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ORIGINAL ARTICLE

Virulence Genes and Antibiograms of *Enterococci* Isolated from Intensive Care Unit Patients in National Liver Institute

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ABSTRACT

Background: *Enterococci are normal flora of human intestine causing several infections such as infective endocarditis, bloodstream infections, and intra pelvic/abdominal abscess especially among patients in ICU. Different virulent factors are produced by the bacterium to enhance their pathogenicity.* ***Objectives:*** *The aim of the study was to evaluate virulence genes and pattern of antibiotic susceptibility of Enterococcus spp. taken from ICU patients admitted at NLI.* ***Methodology:*** *From 140 ICU patients admitted at NLI, samples were taken after 48 hours from admission, and cultured on bile esculin agar, the GP-ID cards of VITEK-2 system used to confirm enterococcal isolation and species identification. Antibiotic susceptibility was done using VITEK2 AST-P592 cards. Multiplex-PCR was used for identification of gelE, asa1, esp genes.* ***Results:*** *esp gene was significantly high in E. faecium (p =0.004). The virulence genes combinations were significant between the enterococcal species (p = 0.004). A significant correlation was found between Enterococci isolates clinical source and esp gene (p =0.012). Antibiotic susceptibilities were variable among enterococcal isolates. The resistance to ampicillin and streptomycin (high level synergy) was significant between E. faecium and E. faecalis (p =0.014, 0.006) respectively. No association was observed regarding antibiotic susceptibility with genes of virulence (p > 0.05).* ***Conclusion:*** *A relationship was found between distribution pattern of virulence genes and the enterococcal species. A correlation was found between Enterococci isolates clinical source and esp gene. Antibiotic resistance was significantly different between the enterococcal species. Surveillance of drug resistance should be done regularly for proper antibiotics selection.*

INTRODUCTION

Enterococci are positive Gram stain, facultative anaerobes and represent commensal bacteria in humans and animals intestinal tracts[1] and are capable of growth under unfavorable conditions like temperature range of 10ºC-45ºC, (6.5%) NaCl, (40%) bile salts, and high pH[2]. *Enterococci* cause infective endocarditis, bloodstream infections, and intra pelvic/abdominal abscess in ICU patients[3]. *Enterococcus faecium* and *Enterococcus faecalis* are the most common enterococcal species representing up to (90%) of enterococcal infections, while other species of *Enterococci* are less frequently known to cause infections of human[4].

Enterococci have virulence factors encoded by virulence genes as aggregation substance encoded by *asa1* gene, enterococcal surface protein encoded by *esp* gene, and gelatinase encoded by *gelE* gene. These virulence factors involved in host tissue colonization, modulation of immune mechanisms and promote invasion[5].

Most *Enterococci* are resistant to glycopeptide and beta-lactam antibiotics, making necessary their simultaneous use with an aminoglycoside for treatment of the most serious enterococcal infections such as endocarditis, meningitis. The efficacy of such drug combination is disturbed with the emergence of strains resistance to some antibiotics, including high resistance to aminoglycosides and glycopeptides[6].

Our study aimed to assess susceptibility of antibiotics pattern and genes of virulence of *Enterococci* collected from patients in ICU at the Hospital of National Liver Institute, Menoufia University.

METHODOLOGY

From December 2020 to January 2022, our study had been conducted involving 140 patients (of both sex) admitted to ICU in National Liver Institute (NLI) Hospital, Menoufia University, which got approval from Ethical Committee Board from NLI, Menoufia University by number NLI IRB 00003413/00364/2022.

Patients' data were collected including demographics, comorbidities, cause of hospital

admission, duration of ICU stay, previous hospitalization and ICU stay, use of corticosteroids or chemotherapy, antibiotics intake, and use of invasive medical devices.

Samples collection:

Samples were taken from ICU patients who were admitted for more than 48 hours developing clinical infection signs.

Samples involved urine, blood, ascitic fluid, tracheal tube, **throat and nasal swabs,** drain, sputum and stool.

Identification of the isolates:

Enterococci were identified by morphology of the colony, positive gram stain, negative catalase test, and esculin hydrolysis[4] then confirmed by VITEK-2 compact system GP-ID cards (bioMerieux, France). Fig (1)

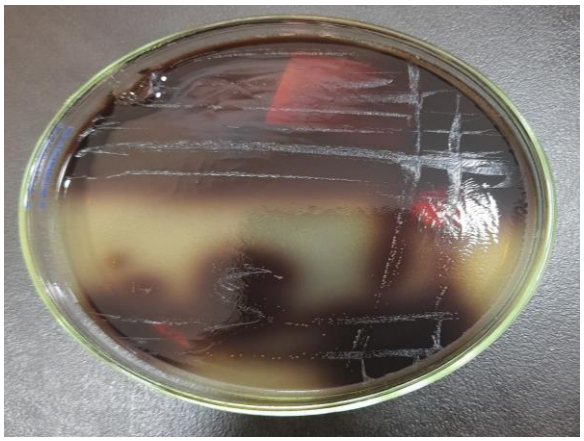

Fig. 1: Culture of *Enterococci* on bile esculin agar

Testing of antibiotic susceptibility:

The susceptibility of tested enterococcal isolates to antibiotics was done using VITEK2 AST-P592 card following the manufacturer's instructions. This card tests these antibiotic drugs: vancomycin, teicoplanin, streptomycin high level synergy, gentamicin high level synergy, tigecycline, tetracycline, linezolid, ampicillin, ciprofloxacin, and erythromycin.

Genotypic identification of virulence genes:

Detection of *gelE, asa1, esp* genes in the enterococcal isolates was done using Multiplex-PCR by primers as shown in table1.

Table 1: the used primers in our study:[4]

Name of primers	Used sequence (5`-3`)	Size of Product (bp)	Temperature of annealing (C)
gelE	F: TATGACAATGCTTTTTGGGAT R: AGATGCACCCGAAATAATATA	213	55
asa1	F: GCACGCTATTACGAACTATGA R: TAAGAAAGAACATCACCACGA	375	55
esp	F: AGATTTCATCTTTGATTCTTGG R: AATTGATTCTTTAGCATCTGG	510	54

Extraction and purification of DNA:

Thermo Scientific gene JET™ genomic DNA Purification Kit was used for purification of DNA according to Manufacturers' instructions.

DNA amplification:

DNA amplification was done using the Primers of the genes (table 1) purchased from Thermo Fisher Scientific USA. Mixtures of PCR contain DreamTaq green PCR Master Mix (2x), 10 µl from DNA Extract, 0.25 µl from each gene forward primer, 0.25 µl from each gene reverse primer.

The Multiplex PCR program was carried out in a thermal condition as follows: initial denaturation at 95°C for 10 min, 35 denaturation cycles at 95°C for 30 sec, temperature of annealing at 55°C for 40 sec, then extension at 72°C for 1 min, and final extensions at 72°C for 7 min.

Amplified products detection:

The amplified products size was visualized using (2%) agarose gels after ethidium bromide staining (Sigma, USA). *gelE* (213bp), *asa1* (375bp), *esp* (510bp) have been determined in comparison to a DNA ladder (100-1000bp) (Fermentas, Germany). Following electrophoresis, visualization was conducted with a UV trans-illuminator and photographed by digital camera. Fig (2)

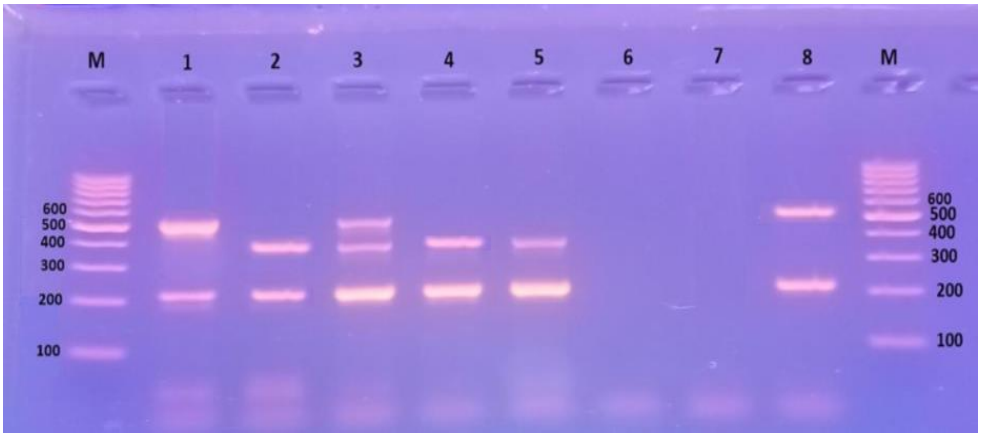

Fig. 2: Multiplex PCR gel electrophoresis of *gelE*, *asa1*, *esp* genes of *Enterococci* spp. isolates, lane M shows DNA ladder (100-1000 bp), lanes 1, 2, 3, 4, 5 and 8 show *gelE* gene at 213 bp, lanes 2, 3, 4, and 5 show *asa1* gene at 375 bp, lanes 1, 3 and 8 show *esp* gene at 510 bp.

Statistical analysis:

Statistical analysis has been calculated by the SPSS- version 13. Quantitative variables described as mean, SD, range with using Student t-test. Qualitative variables described as percentage, and Fisher's exact test or Chi-square test were used. Statistical significance was adjusted at p value <0.05.

RESULTS

Characteristics of Patients:

This study involved 140 patients (53 females and 87 males), their mean age was (60.46±12.91) years of whom (62.14%) were males.

Samples (399) were taken from the patients. Of them, 50 enterococcal isolates (12.53%) collected from different clinical samples; 25 from stool, 11 from urine, 4 from each blood and drain, 3 from ascitic fluid, 2 from throat swab, and one nasal swab, of which *E. faecalis* detected from 22 samples, *E. faecium* from 24 samples, and other species of *Enterococci* from 4 samples (table 2).

Table 2: the isolated organisms distribution regarding the type of samples.

Organism	Ascitic (n=54)	Blood (n=97)	Urine (n=89)	Drain (n=51)	Throat swab (n=27)	Nasal swab (n=22)	Sputum (n=29)	Tracheal Tube (n=5)	stool (n=25)	Total (n=399)
Enterococcus spp	3	4	11	4	2	1	-	-	25	50
E.coli	19	10	21	8	1	1	3	-	-	63
Staphylococcus aureus	12	30	-	5	2	12	5	-	-	66
Klebsiella spp	11	17	11	9	3	-	16	2	-	69
Staphylococcus hominis	-	2	-	-	-	-	-	-	-	2
Acinetobacter	-	-	1	-	-	-	-	1	-	2
Staphylococcus epidermedis	-	3	-	-	-	-	-	1	-	4
Staphylococcus haemolyticus	1	4	2	-	-	-	-	-	-	7
Streptococcus viridans	-	-	-	-	7	-	2	-	-	9
Pseudomonas spp	-	1	1	-	-	-	-	-	-	2
Candida spp	-	-	8	-	2	-	-	-	-	10
No growth	8	26	29	25	6	8	3	-	-	105
Mixed infection: Candida/Ecoli	-	-	1	-	-	-	-	-	-	1
Candida/klebsiella	-	-	4	-	-	-	-	-	-	4
Streptococcus viridans / Candida	-	-	-	-	2	-	-	-	-	2
Klebsiella / Staphylococcus aureus	-	-	-	-	-	-	-	1	-	1
Streptococcus viridans / Staphylococcus aureus	-	-	-	-	2	-	-	-	-	2

According to virulence genes frequency, *esp* gene had been detected significantly high in *E. faecium* (66.67%) (p value=0.004). While no significant difference was detected between the species of *Enterococci* regarding *gelE* and *asa1* genes frequency as shown in table3.

Table 3: virulence genes frequency among the *Enterococci* species

Virulence gene	*Enterococci* species				Test of Sig.	p value
	E. faecalis **(n= 22)**	***E. faecium*** **(n= 24)**	**Other *Enterococci* species (n= 4)**	**Total (n= 50)**		
gelE	18 (81.82%)	19 (79.17%)	4 (100.00%)	41 (82.00%)	Fisher's Exact Test	0.999
asa1	18 (81.82%)	16 (66.67%)	2 (50.00%)	36 (72.00%)	Fisher's Exact Test	0.238
esp	5 (22.73%)	16 (66.67%)	2 (50.00%)	23 (46.00%)	Fisher's Exact Test	0.004*

gelE: gelatinase enzyme**,** *asa1*: aggregation substance**,** *esp*: enterococcal surface protein
* Significant p value

A significant difference was observed among enterococcal species regarding frequency of different combinations of virulence genes (p value= 0.004). The combination of *gelE* + *asa1* genes was more frequent than the other combinations (38%), also more frequent in *E. faecalis* (63.64%) than the other *Enterococci* species (table 4).

Table 4: Frequency of different combination of virulence genes among the *Enterococci* species

Virulence gene	*Enterococci* species				Test of Sig.	p value
	E. faecalis **(n= 22)**	***E. faecium*** **(n= 24)**	**Other *Enterococci* species (n= 4)**	**Total (n=50)**		
Single gene					Fisher's Exact Test	0.004*
gelE	-	-	1 (25.00)	1 (2.00%)		
asa1	-	1 (4.17)	-	1 (2.00%)		
esp	-	-	-	-		
Combined genes						
gelE* + *asa1	14 (63.64%)	4 (16.67%)	1 (25.00%)	19 (38.00%)		
gelE* + *esp	1 (4.55%)	5 (20.83%)	1 (25.00%)	7 (14.00%)		
esp* + *asa1	1 (4.55%)	1 (4.17%)	-	2 (4.00%)		
All genes detected						
gelE* + *asa1* + *esp	3 (13.64%)	10 (41.67%)	1 (25.00%)	14 (28.00%)		

A significant relationship was detected between *esp* gene and *Enterococci* isolates clinical source. While there was no significant relationship between *gelE, asa1* genes and *Enterococci* isolates clinical source (table 5).

Table 5: relationship between virulence genes and *Enterococci* isolates clinical source

Virulence genes	Clinical source							Test of Sig.	p value
	Blood No. (%)	**Urine No. (%)**	**Ascitic fluid No. (%)**	**Throat swab No. (%)**	**Nasal swab No. (%)**	**Drain No. (%)**	**Stool No. (%)**		
gelE	4 (100)	9 (81.82)	2 (66.67)	2 (100)	1 (100)	4 (100)	19 (76)	Fisher's Exact Test	0.855
asa1	4 (100)	10 (90.91)	3 (100)	2 (100)	1 (100)	3 (75)	13 (52)	Fisher's Exact Test	0.105
esp	1 (25)	9 (81.82)	-	1 (50)	1 (100)	3 (75)	8 (32)	Fisher's Exact Test	0.012*

Antibiotic sensitivity of enterococcal isolates:

Testing of enterococcal isolates sensitivity to antibiotics showed that *Enterococci* were highly sensitive to tigecycline (100%) then linezolid, teicoplanin, vancomycin (82%, 80%, 64%) respectively. *Enterococci* were highly resistant to erythromycin (84%) then ciprofloxacin, tetracycline, streptomycin high level synergy, gentamicin high level synergy (70%, 70%, 70%, 62%) respectively as shown in table 6.

Table 6: Pattern of antibiotic sensitivity of *Enterococci* by VITEK 2 compact system

Antibiotic agent	*Enterococci* (no= 50)		
	Sensitive	Intermediate	Resistant
	No (%)	No (%)	No (%)
Vancomycin	32 (64)	2 (4)	16 (32)
Teicoplanin	40 (80)	-	10 (20)
Streptomycin high level synergy	15 (30)	-	35 (70)
Gentamicin high level synergy	19 (38)	-	31 (62)
Tigecycline	50 (100)	-	-
Tetracycline	14 (28)	1 (2)	35 (70)
Linezolid	41 (82)	1 (2)	8 (16)
Ampicillin	25 (50)	-	25 (50)
Ciprofloxacin	12 (24)	3 (6)	35 (70)
Erythromycin	3 (6)	5 (10)	42 (84)

Regarding antibiotic resistance between enterococcal species, a significant difference was detected between *E. faecium* and *E. faecalis* strains regarding their resistance to ampicillin and streptomycin (high level synergy) as strains of *E. faecium* had significant high ampicillin resistance (75%) than *E. faecalis* strains (27.27%) (p value= 0.006) and strains of *E. faecalis* had significant high streptomycin resistance (high level synergy) (90.91%) than *E. faecium* strains (54.17%) (p value= 0.014). Fig (3)

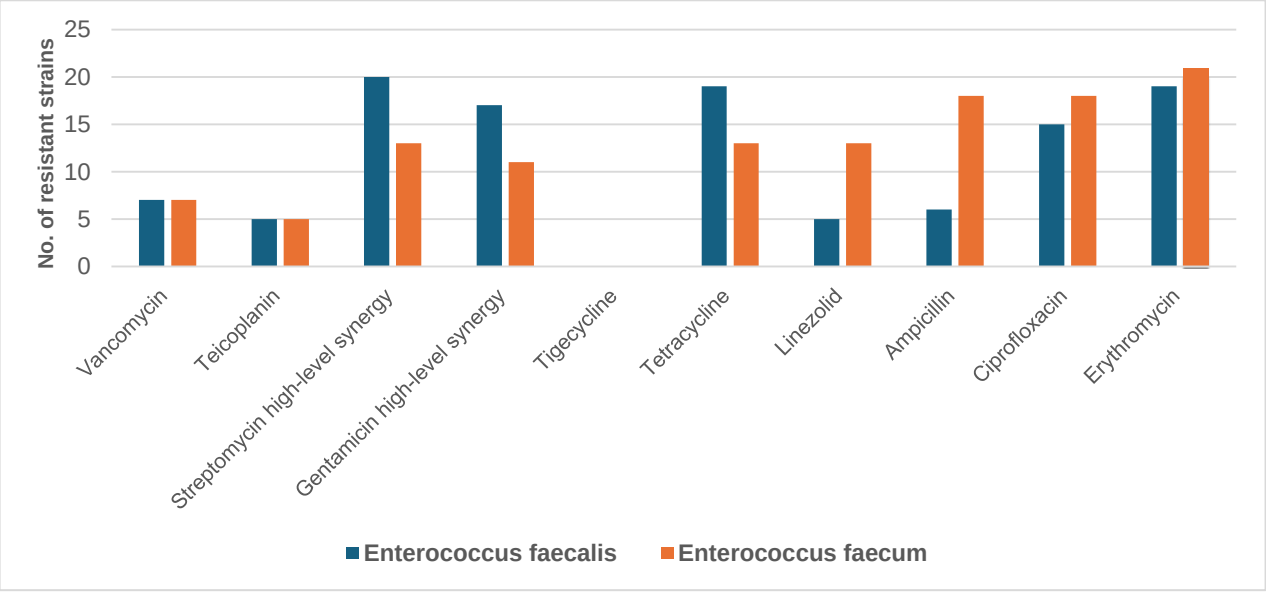

Fig. 3: Comparison between *E. faecium* and *E. faecalis* as regard antibiotic resistance.

Relationship between antibiotic susceptibility and virulence genes:

No relationship was observed between susceptibility of antibiotics of the *Enterococci* isolates with the genes of virulence (table 7).

Table 7: Relationship between antibiotic susceptibility with virulence genes of *Enterococci* isolates:

Antibiotic	Virulence genes						Test of sig.	p value
	gelE n=41		*asa1* n=36		*esp* n=23			
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant		
Vancomycin	27 (65.85)	14 (34.15)	26 (72.22)	10 (27.78)	16 (69.57)	7 (30.43)	X2 = 0.368	0.832
Teicoplanin	32 (78.05)	9 (21.95)	30 (83.33)	6 (16.67)	18 (78.26)	5 (21.74)	Fisher's Exact Test	0.854
Streptomycin high level synergy	14 (34.15)	27 (65.85)	9 (25.00)	27 (75.00)	9 (39.13)	14 (60.87)	X2 = 1.435	0.488
Gentamicin high level synergy	18 (43.90)	23 (56.10)	13 (36.11)	23 (63.89)	10 (43.48)	13 (56.52)	X2 = 0.557	0.757
Tigecycline	41 (100.00)	-	36 (100.00)	-	23 (100.00)	-	-	-
Tetracycline	12 (29.27)	29 (70.73)	11 (30.56)	25 (69.44)	10 (43.48)	13 (56.52)	X2 = 1.498	0.473
Linezolid	34 (82.93)	7 (17.07)	31 (86.11)	5 (13.89)	18 (78.26)	5 (21.74)	Fisher's Exact Test	0.699
Ampicillin	17 (41.46)	24 (58.54)	17 (47.22)	19 (52.78)	4 (17.39)	19 (82.61)	X2 = 5.654	0.059
Ciprofloxacin	14 (34.15)	27 (65.85)	12 (33.33)	24 (66.67)	5 (21.74)	18 (78.26)	X2 = 1.204	0.548
Erythromycin	8 (19.51)	33 (80.49)	5 (13.89)	31 (86.11)	4 (17.39)	19 (82.61)	Fisher's Exact Test	0.792

DISCUSSION

Enterococci are normal flora of human intestine causing infective endocarditis, bloodstream infections, and intra pelvic/abdominal abscess in ICU patients[3]. *Enterococci* have virulence factors encoded by virulence genes as aggregation substance encoded by *asa1* gene, enterococcal surface protein encoded by *esp* gene, and gelatinase encoded by *gelE* gene[5].

One hundred and forty patients participated in our study; their mean age was (60.46 ± 12.91) years of whom (62.14%) were males. Our findings agreed with those of *Iwasa et al.,*[7] in which males made up (64.3%), and with the results of *Tamai et al.,*[8] in which the patients were with mean age (65.5 ±11.9) years. However, in the study of *Lupia et al.,*[9], males made up (38.31%) and in the study of *Birru et al.,*[10], the patients were with mean age (47±13.8) years.

Enterococci isolated in our study at a rate of (12.53%). Our findings agreed with those reported by *Alatrouny et al.,*[11] in which *Enterococci* isolated at a rate of (12%). However, these results were in disagreement with those reported by *Kamel et al.,*[12] in which *Enterococci* isolated at a rate of (45%); this high prevalence because the study was conducted on immunocompromised patients and were in disagreement with results reported by *Li et al.,*[13] in which *Enterococci* isolated at a rate of (5.3%).

In our study, *esp* gene was significantly high in *E. faecium* (66.67%). While no significant difference was observed between species of *Enterococci* regarding *gelE* and *asa1* genes frequency. These findings were in agreement with the study of *Mohanty & Behera*[14] who said that significant difference was observed regarding *esp* gene between *E. faecium* (37.5%) and *E. faecalis* (10.4%), these results were in disagreement with the study of *Kiruthiga et al.,*[15] who observed that no significant difference was observed regarding *esp* gene between *E. faecium* (45.0%) and *E. faecalis* (53.93%).

In our study, a significant difference was observed among *Enterococci* species regarding different combinations frequency of the virulence genes (p value= 0.004). Our results agreed with those reported by *Haghi et al.,*[16] in which a significant difference was observed among *Enterococci* species regarding different combinations frequency of the virulence genes (p value < 0.05). These findings disagreed with those reported by *Çopur et al.,*[17] who observed that no significant difference was observed between species of *Enterococci* regarding different combinations frequency of the virulence genes (p value >0.05).

In our study, a significant correlation was observed between *esp* gene and *Enterococci* isolates clinical source. While no significant correlation was detected between *gelE*, *asa1* genes and *Enterococci* isolates clinical source (p value > 0.05). These findings were similar to those reported by *Strateva et al.,*[18] in which the prevalence of *esp* gene was significantly more in enterococcal non-invasive isolates compared to the invasive isolates. But these findings were in disagreement with those reported by *Çopur et al.,*[17] who reported that no significant correlation was observed between *esp* gene and VRE isolates clinical source.

Regarding the antibiotic susceptibility in our study, we found that *Enterococci* were highly sensitive to tigecycline (100%) then linezolid, teicoplanin, vancomycin (82%, 80%, 64%) respectively. *Enterococci* were highly resistant to erythromycin (84%) then ciprofloxacin, tetracycline, streptomycin high level synergy, gentamicin high level synergy (70%, 70%, 70%, 62%) respectively. These findings were similar to those reported by *Kamel et al.,*[12] in which *Enterococci* were sensitive to vancomycin, and teicoplanin (77.8% for each) and resistant to erythromycin and ciprofloxacin (72.5%, 50%) respectively, and similar to the study of *Chen et al.,*[19] who said that *Enterococci* were resistant to tetracycline (84.6%), and similar to the study of *Mohanty & Behera,*[14] who observed that *Enterococci* were sensitive to linezolid (95.5%), and similar to results reported by *Santimaleeworagun et al.,*[20] in which *Enterococci* were sensitive to tigecycline (100%), and similar to results reported by *Mousavi et al.,*[21] in which *Enterococci* were resistant to high level streptomycin and high level gentamicin (56.7% , 60.3%) respectively. But these findings were in disagreement with those reported by *Chen et al.,*[19] in which *Enterococci* were resistant to tigecycline (92.3%) , and in disagreement with results reported by *Montalbán-López et al.,*[22] in which all *Enterococci* were sensitive to vancomycin (100%), and in contrast to results reported by *Varghese et al.,*[23] in which *Enterococci* were sensitive to teicoplanin and linezolid (100%) for each and resistant to tetracycline (34.7%), high level gentamicin (33.3%), and ciprofloxacin (30.7%), and in contrast to the study of *Rana & Sande,*[24] who observed that *Enterococci* were resistant to high level streptomycin (34%), in contrast to results reported by *Salah et al.,*[25] in which *Enterococci* were resistant to erythromycin (36.24%).

In our study, a significant difference was observed between *E. faecium* and *E. faecalis* strains regarding their resistance to ampicillin and streptomycin (high level synergy) (p value= 0.014, 0.006 respectively). These results were similar to those reported by *Varghese et al.,*[23] in which a significant difference was observed between *E. faecium* and *E. faecalis* strains regarding their resistance to ampicillin (p value =0.04), and similar to those reported by *Schell et al.,*[26] in which strains of *E. faecalis* had significant high streptomycin resistance (high level) (22.7%) than *E. faecium* strains (5.3%), but these results were in disagreement with results reported by *Mousavi et al.,*[21] in which no significant difference was observed between *E. faecium* and *E. faecalis* strains regarding high level streptomycin resistance, and in disagreement with the study of *Georges et al.,*[27] who observed that no significant difference was observed between *E. faecium* and *E. faecalis* strains regarding antibiotic resistance (p value > 0.05).

No association was found between the antibiotic susceptibility and the virulence genes of *Enterococci* (p value> 0.05). These results were similar to those reported by *Fahmy et al.,*[5] in which no relationship was observed between virulence genes and antibiotic resistance of strains of *E. faecium*. These results disagreed with those reported by *Georges et al.,*[27] in which significant association was detected between *esp* and *asa1* genes and resistance of tetracycline (p= 0.0363 and 0.0305, respectively), and between *asa1* and *gelE* genes and resistance of ampicillin (p = 0.0008 and 0.0005, respectively).

CONCLUSION

Our study focused on virulence genes and antibiotic susceptibility pattern of *Enterococci* among ICU patients. As there was a relationship between distribution pattern of virulence genes and the enterococcal species. There was a correlation between *esp* gene and the clinical source of *Enterococci* isolates. The antibiotic resistance was significantly different between the enterococcal species. So, screening of *Enterococci* species isolated from samples should be done routinely and Surveillance of drug resistance should be done regularly for proper antibiotics selection.

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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