

## Detection and Distribution of various HLAR Gene in *Enterococcus faecalis* and *Enterococcus faecium* by Multiplex-PCR

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### ABSTRACT

**Background and Objectives:** In recent years, three new aminoglycoside resistance genes such as *aph* (3')-IIIa and *ant* (4')-Ia, that encode for the APH (3') and ANT (4') have also been identified. The aim of this study was to come up with a multiplex-PCR procedure for detection of *aac*(6')-Ie-*aph*(2'')-Ia, *aph*(3')-IIIa, *ant*(4')-Ia genes in the *Enterococcus* spp. clinical isolates.

**Material and Method:** 100 samples were isolated from various specimens, from various hospitals in Tehran, Iran. The grown colonies were identified by standard biochemical and disc diffusion tests. Multiplex-PCR for *aac* (6')-Ie -*aph*(2'')-Ia , *aph*(3')-IIIa, *ant*(4')-Ia genes amplification were performed in order to confirm bacterial colonies as *Enterococcus* spp.

**Results:** Eighty four (84%) *Enterococcus* spp. isolates were collected from the 100 specimens. The highest and lowest isolates were related to urine (48%) and sputum (2%). Antibiotic susceptibility test results showed that the highest and lowest resistance was related to tetracycline and nitrofurantoin, respectively. Multiplex PCR results revealed that *aac* (6')-Ie-*aph* (2'')-Ia, *ant* (4')-Ia and *aph* (3')-IIIa genes were present in 6% of the isolated bacteria from the urine, 2% from the wound and 1% from the pleural samples. the *aac* (6')-Ie-*aph* (2'')-Ia and *aph* (3')-IIIa genes were present in 25% of the isolated strains from the urine, 3% from the wound and 2% from the plural specimens. Nine percent of the strains were isolated from the urine, 3% from the wound and 1% from the plural were positive for *aac* (6')-Ie-*aph* (2'')-Ia and *ant* (4')-Ia genes.

**Discussion:** we had observed enterococci isolates with phenotypic resistance to HLAR and demonstrated *aac*(6')-Ie-*aph*(2'')-Ia and *aph*(3')-IIIa genes more frequently occurring than other genes. A collection of AMEs are accountable for HLAR status among *Enterococcus* species. The *aac* (6')-Ie-*aph* (2'')-Ia gene was detected more frequently than the other genes.

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### Introduction

Enterococci have progressively appeared as a reason for severe nosocomial and community acquired infections, such as endocarditis and bacteremia in worldwide (1). This variant is also are normal floras of human and animals digestive system. Therefore known as occasional human pathogens responsible for community-acquired and nosocomial infections (2). Common treatment

regiments include a penicillin or a glycopeptide with an aminoglycoside, most usually gentamicin (3). The efficiency of this choice has been challenged by appearance of *enterococcus* strains showing multiple antibiotic resistance, especially those agents' aminoglycosides, penicillin's and glycopeptides (4). Its resistance to wider range of antimicrobial agents particularly, aminoglycosides,

glycopeptides and beta-lactams has increasingly been documented (5).

All enterococci have intrinsic low-level resistance to aminoglycosides, with minimal inhibitory concentrations (MICs) ranging from 4-256 mg/mL. This level of resistance is the result of facultative anaerobic metabolism of enterococci and limited drug uptake (6). High-level aminoglycoside resistance (HLAR) (MIC,  $\geq 512$   $\mu\text{g/ml}$ ) in *enterococci* was first detected in 1980s. They have been then detected with increasing frequency in specimens from hospitalized patients, and in the intestinal tract of humans and animals (7). HLAR in *enterococci* is mediated by aminoglycoside-modifying enzymes (AMEs), which remove the synergic bactericidal effect between anti-cell wall agents, including b-lactams or glycopeptides and aminoglycosides, such as gentamicin, tobramycin, netilmicin, kanamycin and amikacin (1). The most common AMEs in *Enterococcus* spp. are the AAC (6') (aminoglycoside acetyltransferase), -APH (2'') (aminoglycoside phosphotransferase), which inactivates gentamicin, kanamycin, tobramycin, netilmicin and amikacin; APH (3'), which inactivates kanamycin and amikacin; ANT (4') (aminoglycoside nucleotidyltransferase or adenyltransferase), which inactivates kanamycin, amikacin and tobramycin; and ANT (6'), that inactivates streptomycin. Furthermore, *E. faecium* strains produce a chromosomally encoded aminoglycoside acetyltransferase, AAC (6')-Ii, that inactivates tobramycin, netilmicin, kanamycin and sisomicin (8).

Epidemiologic studies which detected the presence of *aac* (6'')-Ie, *aph* (2'')-Ia, *aph* (2'')-Ib, *aph* (2'')-Ic, and *aph* (2'')-Id genes in clinical and animal enterococcus isolates have either used single PCR primer pair for each gene in separate reactions or, at greatest, used PCR primers for two of these gentamicin resistance genes in one reaction (9).

## 2. Objectives

This study aimed to investigate the true prevalence of high level aminoglycoside resistance (HLAR) strains among clinically important isolates of enterococcal strains and to examine

susceptibility of *E. faecalis* and to various antimicrobial agents including ampicillin (10 $\mu\text{g}$ ), penicillin (10 $\mu\text{g}$ ), gentamicin (10 $\mu\text{g}$ ), streptomycin (10 $\mu\text{g}$ ), vancomycin (30 $\mu\text{g}$ ), ciprofloxacin (5 $\mu\text{g}$ ), nitrofurantoin (300- $\mu\text{g}$ ) and tetracycline (30 $\mu\text{g}$ ). In addition, the distribution of *aac*(6'')-Ie-*aph*(2'')-Ia, *aph*(3'')-IIIa, *ant*(4'')-Ia genes was investigated. Furthermore, the MIC and dispensation of the antimicrobial-resistance AEM's genes were compared.

## 3. Material and method

### 3.1. Bacterial strains

In this Experimental study 100 clinical isolates of enterococci were obtained from variety of clinical specimens, including urine, wound, sputum, abscess and tissue were obtained from inpatient and outpatient samples sent to Noor Clinical and Pathobiological Laboratory, Iran in all age groups during a one year (Jul 2013 - Jul 2014). Appropriate inpatient details were collected and recorded to avoid identical isolated from the same patient. An Institutional ethical clearance was obtained for conducting this study (reference number: 1168). All samples were cultured on Blood-Agar plates (Merck Co., Germany) and incubated at 37°C for 24h. Then, all suspected grown colonies were evaluated by standard biochemical and microbiological tests, including, gram staining, catalase, growth at 10-45 °C, tolerance in 60°C, bile-esculin test, growth in 6.5% NaCl, L-arabinose fermentation and pyrrolidonylarylamidase. *E. faecalis* ATCC 29212 and *E. faecium* ATCC 51559 were used as reference strain. Therefore Sample size calculated from Prashant Kadam study formula (10).

### 3.2. Antimicrobial susceptibility tests

Susceptibility tests were performed by disc diffusion method on the Mueller- Hinton Agar (Merck Co., Germany) plates according to Clinical and Laboratory Standards Institute (CLSI) guideline (27) for the following antibiotics: ampicillin (10 $\mu\text{g}$ ), penicillin (10 $\mu\text{g}$ ), gentamicin (10 $\mu\text{g}$ ), streptomycin (10 $\mu\text{g}$ ), vancomycin (30 $\mu\text{g}$ ), ciprofloxacin (5 $\mu\text{g}$ ), nitrofurantoin (300- $\mu\text{g}$ ) and tetracycline (30 $\mu\text{g}$ ). *E. faecalis* ATCC 29212 was

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used as reference strain for antibiotic susceptibility testing.

### 3.3. Minimum Inhibitory Concentration for HLAR

The samples were confirmed as high level aminoglycoside resistant (HLAR) enterococci by considering growth  $\geq 512 \mu\text{g/mL}$  for gentamicin and  $\geq 1024 \mu\text{g/mL}$  for streptomycin. The 18h bacterial cultures were adjusted to 0.5 McFarland's turbidity and the inoculum was spot inoculated on the surface of BHI agar with increasing concentrations of gentamicin and streptomycin antibiotics (Merck Co., Germany). The plates were incubated at 37°C for 24 hrs and inspected for more than one colony forming units in the patterned area. *E. faecalis* ATCC 29212 was used as a negative control strain.

### 3.4. Multiplex-PCR for Analysis of Aminoglycoside Modifying Genes

Multiplex-PCR was done using the DNA amplification instrument master cycler gradient (Eppendorf, Germany) for detection of the *aac(6')-Ie -aph(2'')-Ia*, *aph(3')-IIIa*, *ant(4')-Ia* genes. Genomic DNA were obtained from *E. faecalis* colonies grown overnight on blood agar (Merck Co., Germany) plates by DNA extraction kit (Bioneer Co., Korea) according to manufacturer's instruction. Specific primer pairs for amplification of these genes are listed in Table 1 (10). A volume of 1.5  $\mu\text{l}$  of extracted genomic DNA (20 ng) was added to Master mix (Amplicon Co., Denmark) including 2.5  $\mu\text{l}$  of 10X PCR buffer, 1.5  $\mu\text{l}$  MgCl<sub>2</sub> (50 mM), 0.5  $\mu\text{l}$  dNTPs (10 mM), 1.25  $\mu\text{l}$  of each primer (10 pmol), 0.5  $\mu\text{l}$  of Taq DNA polymerase (5 U/ $\mu\text{l}$ ) and 16  $\mu\text{l}$  sterile distilled water, a total volume of 25  $\mu\text{l}$  PCR reaction mixture. The reaction mixture was performed in a thermal gradient cycler (Eppendorf, Germany) with the denaturation at 94°C for 4 min, 30 cycles with denaturation at 94°C for 31 s, annealing at 52°C for 31 s, extension at 72°C for 45 s and final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis in 1.5% agarose gel. *Staphylococcus aureus* ATCC 29213 was used as a negative control for M-PCR therefore DNAs from

*E. faecium* SF11770, *E. faecalis* WBH18190 used as positive controls.

### 3.5 Statistical analysis

Results were analyzed as negative or positive polymerase chain reaction amplification reaction for each bacteria, as well as 2 bacteria simultaneously. Descriptive analysis were performed and results presented as number (%). Also Statistical evaluation of the result of antibiotic sensitivity test was done using 'Z' test for proportions. *P value* was measured by the  $\chi^2$  and ANOVA test with Yates' correction

## 4. Results

### 4.1. Bacterial isolates

A total of 84 *Enterococcus* strains were isolated from the different clinical samples, including; urine, wound, sputum, abscess and tissues. Respectively 60% and 40% of the samples were obtained from women and men. Most isolates (n=66, 66%) were recovered from urine samples. various species of *Enterococcus* were isolated in all, 70 samples (83.3%) *E. faecalis* and 14 samples (16%) *E. faecium* isolates were detected, respectively (Diagram 2). The male-female ratio was 1.25:1. Age distribution show in diagram 2.

### 4.2. Antibiotic Resistance Profiles

Kirby-Bauer antibiotic tests were performed for the *E. faecalis* isolates which indicated the percentage of resistance to ampicillin, penicillin, gentamicin, streptomycin, vancomycin, ciprofloxacin, nitrofurantoin and tetracycline were 9%, 12%, 43%, 47 %, 7%, 39%, 1% and 84%, respectively (Diagram 2). The highest and lowest resistance rates were towards tetracycline and nitrofurantoin with 84% and 1% frequency, respectively. Except for one strain, all *E. faecalis* isolates were susceptible to nitrofurantoin. The results of high-level gentamicin and streptomycin resistance test in *E. faecalis* showed 33 (44%), 5 (5.9%), 45 (53.5%) were sensitive (S), intermediate (I) and resistance (R) to gentamicin and 32 (38%), 5 (5.9%) and 47 (55.9%) were S, I and R to streptomycin, respectively. High-level resistance test in *E. faecium* revealed 3 (18.7%) and 7 (43.7%) were I and 13 (81.2%) and 9

(56.2%) were R to gentamicin and streptomycin, respectively. We did not find any gentamicin and streptomycin sensitive *E. faecium* strains.

#### 4.3. MIC for HLAR

Aminoglycoside antibiotics are considered efficient in treating serious infections caused by both gram negative and gram positive bacteria. Due to acquisition of extrinsic resistance to high level aminoglycoside antibiotics in enterococci, these strains gain importance in clinical settings. A total of 84 enterococcal isolates were separated by MIC method, 35/84 (~43%) were HLGR (MIC  $\geq$  512  $\mu\text{g}/\text{mL}$ ) for gentamicin and 28/84 (~30%) were HLSR (MIC  $\geq$  1024  $\mu\text{g}/\text{mL}$ ) for streptomycin. Although the present study revealed HLSR strains.

#### 4.4. Multiplex-PCR Analysis of Aminoglycoside Modifying Genes Results

All 84 enterococcal isolates were analyzed for the presence of aminoglycoside modifying enzyme coding genes. Identification of aminoglycoside resistance genes was accomplished by detection of *aac(6')-Ie-aph(2'')-Ia*, *aph(3')-IIIa*, *ant(4')-Ia* genes in the *E. faecalis* strains by Multiplex PCR method. The distribution of *aac(6')-Ie-aph(2'')-Ia*, *ant(4')-Ia*, *aph(3')-IIIa* genes were 59%, 30%, and 49%, respectively (Diagram 3). The PCR-amplified DNA products of these genes are shown in figure 1.

#### 5. Discussion

In the meantime early 1970s, Enterococci were considered as nosocomial pathogens. The character of enterococci as a causal agent of numerous infections has become noticeably significant, not only for their known pathogenic potential but also due to increasing antimicrobial resistance (11, 12). The prevalence of *E. faecalis* and *E. faecium*, is approximately 75% and 20% of all enterococcal-related infections, respectively (13). In this study, 84 *Enterococcus* strains were collected from urine, wound, ascites, biopsy, CSF, pleura, sputum, synovial fluid, BAL, prostate, testes, blood, vagina, sinus secretions, placenta, pericardium and abscess specimens (100 samples). The frequency of *E. faecalis* and *E. faecium*

isolation rate were 16% and 70 samples (83.3%), respectively. Out of 16 *E. faecium* strains, 6 and 10 isolates were obtained from men and women, respectively. Of the 70 *E. faecalis* strains, 28 and 42 isolates were collected from male and female patients, respectively. ANOVA statistical analysis showed that there was a significant difference between *Enterococcal* infections and patients sex, with women being more infected than men ( $P < 0.05$ ). Gordon et al also showed *Enterococci* infections are similarly disseminated between sexes and urinary tract infections are more common in healthy women than men. (14, 15). This results according to our information.

Indiscriminate use of antibiotics such as aminoglycoside, cephalosporin, aztreonam, trimethoprim and other antibiotics has resulted in increased enterococcal infections (16). Antibiotic susceptibility test showed 75%, 42%, 72%, 82%, 11% and 7% of the isolates were sensitive to vancomycin, ciprofloxacin, penicillin, nitrofurantoin, gentamicin and streptomycin, respectively. The highest resistance rate (84%) was related to tetracycline. High level gentamicin resistance is mainly due to the presence of functional enzyme *aac(6')-Ie-aph(2'')-Ia* which also confers high level resistance to tobramycin, kanamycin, amikacin, dibekacin, and netilmicin not including streptomycin (17).

Aminoglycosides are commonly used in synergy with anti-cell wall agents for severe enterococcal infections such as endocarditis and bacteremia. The synergic effect is a result of disruption of bacterial cell wall by the B-lactam antibiotic which permits the aminoglycoside to enter and apply their bactericidal effects (18, 19). The loss of synergistic effect between aminoglycosides and cell wall active agents has caused problems in selecting suitable treatment for HLAR infections. This outcome lead to a lack of satisfactory treatment of HLAR enterococci. Low-level aminoglycoside resistance is an inherent characteristic in all enterococcal species; although, HLAR can occur due to numerous AMEs (20, 21). In this study, HLGR was observed in 33 *E. faecalis* strains (50%) which was consistent and similar with the reported by Behnood (32.43%) (22) and Feizabadi (65%) (23). Frequency of

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HLGR was stated to be 46.15% in Italy (24), 45.5% in Brazil (25), 82.3% in Michigan (9) and in 46% in South Africa (26). The lowest rate (15.7%) for HLGR was reported in Greece (27). There appears to be significant for HLGR isolation rate in various geographic regions. The distribution of *aac (6')-Ie -aph (2'')-Ia*, *ant (4')-Ia*, *aph (3')-IIIa* genes (GenBank Accession number: KF550184) were 59%, 30%, and 49%, respectively. This finding are in agreement with Emaneini et al (11) but contradicted with those reported by Padmasini et al (1). The contrast may be due to the expression of genes other than genes analyzed in this study. HLAR genes are located on plasmids and most frequently on conjugative transposons which can lead to horizontal transfer of resistance factors (28).

### 6• Conclusions

In conclusion we had observed enterococci isolates with phenotypic resistance to high level gentamicin and demonstrated *aac(6')-Ie-aph(2'')-Ia* and *aph(3')-IIIa* genes more frequently occurring than other genes. A collection of AMEs are accountable for HLAR status among *Enterococcus* species. The *aac (6')-Ie-aph (2'')-Ia* gene was

detected more frequently than the other genes. This highlights the limited gene spreading and transfer of resistant genes within a geographical region. Henceforth, surveillance studies can be conducted among *Enterococcus* isolates from various sources in any given geographical region to document the AME gene patterns. Recent studies also indicated HLGR to be more common in all species of enterococci than HLSR.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

### Acknowledgement

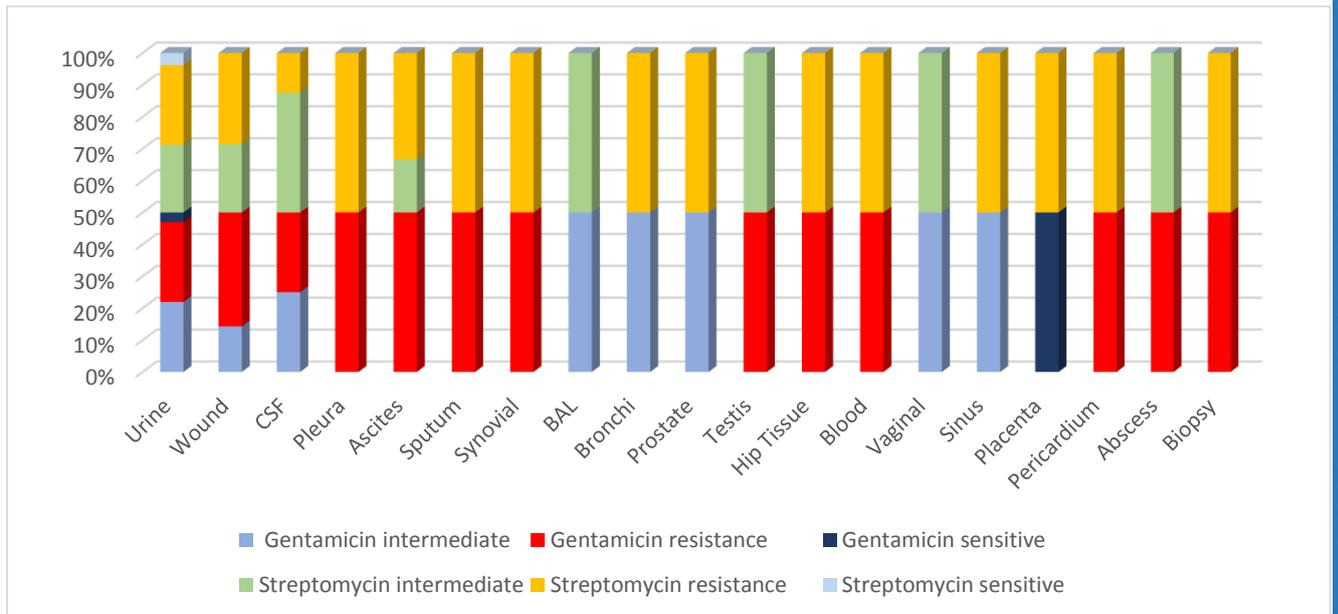
We would like to appreciate Noor clinical Laboratory for their corporation in bacterial sampling.

### Ethical approval

This is experimental study and no need ethical approval therefore An Institutional ethical clearance was obtained for conducting this study (reference number: 1168).

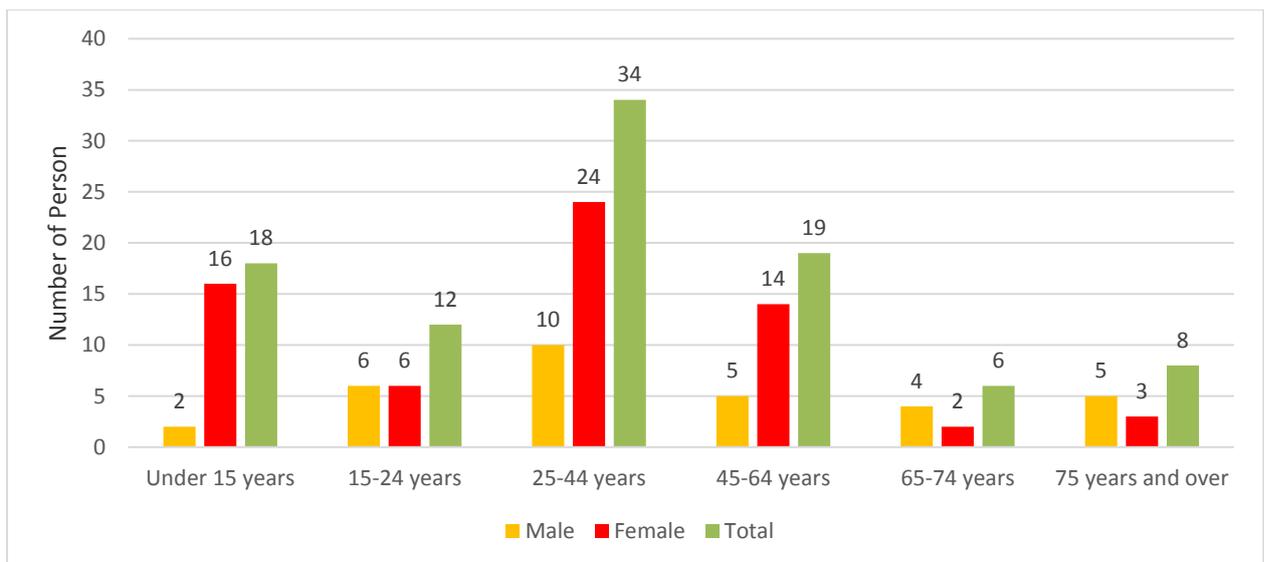
**Table 1.** Oligonucleotide primer sequence target gene and PCR product size (bp) for multiplex PCR (28)

target gene	primer sequence (5'→3')	PCR product size (bp)
<i>aac(6')-Ie -aph(2'')-Ia</i>	5'-CAGGAATTTATCGAAAATGGTAGAAAAG-3' 5'-CACAATCGACTAAAGAGTACCAATC-3'	369
<i>aph(3')-IIIa</i>	5'-GGCTAAAATGAGAATATCACCGG-3' 5'-CTTTAAAAAATCATACAGCTCGCG-3'	523
<i>ant(4')-Ia</i>	5'-CAAAGTCTAAATCGGTAGAAGCC-3' 5'-GGAAAGTTGACCAGACATTACGAACT-3'	296



**Diagram 1.** Bacterial strains and their antibiotic susceptibility profiles

I; intermediate, S; sensitive, R; resistance, F; female, M; male, CSF; Cerebrospinal fluid, BAL; bronco-alveolar lavage fluid



**Diagram 2.** The results of the separate age groups infected with Enterococcus

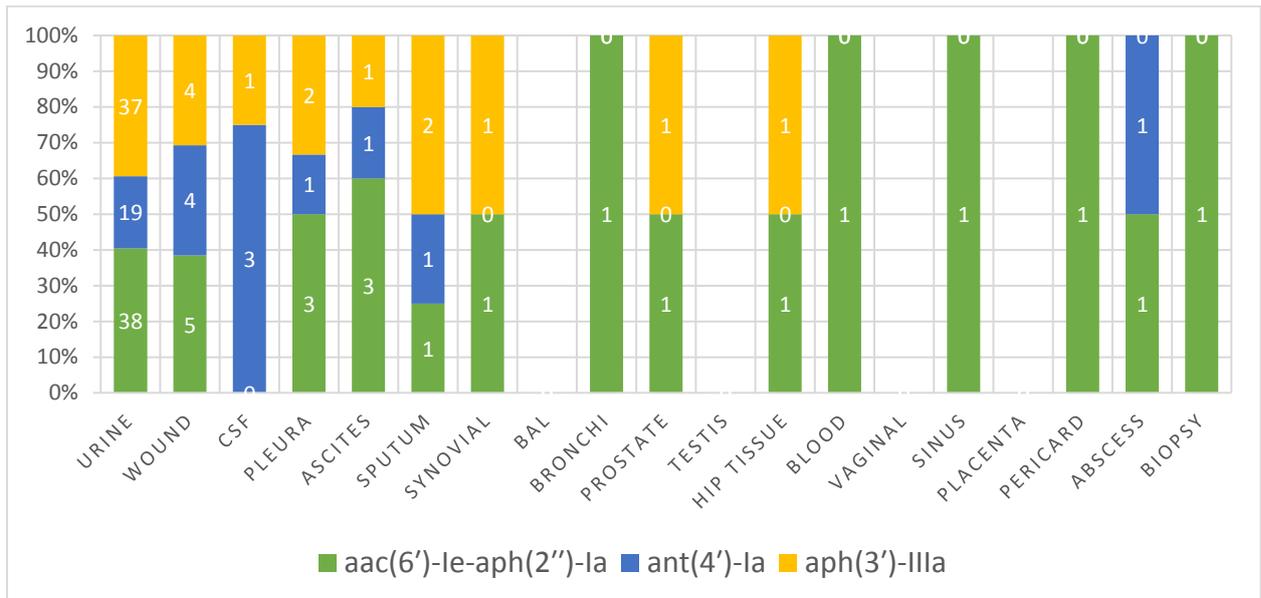


Diagram 3. Distribution of various resistance genes in different samples

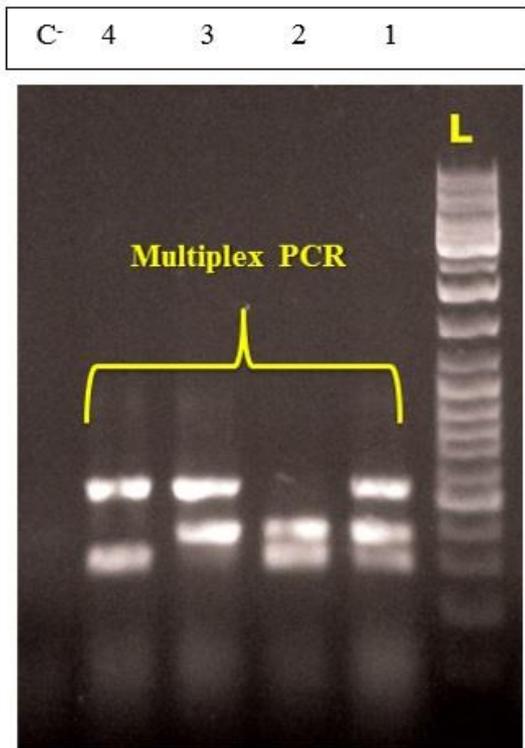


Figure 1. PCR amplification of *aac(6')-Ie - aph(2'')-Ia*, *aph(3')-IIIa* and *ant(4')-Ia* genes in four selected isolates of *E. faecalis*. L: 100 bp DNA size marker.

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