transcription of genes related to toxin structure and function) and P_{REG} (involve in transcription of regulatory genes) and present near *esp* gene [69]. Like gelatinase, expression of Cyt is quorum sensing dependent and regulated by two component systems [70]. The regulatory system of Cyt consists of two open reading frames (ORFs) namely *cylR1* and *cylR2* which encodes a transmembrane protein of unknown function (cylR1) and a helix-turn-helix DNA binding protein (cylR2) [71]. The Cyt operon is either present on conjugative pheromone responsive plasmid such as pAD1 [72] or encoded by chromosome within 150 kb PAI [73, 74]. Todd et al. [75] conducted the first comprehensive study on hemolysin molecule after the observation of hemolysis zones on blood agar plates produced by *E. fl*. Increased virulence due to Cyt in *E. fl* was first described in the study of Ike and colleagues [76] through dose dependent intraperitoneal injections of *E. fl* strains harboring plasmid pAD1 which encodes Cyt. Later, various researchers showed the lyses of mouse erythrocytes, macrophages, and PMNs or death of experimental animals/organism like mouse, rabbits and *C. elegans* with Cyt [58, 73, 77–80]. Self-lysis of Cyt producing cells is prevented by an unknown mechanism. However, immunity proteins or ABC transporters protects other lantibiotic producing bacteria from self-lysis [81, 82]. In *E. fl*, a zinc metalloprotease and transmembrane protein, CylI (immunity factor) is shown to protect from Cyt mediated bacterial cell death [83].

Despite having a virulence face, Cyt can also act as beneficiary trait for both *E. fl* and its host. Possible beneficial activities might include, acting as colonization factor, providing self-defense against something which is more harmful (probably an intestinal parasite), facilitating nutrient acquisition from prokaryotic or eukaryotic sources, function as signaling molecule to monitor bacterial population size and probe the environment for target cells and last but not the least, bacteriocin activity of Cyt allows *E. fl* to occupy a novel host niche which non-cytolytic bacteria cannot access [68, 84, 85].

3.5 Enterococcal surface protein (*esp*)

Esp, a putative virulent factor is found in both *E. fl* and *E. fm*. It is located on pathogenicity island (PAI) at the surface of the bacterium [56]. It was initially

identified in a highly virulent gentamicin resistant strain of *E. fl* [69]. *Esp* shares global structural similarity with *Streptococcus agalactiae* Rib [86], *S. pyogenes* R28, C-alpha protein, and *S. aureus* Bap (biofilm associated protein) [87]. These similarities are restricted to a highly conserved region within the C repeat units of *Esp* proteins and group A and B of streptococcal proteins of streptococci and nonrepeat N-terminal region of Bap protein [71]. Bap protein from *S. aureus* is associated with biofilm formation and shares a sequence and structural similarity with *Esp*. *Esp* is also associated with *E. fl* biofilm formation on different surfaces, like polysterylene plates and hospital equipment like catheters, prosthetic heart valves, orthopedic appliances, artificial cardiac pace makers [47], ureteral stents [88], intravascular catheters [89], silicone gastrostomy devices [90], and biliary stents [91].

A variant of *Esp* is also reported in *E. fm* isolates [92]. *E. fm esp* is predominantly present in nosocomial settings in contrast to *E. fl esp* which is widely distributed among environmental strains [93, 94]. Expression of *esp* is affected by environmental conditions like temperature (maximum at 37°C) and availability of oxygen, i.e., under anaerobiosis [56]. Several research groups demonstrated the role of *E. fm esp* in pathogenesis of experimental endocarditis, UTIs, and bacteremia. While no specific role of *esp* was found in peritonitis, and colonization of GIT [95, 96]. Role of *esp* was also established by a genetic approach. In a study conducted by Tendolkar *et al.* [97], *esp*-lacking *E. fl* strains produced biofilm in large amounts after successful induction and expression of *esp* gene. In contrast, several studies suggest that *esp* is not necessary for biofilm formation [98, 99]. Study conducted by Kristich *et al*. [100] demonstrated that *E. fl* OG1RF produced biofilms not only in the absence of *esp* and entire PAI that harbors it. In other studies, conducted on clinical enterococcal isolates, majority of the *esp*-negative isolates produced biofilms and no correlation was found among *esp* gene and biofilm forming capacity [89, 101].

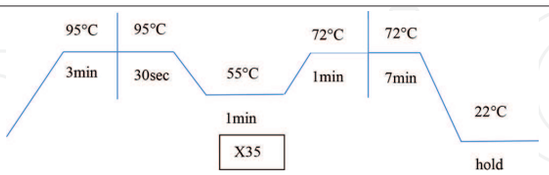
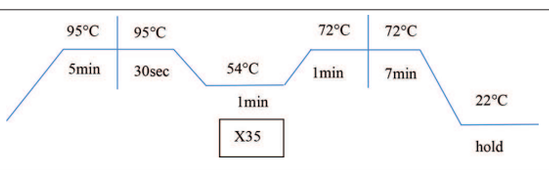
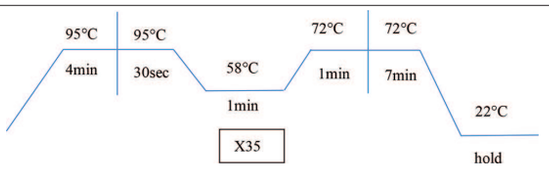
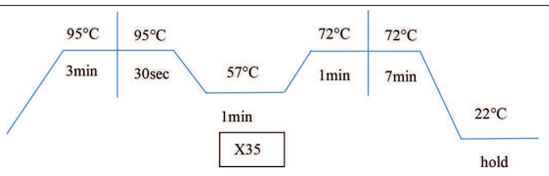
3.6 Aggregation substance (AS)

AS is a group of proteins encoded by pheromone-induced conjugative plasmids. AS directed bacteria to aggregate which results in close cell contact between donor and recipient. Several studies showed that AS mediated internalization of *E. fl* by cultured human intestinal epithelial cells and increased in vitro adhesion to cultured renal tubular cells [102]. Among the best studied AS proteins are Asa I, Asp I, and Acs 10 encoded by *asa1*, *aspI* and *prgB* genes of conjugated plasmids *pAD1* and *pCF10*, respectively, and shows >90% sequence identity [56, 66]. These proteins contain an N-terminal domain, a central domain, a variable region and two Arg-Gly-Asp (RGD) motifs which are also found in fibronectin and associated with integrin binding proteins [102, 103]. Apart from their function in conjugation transfer, these RGD motifs are also involve in eukaryotic cell binding and binding to renal epithelial cells [102]. It is demonstrated in a study that central domain and N-terminal domain are responsible for aggregation of Asc 10 [104]. Beside its role in conjugation, AS also serves as a virulence factor in *E. fl* by promoting cell-cell contact, adhesions to host cells and ECM proteins (including thrombospondin, fibronectin, vitronectin, and collagen type I), increased vegetation in experimental endocarditis, resistance to killing by polymorphonuclear leukocytes (PMNs) by inhibition of respiratory burst (production of ROS) in the macrophages, increased cell surface hydrophobicity [71]. All the proteins aid in the pathogenesis of AS in *E. fl,* like Asa I increases adherence to human macrophages and renal tubular cells, Asc 10 facilitates internalization and intracellular survival in PMNs [74, 103, 105]. Both

Virulent factors	Gene	Primer sequence (5′-3′)	Product length (bp)	Reference
Biofilm associated genes	*espTIM*	CTT-TGA-TTC-TTG-GTT-GTC-GGA-TAC TCC-AAC-TAC-CAC-GGT-TTG-TTT-ATC	475	[111]
	agg	AAG-AAA-AAG-AAG-TAG-ACC-AAC AAA-CGG-CAA-GAC-AAG-TAA-ATA	1553	[112]
	acm	GGC-CAG-AAA-CGT-AAC-CGA-TA CGC-TGG-GGA-AAT-CTT-GTA-AA	353	[113]
	efaAfm	AAC-AGA-TCC-GCA-TGA-ATA CAT-TTC-ATC-ATC-TGA-TAG-TA	735	[92]
	efaAfs	GAC-AGA-CCC-TCA-CGA-ATA AGT-TCA-TCA-TGC-TGT-AGT-A	705	
	asa	GCA-CGC-TAT-TAC-GAA-CTA-TGA TAA-GAA-AGA-ACA-TCA-CCA-CGA	375	[114]
	ace	AAA-GTA-GAA-TTA-GAT-CCA-CAC TCT-ATC-ACA-TTC-GGT-TGC-G	320	[115]
	ccf	GGG-AAT-TGA-GTA-GTG-AAG-AAG AGC-CGC-TAA-AAT-CGG-TAA-AAT	542	[112]
	cpd	TGG-TGG-GTT-ATT-TTT-CAA-TTC TAC-GGC-TCT-GGC-TTA-CTA	782	
	cob	AAC-ATT-CAG-CAA-ACA-AAG-C TTG-TCA-TAA-AGA-GTG-GTC-AT	1405	
	eep	GAG-CGG-GTA-TTT-TAGTTC-GT TAC-TCCAGCATTGGATGCT	937	
Gelatinase operon genes	*gelE*	ACC-CCG-TAT-CAT-TGG-TTT ACG-CAT-TGC-TTT-TCC-ATC	419	[116]
	sprE	TTG-AGC-TCC-GTT-CCT-GCC-GAA-AGT-CAT-TC TTG-GTA-CCG-ATT-GGG-GAA-CCA-GAT-TGA-CC	591	
	fsrA	ATG-AGT-GAA-CAA-ATG-GCT-ATT-TA CTA-AGT-AAG-AAA-TAG-TGC-CTT-GA	740	
	fsrB	GGG-AGC-TCT-GGA-CAA-AGT-ATT-ATC-TAA-CCG TTG-GTA-CCC-ACA-CCA-TCA-CTG-ACT-TTT-GC	566	
	fsrC	ATG-ATT-TTG-TCG-TTA-TTA-GCT-ACT CAT-CGT-TAA-CAA-CTT-TTT-TAC-TG	1343	
Cytolysin operon genes	$cylL_L$	GAT-GGA-GGG-TAA-GAA-TTA-TGG GCT-TCA-CCT-CAC-TAA-GTT-TTA-TAG	253	[117]
	$cylL_s$	GAA-GCA-CAG-TGC-TAA-ATA-AGG GTA-TAA-GAG-GGC-TAG-TTT-CAC	240	
	cylM	AAA-AGG-AGT-GCT-TAC-ATG-GAA-GAT CAT-AAC-CCA-CAC-CAC-TGA-TTC-C	2940	
	cylB	AAG-TAC-ACT-AGT-AGA-ACT-AAG-GGA ACA-GTG-AAC-GAT-ATA-ACT-CGC-TAT-T	2020	
	cylA	ACT-CGG-GGA-TTG-ATA-GGC GCT-GCT-AAA-GCT-GCG-CTT	688	

Virulent factors	Gene	Primer sequence (5′-3′)	Product length (bp)	Reference
Enterocin genes	*entA*	ATG-AAA-CAT-TTA-AAA-ATT-TTG-TCT-ATT-AAA-G TTA-GCA-CTT-CCC-TGG-AAT-TGC-TCC	1770	[118]
	entB	AGA-CCT-AAC-AAC-TTA-TCT-AAA-G GTT-GCA-TTT-AGA-GTA-TAC-ATT-TGC	126	
	entP	ATG-AGA-AAA-AAA-TTA-TTT-AGT-TTA-GCT-CTT-ATT-GG TTA-ATG-TCC-CAT-ACC-TGC-CAA-ACC-AG	216	
	Ef1097	GGC-GAT-GGC-ATT-ACT-AAT-GAC-ATT-AGG CTT-AGC-CCA-CAT-TGA-ACT-GCC-CAT-AAA-GC	408	
	enlA	CGA-TTT-CTG-TTG-TAG-GAA-CC GTA-CAT-CTC-CAT-ATA-CTT-TTC-C	1405	
Insertion sequence element gene	*IS16*	CATG-TTC-CAG-CAA-CCA-GAG TCA-AAA-AGT-GGG-CTT-GGC	547	[111]
Hyaluronidase gene	*hyl*	ACA-GAA-GAG-CTG-CAG-GAA-ATG GAC-TGA-CGT-CCA-AGT-TTC-CAA	276	[119]
Catalase gene	*kat*	ACC-CCG-TAT-CAT-TGG-TTT ACG-CAT-TGC-TTT-TCC-ATC	419	[110]
Lipase gene	*Lip-fm*	TTG-AGC-TCC-GTT-CCT-GCC-GAA-AGT-CAT-TC TTG-GTA-CCG-ATT-GGG-GAA-CCA-GAT-TGA-CC	591	[108]
	Lip-fl	ATG-AGT-GAA-CAA-ATG-GCT-ATT-TA CTA-AGT-AAG-AAA-TAG-TGC-CTT-GA	740	

Table 1.
List of primers reported for the genotypic assessment of major virulence factors.

	Thermal cycler programme	Gene name (Reference)
A		*gelE*, *sprE*, *fsrA*, *fsrB*, *fsrC* genes [116]
B		*lip-fm* and *lip-fl* genes [108]
C		*kat* gene [110]
D		$cylL_L$, *cylM*, *cylB*, *cylA* [117]

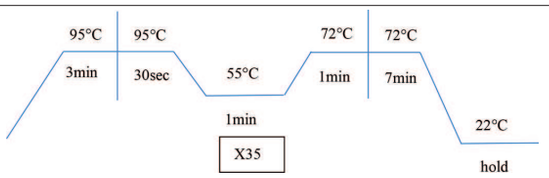
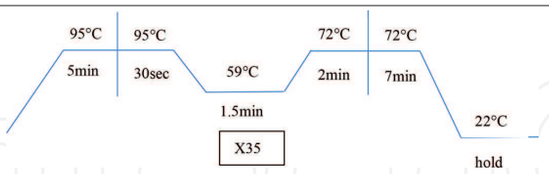
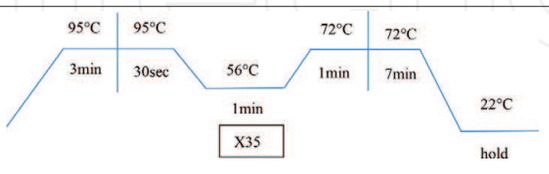
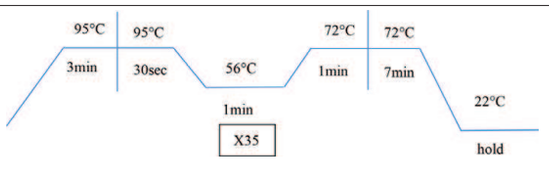
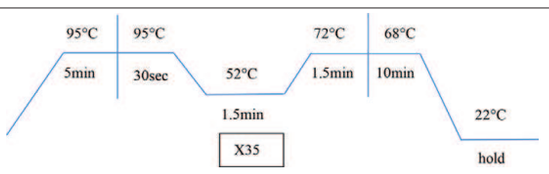
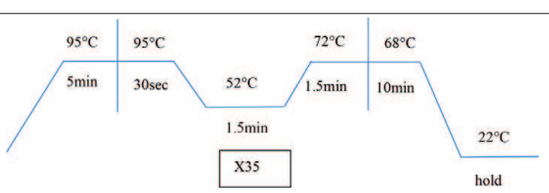
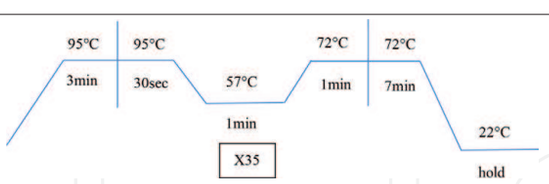
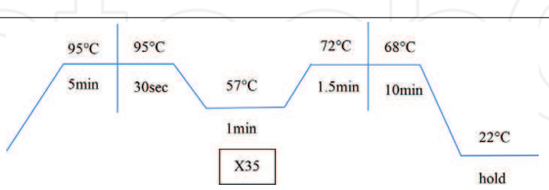
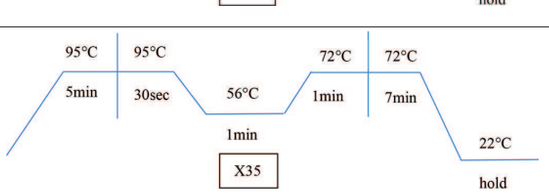
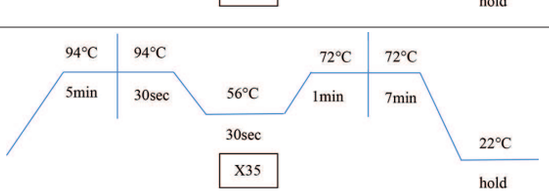
	Thermal cycler programme	Gene name (Reference)
E		*efaAfs* and *efaAfm* [92]
F		*espTIM* [111]
G		*acm* gene [113]
H		*ace* and *asa1* gene [114, 115]
I		*agg* or *AP* gene [112]
J		*ccf*, *cpd*, *eep*, *cob* genes [112]
K		*entP, entA, entB, ef1097* gene [118]
L		*enlA* gene [118]
M		*IS16* gene [111]
N		*Hyl gene* [119]

Table 2.
Illustrations for the PCR conditions for the amplification of various putative virulence genes.

Asa I and Asc 10 increase virulence of *E. fl* in rabbit endocarditis model by increasing adherence to certain ECM proteins [79, 106].

4. Conclusions

In conclusion, acquired resistance to certain antibiotics is an important feature of the genus *Enterococcus*. Persistent use of antibiotics in humans and animals for therapy and as growth promoters plus the presence of insertion sequences, transposons, integerons and plasmids make them large reservoirs of transferable antibiotic resistance and virulence genes in various ecosystems including soil, water, and food. Due to its rapid popularity, as resistant bacteria, ENT serves as an important key indicator in the surveillance of many humans and veterinary resistance profile. Adherence capability plus antibiotic resistance make them more problematic for effective therapeutic decisions. Till now only food consumption is considered as an option for the spread of antibiotic resistant bacteria to humans but the detection of resistant bacteria in soil opens a new route for the exposure of environmental antibiotic resistance to humans. Results of different studies from our lab concludes that soil, poultry, animals and birds carried high burdens of ENT which are fully armed with potential virulent and antibiotic resistance genes [107–110]. In Pakistan, there is paucity of information regarding prevalence, types and genetic characteristics of enterococci along with their resistance/virulence genes and clones especially from clinical and other environmental sources. In this respect, regular environmental monitoring using most advance molecular genotyping (**Tables 1** and **2**) as routine testing is recommended. Genes mirror the requirements of life. As our understanding of enterococcal genomics grows, bacterial genomics will become an important tool for providing new insights into the nature, biology and habitats of the enterococci. Presence of insertion sequence (IS16) gene in soil isolates verified the dissemination of hospital associated ENT into the environment via inappropriate handling of hospital wastes [108]. It is therefore also recommended to dispose clinical/hospital waste properly and appropriately.

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