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# Phylogenetic Analysis of the Virulence and Antibiotic Resistance Genes in *Enterococcus* Species *in-silico.*

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**ABSTRACT**

The presence virulence determinants and antibiotic resistance patterns in 13 *Enterococcus* species were investigated through *in silico* tools. Isolate NC_017312*Enterococcus faecalis* 62 was found to harbour the *esp* gene that might be responsible for persistence in urinary tract epithelial cells. About 30.77% (n=4) isolates harboured aggregation substance, *agg* gene. The *ace* gene, responsible for endocarditis, was found in only one isolate. About 38.46% (n=5) isolates were found to harbour *efaA* gene which is known to enable the adherence to biotic and abiotic surfaces and evade immune response. As for adhesins, *efaA* was more prevalent (38.46%) gene followed by *agg* (30.76%), *ace* and *esp* (7.69%). Four isolates (30.76%) had *pilB* gene while 2 isolates (15.38%) had *pilA* gene. Very low prevalence of putative glycosyltransferase, *hyl* gene was found that may play insignificant role in the pathogenicity of enterococcal infections. Three isolates (23.08%) were found to harbour both *sgrA* and *ecbA* genes. The genes for *cylL*$_L$and *cylM* were each present in one isolate only. None of the isolate harboured *cylA* and *cylB*that may result in lack of genes in *cyl* operons and produce haemolytic-negative strains. High level of erythromycin, *ermB* (30.77%) and tetracycline, *tetM* (23.08%) resistance genes were encountered. Isolate NC_004668 *Enterococcus faecalis* V583 had the *aac(6')-Ie-aph(2")-Ia*gene while no isolate was detected to harbour *vanA* gene. Adhesin genes were not detected in genotype 1 that contained *E. faecium* strains only. Cytolysin genes were present in genotype 3 while other virulence genes were present in only genotype 1. Genotype 2 harboured no antibiotic resistance genes. So, antibiotic resistance genes were randomly distributed within the genotypes while virulence gene distribution patterns were dependent on genotype. The data generated here may serve as a basis for additional surveillances studies of infections caused by *Enterococcus* species and help to choose effective antibiotic for the treatment of enterococcal infections.

**Keywords:** *Enterococcus*, Virulence genes, Antibiotic resistance genes, Genotyping.

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## INTRODUCTION

Gastrointestinal tract of humans and animals are the reservoir of *Enterococcus* species [1]. Anatomical sites such as the vagina and oral cavity also harboured enterococci was previously reported [2]. *Enterococcus* species mainly *E. faecium* and *E. faecalis* are the leading cause of urinary tract infections, postsurgical wound infections, abdominal infections, endocarditis, peritonitis along with others [3,4] and are mainly responsible for 90% enterococcal infections [5]. Previous report stated that *E. faecalis* are mainly responsible for enterococcal infections but recent reports documented that 20-36% enterococcal infections are caused by *E. faecium* in U.S. hospitals [6-9]. Hospital-associated *E. faecium* strains also responsible for emerging infection in European countries was previously documented [10]. Treatment of enterococcal infections is hampered due to their adaptability in the hospital environment and intrinsic antimicrobial resistance property [11]. Virulence determinants of *Enterococcus* species help to adhere, colonize and invade into the host tissues and also produce enzyme and toxins that increase the severity of the enterococcal infections [12]. Biofilm formation capacity of *Enterococcus* species aids in disease sustenance since antimicrobial substances are unable to penetrate the biofilm forming *Enterococcus* [13]. Commensal enterococcal species can become opportunistic pathogens by transferring the virulent and antibiotic resistant genes [14]. *E. faecium* infections are difficult to treat due to the spread of the strain resistant to three major classes of antibiotics, aminoglycosides, β-lactams, and glycopeptides[5]. *E. faecium* also conferred resistance gene to other newly developed antibiotics and therefore decreased the therapeutic options [15].

The aim of the present study is to identify the virulence and antibiotic resistance genes of 13 *Enterococcus* species by *in silico* PCR and to find the distribution patterns in genotypes based on pulsed-field gel electrophoresis (PFGE).

## MATERIALS AND METHODS

**Strains used in the study:** Strains used in the study are summarized in Table 1.

**Primer used in the study:** Primers used for virulence and antibiotic resistance genes detection in the study are summarized in Table 2 and 3.

**PCR amplification:** *In silico* PCR amplification was done in the website http://insilico.ehu.eus/PCR/ [22,23].

**PFGE digestion:** Pulsed-field gel electrophoresis (PFGE) digestion was done in the websitehttp://insilico.ehu.es/digest/ [22,23]. The enzyme used for the digestion was *Mre*I. Banding patterns were scored in a binary matrix and a dendrogram was constructed using Complete Linkage method by SPSS 16 (IBM, USA).

**Table 1: Name of the isolates**

| | |
|---|---|
| 1 | NC_020995 *Enterococcus casseliflavus* EC20 |
| 2 | NC_017312 *Enterococcus faecalis* 62 |
| 3 | NC_018221 *Enterococcus faecalis* D32 |
| 4 | NC_017316 *Enterococcus faecalis* OG1RF |
| 5 | NC_004668 *Enterococcus faecalis* V583 |
| 6 | NC_019770 *Enterococcus faecalis* str. Symbioflor 1 |
| 7 | NC_017022 *Enterococcus faecium* Aus0004 |
| 8 | NC_021994 *Enterococcus faecium* Aus0085 |
| 9 | NC_017960 *Enterococcus faecium* DO |
| 10 | NC_020207 *Enterococcus faecium* NRRL B-2354 |
| 11 | NC_018081 *Enterococcus hirae* ATCC 9790 |
| 12 | NC_022878 *Enterococcus mundtii* QU 25 |
| 13 | NC_021023 *Enterococcus* sp. 7L76 draft genome |

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**Table 2: Primers for virulence genes detection**

| Gene | Primer sequence 5' to 3' | Amplicon size (bp) | References |
|---|---|---|---|
| *esp* | TTGCTAATGCTAGTCCACGACC<br>GCGTCAACACTTGCATTGCCGAA | 933 | [3] |
| *agg* | AAGAAAAAGAAGTAGACCAAC<br>AAACGGCAAGACAAGTAAATA | 1553 | [15] |
| *ace* | GAGCAAAAGTTCAATCGTTGAC<br>GTCTGTCTTTTCACTTGTTTCT | 1003 | [17] |
| *efeA* | GACAGACCCTCACGAATA<br>AGTTCATCATGCTGTAGTA | 704 | [15] |
| *pilA* | AAAACGCCACCAGAGAAGGT<br>CATTGGCGCAATCACAACCA | 459 | [18] |
| *pilB* | GATACCCAGCTGACGGCTTT<br>GGTACTGCCGAAAACGAAGC | 959 | [18] |
| *hyl* | CCCTGGACACATGAAATGCG<br>AGCATCGGCCGTTGATAGAC | 605 | [18] |
| *srgA* | CTGATCGGATTGTTTATGA<br>AATAAACTTCCCCAATAACTT | 150 | [18] |
| *ecbA* | GGAGTGAGGCTTTTAAACCAGA<br>GGAAACAGGGTACTTTG | 182 | [18] |
| *cylA* | TGGATGATAGTGATAGGAAGT<br>TCTACAGTAAATCTTTCGTCA | 517 | [15] |
| *cylB* | ATTCCTACCTATGTTCTGTTA<br>AATAAACTCTTCTTTTCCAAC | 843 | [15] |
| *cylL*$_L$ | GATGGAGGGTAAGAATTATGG<br>GCTTCACCTCACTAAGTTTTATAG | 254 | [19] |
| *cylM* | CTGATGGAAAGAAGATAGTAT<br>TGAGTTGGTCTGATTACATTT | 742 | [15] |

**Table 3: Primers for antibiotic resistance genes detection**

| Gene | Primer sequences (5'-3') | Amplicon size (bp) | References |
|---|---|---|---|
| *erm(B)* | CATTTAACGACGAAACTGGC<br>GGAACATCTGTGGTATGGCG | 405 | [20] |
| *aac(6')-Ie-aph(2")-Ia* | CAGAGCCTTGGGAAGATGAAG<br>CCTCGTGTAATTCATGTTCTGGC | 348 | [20] |
| *tet(M)* | GGACAAAGGTACAACGAGGAC<br>GGTCATCGTTTCCCTCTATTACC | 446 | [21] |
| *vanA* | GTAGGCTGCGATATTCAAAGC<br>CGATTCAATTGCGTAGTCCAA | 231 | [20] |

## RESULTS AND DISCUSSION

*In silico* pulsed-field gel electrophoresis (PFGE) analysis was performed with *Mre*I restriction digestion. CG'CCGG_CG was the recognition sequence. Twenty-six different band sizes were observed upon gel electrophoresis. Lambda ladder was used to compare the band size. Dendrogram was constructed by SPSS software (Fig 1) and grouped thirteen isolates into four genotypes. Genotype 1 and 4 contained about 30.77% of the isolates while genotype 2 and 3 contained about 15.38% and 23.08% of the isolates (Fig 2).

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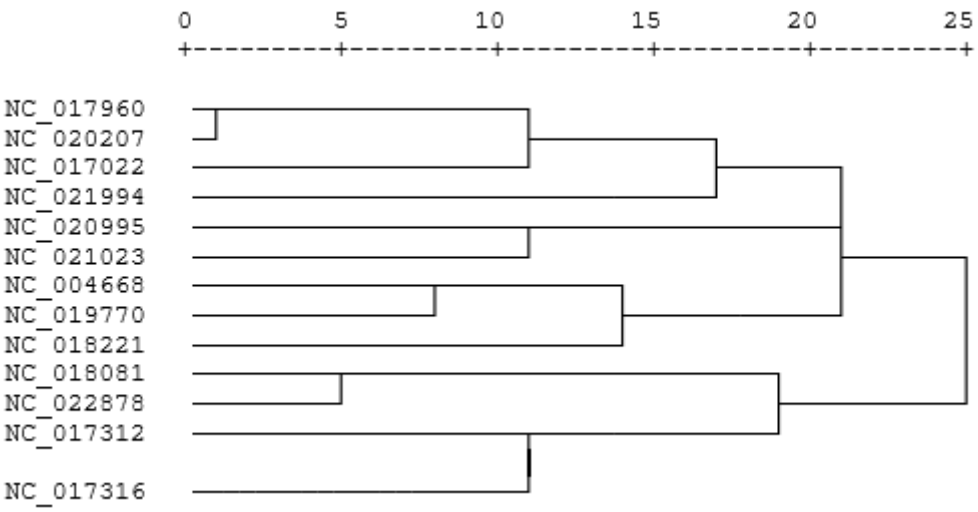


**Fig 1: Phylogenetic diversity of *Enterococcus* species identified by PFGE.**

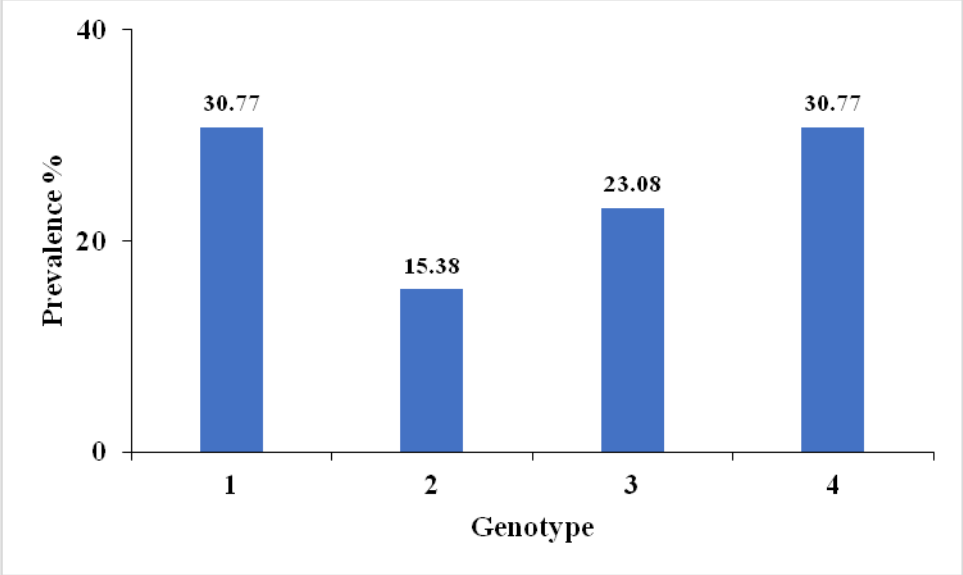


**Fig 2: Prevalence of genotypes.**

Enterococcal surface protein, *esp*(202 kDa) is a surface exposed protein that plays role in adhesion and colonizes to urinary tract epithelial cells [24,25]. Enterococcal surface protein, *esp* of *E. faecalis* was able to form biofilm on a polystyrene surface [25].They also concluded that isolate harbouring no *esp* gene was unable to form biofilm. Only one isolate NC_017312*Enterococcus faecalis* 62 had the *esp* gene which gave 933 bp gene product in the present study. So, this isolate might be able to persist in urinary tract epithelial cells and might be involved in infections [26].A recent study found that there was no correlation was between biofilm formation capacity and the presence of *esp* gene [27].*E. faecalis* required aggregation substance *agg* for cell-to-cell contact was documented by [27]. Several findings reported that aggregation substance, *agg* is required for the adherence of bacterial proteins to extracellular matrix and also to increase cell surface hydrophobicity[28,29].Aggregation substance, *agg* isolated from food sample should be undesirable since it mediates contact between bacterial and host cells and contributes to the acquisition of these virulence genes [27]. Four isolates were found to harbour *agg* gene with 1553 bp PCR product. Hence the prevalence was 30.77%. About 57.9% clinical isolates carried *agg* gene [27]. Enterococcal surface adhesion, *ace* was found to be involved in the bacterial association to the host cell matrix proteins, collagen I and IV and laminin [27].The *ace* gene might also be involved in the pathogenesis of endocarditis [29,30,31]. NC_004668 *Enterococcus faecalis* V583 had the *ace* gene and produced 1003 bp gene product. Isolates from human samples and dairy products harboured *ace* gene [28]. It was also documented that *E. faecalis* harbouring the *ace* gene caused

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aortic valve endocarditis [32].Several studies found that *Enterococcus faecalis* antigen A, *efaA* was able to adhere biotic and abiotic surfaces and evade immune response [3,33,34].A recent study found *efaA* positive isolates in 94.3% UTI patients and 72.9% wound samples [35]. Five isolates (38.46%) were found to harbour *efaA* gene with 704 bp gene product. As for adhesins, *efaA* was more prevalent (38.46%) than *agg* gene (30.76%). The *ace* and *esp* were found in 7.69% of the isolates. These four genes were absent in genotype 1. Fifty percent isolates present in genotype 2 expressed *agg* and *efeA* genes (Fig 3). All the isolates present in genotype 3 harboured *efeA* gene while about 66.67% and 33.33% of the isolates present in genotype 3 carried *agg*, *ace* and *efeA* genes, respectively. Twenty-five percent isolates in genotype 4 harboured *esp* and *agg* genes and 50% isolates present in genotype 4 expressed *efeA* gene.

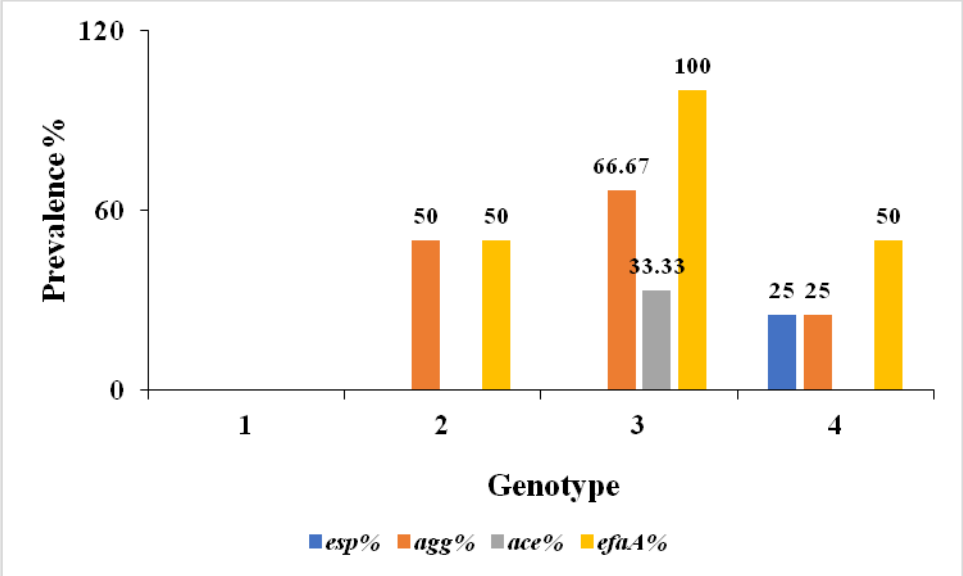


**Fig 3: Genotypic distribution of *esp*, *agg*, *ace* and *efeA* genes.**

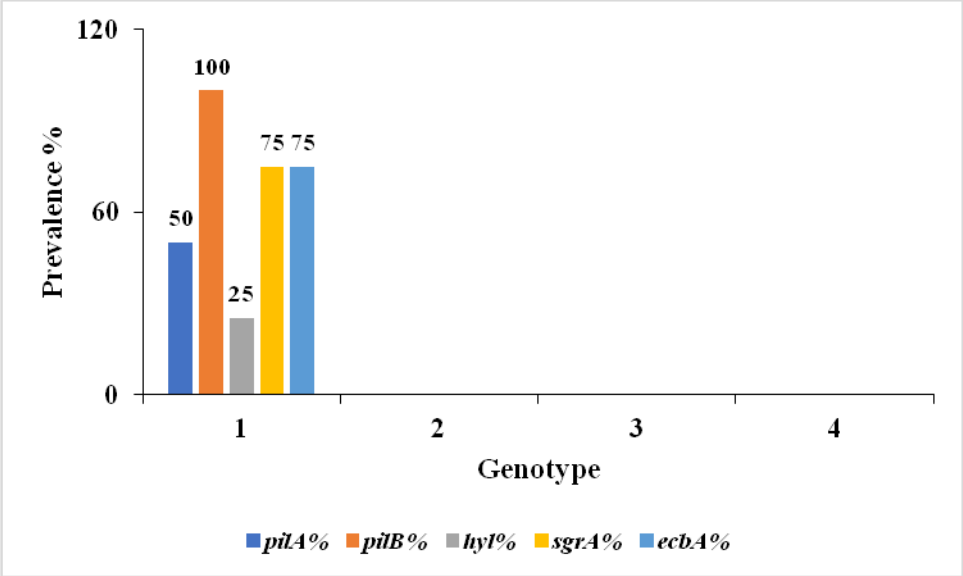


**Fig 4: Genotypic distribution of *pilA*, *pilB*, *hyl*, *sgrA* and *ecbA* genes.**

Surface organelle pili involved in biofilm formation and developed endocarditis was reported by [36]. The previous study reported that pili binds to epithelium and skin cells [37]. Clinical *E. faecium* and *E. faecalis* isolates were observed that harbour *pilB* gene (100%). About 26.6% *E. faecalis* isolates were found that didn't harbour *pilA* gene [18]. Present study found that four isolates (30.76%) had *pilB* gene with 959 bp gene product while 2 isolates (15.38%) had *pilA* gene with an approximate amplicon length of 459 bp gene

product. Recent study found that very low prevalence of *pilA* gene and explained that temperature for *pilA* gene expression and lack of horizontal gene transfer might be the reason [18]. Plasmid harbouring gene, putative glycosyltransferase (*hyl*) colonized the gastrointestinal tracts of mice and contributed to the pathogenicity of *E. faecium* strains [38]. Previously 32 hospitals sample of *E. faecalis* were analyzed and found that 23% isolates had the *hyl* gene [39]. Only 2.1% isolates had the *hyl* gene isolated from Australian haematology patients [40]. Only NC_017960 *Enterococcus faecium* DO was found to harbour *hyl* gene and produced 605 bp gene product. Low prevalence of *hyl* gene was encountered in the present study. So, they might play an insignificant role in the pathogenicity of *Enterococcus* infections in comparison with other virulent genes [18].Surface adhesion, *sgrA* is involved in biofilm formation. Only three *E. faecium* isolates expressed *sgrA* gene and gave a 150 bp gene product. On the other hand, *ecbA* mediates the binding of *E. faecium* to collagen type V and fibrinogen, both of which were prevalent in clinical *E. faecium* strains [41]. The *sgrA* positive isolates were also found to be positive for *ecbA* gene and gave a 182 bp gene product. One study found more *sgrA* gene (100%) positive isolates than *ecbA* (81%) [18]. All these genes were present in only genotype 1 (Fig 4). All the isolates present in genotype 1 expressed *pilB* genes while 75% isolates in genotype 1 harboured *sgrA* and *ecbA* genes. Fifty percent and twenty-five percent isolates in genotype 1 carried *pilA* and *hyl* genes, respectively.

Enterococcal toxin, encoded by cytolysin operon, *cyl,*hasβ-haemolytic properties in humans[27]. Two small antibiotic-like peptides, $cylL_L$ and $cylL_S$ are modified by the products of *cylM* gene. Only NC_004668 *Enterococcus faecalis* V583 was found to harbour $cylL_L$ and *cylM* genes. ATP-binding cassette transporter, *cylB* gene, transported the peptides out of the cells and *cylA* gene processes and activates the peptides to produce cytolysin [42]. No isolate was found to harbour *cylA* and *cylB* gene in the present study. Some clinical enterococci isolate found in one study that harboured *cyl* gene but being non-haemolytic [43]. Lack of genes in *cyl* operons or the presence of silent *cylA* gene may result in *cylA*-positive /haemolytic-negative strains [29,42]. Genotypic distribution of cytolysin gene $cylL_L$and *cylM* found that they were present in only genotype 3 (Fig 5). Other genotype carried no cytolysin gene. About 33.33% isolates in genotype 3 expressed $cylL_L$and *cylM* genes, respectively.

Previous study documented that transferable genetic element like plasmids mediates the transfer of tetracycline resistance gene in *Enterococcus* species [44]. The protein encoded by *tetM* gene has a similar function and sequence homology to elongation factors (EFs). This protein mediates the hydrolysis of GTP like EFs in the presence of ribosome and after ribosomal modification displays the tetracycline molecules [44]. About 31.6% *E. faecalis* isolates had the *tetM* gene in China [45]. One *E. faecalis* and two *E. faecium* were found to harbour *tetM* gene with 446 bp gene product. Recent study found 92% of the strains isolated from urine and about 82.5% of the strains from feces harboured *tetM* gene [21]. One study concluded that indiscriminate use of antibiotics increased the high prevalence of tetracycline resistance gene in these patients in Iran [46].The *vanA* gene conferred resistance to vancomycin and teicoplanin which could be transmitted to other bacteria [47]. Destruction of D-Ala-ending pentapeptide precursors developed the glycopeptide-resistant *Enterococcus*species [48]. Previous survey on inpatients and outpatients with UTIs in America and Canada reported that about 56.8% of the *E. faecalis* isolates had the *vanA* resistance gene due to the extensive use of vancomycin in these countries [49]. Present study found no *vanA* gene in *Enterococcus* species. A recent study also displayed no *vanA* resistance gene [21]. Previously avoparcin, used in feed, was banned in Korea which reduces the vancomycin-resistant enterococci among the food animals [50]. So, the prohibition of avoparcin was an effective measure in food animals. The bifunctional modifying enzyme, *aac(6')-Ie-aph(2")-Ia*gene conferred resistance to gentamicin antibiotics. Previous study stated that this gene conferred resistance to all aminoglycosides except streptomycin since this gene displayed both 6'-acetyltransferase and 2'-phosphotransferase activities [51]. Isolate NC_004668 *Enterococcus faecalis* V583 had the *aac(6')-Ie-aph(2")-Ia*gene with 384 bp gene product. Several studies reported that mPCR assay detected the *ermB* gene, encoded by ribosomal methylases in enterococci [52,53]. A recent study found three erythromycin resistance genes including *ermB* in hospitals [54]. Four isolates were found to express *ermB* gene with 425 bp gene product. High prevalence of erythromycin and tetracycline resistance genes were encountered in all sources since they were frequently used in human and animal therapy [55]. Present study also found the high rate of erythromycin (30.77%) and tetracycline (23.08%) resistance genes. Isolate NC_021994 *Enterococcus faecium* Aus0085 harboured both *ermB* and *tetM* genes.Fifty percent isolates present in genotype 1 harboured *tetM* and *ermB* genes (Fig 6). Genotype 2 carried no antibiotic resistance genes. About 66.67% and 33.33% of the isolates in genotype3 carried *ermB* and *aac(6')-Ie-aph(2")* genes while twenty-five percent isolates present in genotype 4 expressed *tetM* genes.

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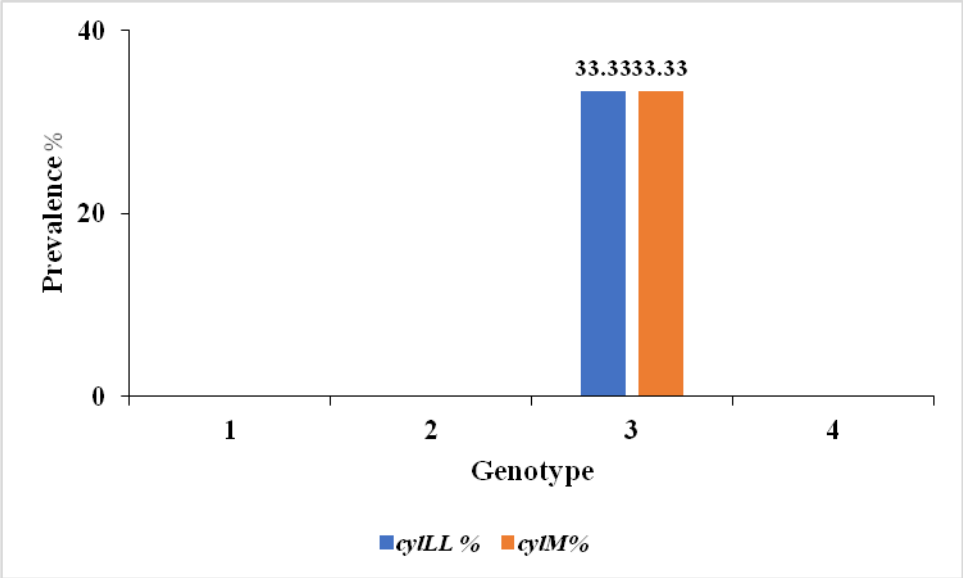


**Fig 5: Genotypic distribution of *cylL*$_L$ and *cylM* genes.**

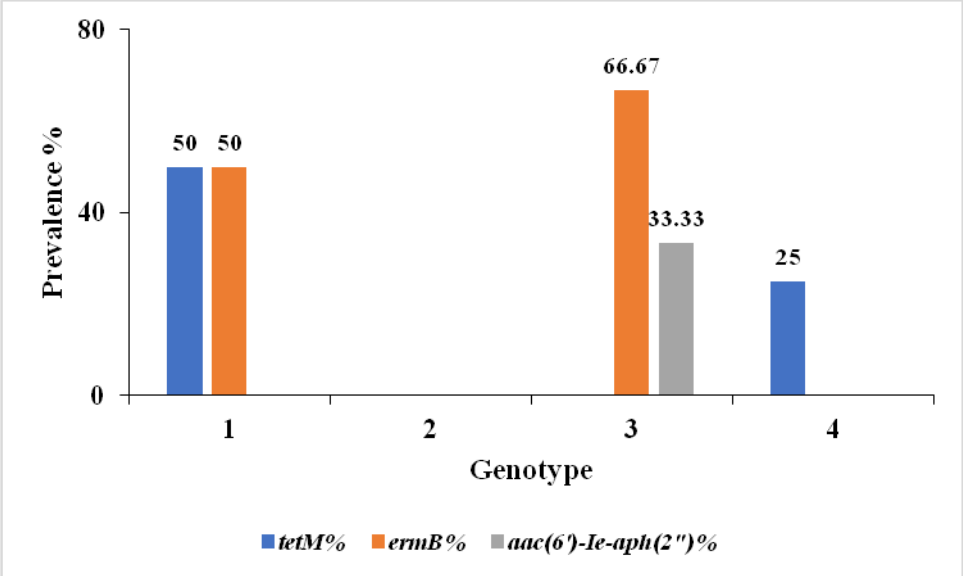


**Fig 6: Genotypic distribution of *tetM*, *ermB* and *aac(6')-Ie-aph(2")* genes.**

## CONCLUSION

We concluded that virulence genes were widely distributed in *Enterococcus* species and might play important role in *Enterococcus* pathogenesis. This data summarized the association of virulence and antibiotic resistance genes in *Enterococcus* species. Vancomycin may be the last alternative antibiotics used for the treatment of enterococcal infections as isolates didn't harbour the *vanA* gene. Further investigation is needed to establish the defense mechanisms of *Enterococcus* infections.

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