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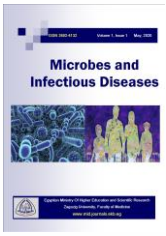

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Original article

Characterization of Enterococci isolated from intensive care unit (ICU); Distribution of virulence markers, virulence genes and antibiotic resistance pattern

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ABSTRACT

Background: Enterococci are considered as the third most common cause of nosocomial infections. Enterococci acquire antibiotic resistance by gene transfer. Virulence factors facilitate colonization and evasion from the immune system. Our objectives were to evaluate the distribution of virulence markers and genes among enterococci isolated from intensive care unit (ICU) in Sohag University Hospital and to determine the antibiotic resistance pattern of enterococci. **Methods:** Virulence markers were detected by gelatinase test, caseinase test, slime layer production and modified micro titer plate method. Polymerase chain reaction is used for identification of enterococcal species and detection of virulence genes as *gel E, asa 1, esp, hyl* and *cyl A*. Antibiotic sensitivity tests were performed by disc diffusion method by using ampicillin, vancomycin, tetracycline, erythromycin and teichoplanin. Vancomycin minimum inhibitory concentrations (MICs) were measured by E-test. **Results:** Vancomycin resistant enterococci (VRE) was detected in 38.4 % of isolates. There was no significant difference in the distribution of all virulence markers between *Enterococcus faecalis* (*E. faecalis)* and *Enterococcus faecium* (*E. faecium)*. The *hyl* gene was more commonly detected in *E. faecalis* (*p*-value= 0.01). Enterococci isolated from cases of surgical site infection (SSI) ,pneumonia and sepsis contain multiple virulence genes with the highest percentage. Vancomycin resistance was higher in *gel E* positive and asa1 positive *E. faecalis* than negative *E. faecalis*. **Conclusion:** Multi-drug resistance (MDR) was detected in 57.6% of enterococci. *Enterococcus faecalis* and *E. faecium* have the same degree of virulence. An association was noted between the *esp* & *asa 1* genes and biofilm formation**.**

Introduction

There are many species of enterococci which can produce infections in human, *Enterococcus faecalis (E.faecalis)* is the commonest species with the highest virulence followed by *Enterococcus faecium* (*E.faecium)* which exhibits multidrug resistance (MDR) pattern and high prevalence [1].

Enterococci show intrinsic resistance to multiple antibiotics as cephalosporins,

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sulphonamides and aminoglycosides [2]. Acquired resistance can be acquired easily by horizontal gene transfer, some strains develop resistance to three or more groups of antimicrobials, these strains are known as MDR strains [3]. Vancomycin and teicoplanin are important therapeutic options against MDR strain of enterococci, so resistance to these antibiotics is of great and special concern. Extensive use of vancomycin resulting in emergence of vancomycin resistant enterococci "VRE" which have an important role in spread of antimicrobial resistance genes among species [3].

Nosocomial infections which are caused by enterococci especially VRE are associated with prolonged hospitalization and high mortality rate [4]. World Health Organization in 2017 placed vancomycin resistant *E. faecium* in global priority pathogens list (global PPL) of the antibiotic resistant bacteria in a special category known as “HIGH PRIORITY" to help in prioritizing and focusing the research toward development of antibiotic treatments which are new and effective [5].

Enterococci possess virulence genes coding for virulence factors, for example: *ace* gene for collagen binding protein, *asa1* gene for aggregation substance, *cyl A* gene for cytolysin, *efa A* gene for endocarditis antigen, *esp* gene for enterococcal surface protein, *gel E* for gelatinase and *hyl* gene for hyaluronidase [6]. These virulence factors correlate with colonization of host tissue, modulation of host defense mechanisms and facilitate invasion [7].

Several studies found an association between the degree of virulence of enterococci and the pattern of resistance to antimicrobial agents while other studies reported that there was no relation between them, therefore increased virulence was not always associated with increased antimicrobial resistance [8].

The aim of the study was to detect the distribution of the virulence markers and the virulence genes among the isolated species of enterococci, determine the relation between virulence markers and virulence genes and study antibiotic resistance pattern of enterococci.

Methods

Sample collection

This is a cross-sectional laboratory based study conducted in Microbiology and Immunology Department, Central research lab, Sohag University hospital in the period from February 2020 to February 2021. Clinical data of the study participants were collected from their medical records. Clinical samples were collected from patients admitted to ICU at least 48 hrs after hospital admission including urine, pus swabs, sputum from non-invasive infections (urinary tract infection, surgical site infection, pneumonia respectively) and blood from invasive infections (sepsis and endocarditis) [9]. Informed consents were taken from all the patients or their relatives. Ethical approval was obtained from the ethical committee of Faculty of Medicine, Sohag University and the study was retrospectively registered on Clinical Trials.gov (identifier: NCT04777240).

Identification of enterococci

Clinical samples were transported rapidly, pus and sputum samples were inoculated directly on bile esculin azide agar (BEA) (CONDA, Spain). Urine samples were inoculated by calibrated loop (1 µL) on BEA agar, the agar plates were incubated at 37^{o}C for 24-48 hours. Blood samples were inoculated on blood culture bottles and incubated at 37^{o}C for 14 days, subcultures on BEA were performed each other day. On BEA agar enterococci had the ability to hydrolyse esculin to esculetin and dextrose, which reacts with ferric citrate producing a brownish black precipitate around the colonies. The growing colonies on BEA were examined microscopically after staining by Gram stain, enterococci appeared as Gram positive diplococcic or arranged in short chains, enterococci were catalase negative. Salt tolerance test was used to differentiate enterococci from group D streptococci: 2-3 colonies of the tested organism from 24 hrs BEA culture were inoculated on nutrient broth containing sodium chloride NaCl 6.5% and were incubated at 37^{o}C for 72hr. Enterococci can tolerate and grow in high concentration of NaCl (6.5%). Visible turbidity in the broth indicated growth of the organism.

Phenotypic detection of virulence markers of enterococci

1-Detection of gelatinase activity

This test was performed by stab inoculation of the colonies of enterococci on nutrient agar containing gelatin 3 % (Himedia, India), incubated for 24 hrs at 37^{o}C, then the tubes were refrigerated for 30 minutes at 4^{o}C. Positive results appeared as liquefaction of gelatin.

2- Detection of caseinase production

This test was performed by streaking colonies of enterococci on Muller hinton agar (Himedia, India)

containing skimmed milk 3%, plates were incubated for 24 hrs at 37ºC, appearance of transparent zone around colonies indicated positive results [10].

3- Formation of slime layer

This test was performed by inoculation of enterococci on brain heart infusion agar (LAB, United Kingdom) containing 5% sucrose, plates were incubated for 24 hrs at 37ºC. This test was used to detect the ability of enterococci to produce extracellular polysaccharide, positive strains gave mucoid and slimy colonies [10].

4-Modified microtiter plate method for detection of Biofilm

Enterococci were grown in brain heart infusion "BHI" broth (Himedia, India) supplemented with 0.25% glucose, incubated at 37ºC overnight, then the culture was diluted with non-inoculated BHI broth 1:20. Sterile polystyrene microtitre plate was inoculated with 200 µL of the diluted bacterial suspensions. The plate was incubated at 37ºC for 24 hrs, phosphate buffered saline was used for washing the wells, then dried and stained with crystal violet stain (1%) for 15 min. The wells of the plate were rinsed with distilled water and finally solubilized in ethanol/acetone(80/20). The absorbance was measured by ELISA reader at 630 nm. Biofilm formation was classified as follow: non biofilm forming (-), weak (+), moderate (++), strong (+++) corresponding to the A_{630} values ≤1, 1 - ≤2, 2-≤3 and >3 respectively [11].

Antibiotic sensitivity test

1-Disc diffusion test

Antibiotic sensitivity testing was performed by disc diffusion method on Muller Hinton agar (Himedia, India), results were interpreted according to CLSI 2018 [12].

2- Epsilometer-test (E-test)

Minimal inhibitory concentration (MIC) of vancomycin was measured by E-test for isolates which showed resistance or intermediate resistance to vancomycin by disc diffusion method. The test was performed by inoculating the surface of Muller Hinton agar plate (Himedia, India) with swab dipped in bacterial suspension adjusted to 0.5 McFarland standard turbidity followed by placing vancomycin strip on the surface of the agar plate which was incubated at 37ºC for 24 hours. Results were interpreted according to CLSI guidelines, vancomycin MIC breakpoints to (≤4µg/mL susceptible, 8–16 µg/mL intermediate, ≥32 µg/mL resistant) [12].

Molecular characterization of enterococci

1- DNA extraction

Bacterial DNA was extracted by boiling, 3-5 colonies of enterococci were emulsified in 100 µl of sterile distilled water, heated at 100ºC for 20 minutes and centrifuged at 10.000 rpm for 10 minutes. The extracted DNA was stored at -20ºC to be used for amplification of the target genes by polymerase chain reaction (PCR) [13].

2- Polymerase chain reaction

- ***Identification of enterococcus species***

Genotypic species identification of enterococci was performed by conventional gene specific uniplex PCR for *E. faecalis* and *E. Faecium* in thermal cycler (T Gradient-Biometra) with 25 µl total reaction volume including 5 µL of purified bacterial DNA, 1.25 µL of the forward primer, 1.25 µL of the reverse primer **(Table 1)**, 12.5 µl of master mix (Willowfort, UK) and 5 µl of molecular water. Cyclic conditions of the PCR reaction are listed in **table (2)**.The reaction was stopped by cooling at 4°C.

- ***Detection of virulence genes***

Identification of virulence genes; *gel E* (gene for gelatinase), *asa1* (gene for aggregation substance), *cyl A* (gene for cytolysin activator), *esp* (gene for Enterococcal surface protein) *hyl* (gene for Hyaluronidase) of *E. faecalis* and *E. faecium* was performed by uniplex PCR duo to variation in the melting temperature of the primers. Twenty-five µl total reaction volume including 5 µL of purified bacterial DNA, 1.25 µL of the forward primer, 1.25 µL of the reverse primer **(Table 1)**, 12.5 µl of Master mix (Willowfort, UK) and 5 µl of Molecular water. Cyclic conditions of the PCR reaction are listed in **table (2)**.The reaction was stopped by cooling at 4°C. The amplified products were visualized on 2% agarose gel stained with ethidium bromide. The stained gels were visualized and documented with a gel documentation system (InGenius 3, UK), and analyzed visually to determine the size of PCR amplicons of the target genes directly by comparison with DNA ladder (Willowfort BERUS 100bp, WF-WF10407001, UK) as shown in **table (1)**.

Statistical analysis

Data was analyzed using STATA version 14.2 (Stata Statistical Software: Release 14.2 College Station, TX: StataCorp LP.). Qualitative data was presented as number and percentage and compared using either Chi square test or fisher exact

test. *p* value was considered significant if it was less than 0.05.

Results

Enterococci species

During the study period, 52 isolates of enterococci were identified by colony morphology on BEA agar and their ability to grow in presence of NaCl 6.5%

Enterococci were identified up to species level by PCR **(Figure 1)** as follow: 30 isolates (57.7 %) were *E. faecalis* and 22 isolates (42.3 %) were *E. faecium*. According to the results of antibiotic sensitivity test, 20 isolates (38.4 %) were classified as VRE (12 *E. faecalis* and 8 *E.faecium*) and 32 isolates (61.6%) were VSE (18 *E. faecalis* and 14 *E.faecium*).

Enterococci were isolated from different types of infection, the non-invasive infections include the following; 18 from surgical site infections (SSI), 14 from urinary tract infections (UTI), 4 from pneumonia and invasive infections as follow: 12 from sepsis and 4 from endocarditis. There was no significant difference in the distribution of enterococci species according to the type of infection (*p*-value= 0.11).

There was significant difference between the association of some risk factors with the type of infection. The invasive diseases were more common with certain risk factors as central venous catheter, urinary catheter and diabetic patients than non-invasive diseases and these results were significant as shown in **table (3)**. There was no significant association between any risk factor with infection with VRE.

Virulence markers

The prevalence of virulence markers among the isolates of enterococci were as follow; caseinase (73%), biofilm formation (73%), gelatinase (34.6%) and slime layer production (26.9%).

In this study, according to the results of the phenotypic methods used for detection of virulence markers, there was no statistically significant difference in the distribution of all detected virulence markers between *E. faecalis* and *E. faecium*. Also, there was no significant difference in the distribution of of all virulence markers of enterococci in invasive and non-invasive infections.

Virulence genes

Virulence genes were detected among the isolates of enterococci as follow; *asa1* (84.6%), *esp* (80.7%) , *gel E* (73%), *hyl* (38.4%) and *cyl A* (30.7%) **(Figure 1)**. According to the results of PCR, among the identified species of enterococci, we found that only the *hyl* gene was more commonly detected in *E. faecalis* (53.33%) and less prevalent in *E. faecium* (18.18%) and these results were significant **(Table 4)**.

Among non-invasive infections, we found that enterococci isolated from cases of surgical site infections and pneumonia contain *gel E* and *asa1* genes with the highest percentage. While in invasive infections, enterococci isolated from cases of sepsis contain *gel E* and *esp* genes with the highest percentage **(Table 5)**.

Relation between virulence markers and virulence genes

In this study, 90.48% of enterococci carry the *esp* gene were biofilm producers and 86.36% of enterococci carry the *asa1* gene were biofilm producers **(Table 6)**.

Antibiotic resistance in *E. faecalis* and *E.faecium*

Overall antibiotic resistance in enterococci was as follow: tetracycline (92.3%), ampicillin (76.9%), erythromycin (73%), vancomycin (38.4%) and teichoplanin (15.3%). In this study 57.6% of enterococci were MDR. Multi-drug resistant strains were more commonly detected in *E. faecium* (72.73%) than *E. faecalis* (46.67%).

In this study 80% of MDR strains were biofilm producers, MDR strains of *E. faecium* (87.50%) were more biofilm producers than MDR strains of *E. faecalis* (71.43%).

Vancomycin resistance was higher in *gel E* positive and *asa1* positive *E. faecalis* than negative *E. faecalis* (*p*-value= 0.02, *p*-value= 0.02). There was no association between *hyl*, *esp* and *cyl A* genes and vancomycin resistance.

There was no relationship between ampicillin, erythromycin, teichoplanin and tetracycline resistance and virulence genes in *E. faecalis*. There was no relationship between antibiotic resistance and virulence genes in *E. faecium*.

Table 1. Sequence of primers used to identify Enterococcus species and to detect the virulence genes.

Target gene	Primer Sequence from 5′ to 3′	Molecular size
E. faecalis	5′ATCAAGTAC AGTTAGTCT-3′ 5′-ACGATTCAAAGCTAACTG-3′	941 bp [14]
E. faecium	5′-TTGAGGCAGACCAGA TTGACG-3′ 5′-TATGAC AGCGACTCCGATTCC-3′	685 bp [14]
gel E	5′- TATGACAATGCTTTTTGGGAT-3′ 5′- AGATGCACCCGAAATAATATA-3′	100 bp [15]
asa1	5′- GCACGCTATTACGAACTATGA-3′ 5′- TAAGAAAGAACATCACCACGA-3′	375 bp [15]
hyl	5′- ACAGAAGAGCTGCAGGAAATG-3′ 5′- GACTGACGTCCAAGTTTCCAA-3′	200 bp [15]
esp	5′-TTGCTAATGCTAGTCCACGACC-3′ 5′-GCGTCAACACTTGCATTGCCGA-3′	932 bp [16]
cyl A	5′-GACTCGGGGATTGATAGGC-3′ 5′-GCTGCTAAAGCTGCGCTTAC-3′	688 bp [16]

gel E; gelatinase, *asa1*; aggregation substance , *hyl*; hyaluronidase , *esp*; enterococcal surface protein, *cyl A*; cytolycin A.

Table 2. Cyclic conditions of uniplex PCR reactions for each target gene.

Target gene	Cycling conditions (30 cycles)				
	Initial denaturation	**Denaturation**	**Primer annealing**	**Primer extension**	**Final extension**
E.faecalis	94ºC for 5 min	94ºC for 30 sec	42ºC for 30 sec	72ºC for 48 sec	72ºC for 10 min
E.faecium	94ºC for 5 min	94ºC for 30 sec	56ºC for 30 sec	72ºC for 35 sec	72ºC for 10 min
gel E	94ºC for 5 min	94ºC for 30 sec	49ºC for 30 sec	72ºC for 5 sec	72ºC for 10 min
asa1	94ºC for 5 min	94ºC for 30 sec	50ºC for 30 sec	72ºC for 25 sec	72ºC for 10 min
hyl	94ºC for 5 min	94ºC for 30 sec	54ºC for 30 sec	72ºC for 10 sec	72ºC for 10 min
esp	94ºC for 5 min	94ºC for 30 sec	61ºC for 30 sec	72ºC for 48 sec	72ºC for 10 min
cyl A	94ºC for 5 min	94ºC for 30 sec	55ºC for 30 sec	72ºC for 35 sec	72ºC for 10 min

Table 3. Relation between risk factors and type of infection.

Risk factor	Invasive N=16	Noninvasive N=36	*p* value
Age/year			
Mean ± SD	43.81±10.21	46.19±10.76	0.46
Median (range)	44 (22:62)	46.5 (19:95)	
Gender			
Female	9 (56.25%)	23 (63.89%)	0.60
Male	7 (43.75%)	13 (36.11%)	
Hypertension	2 (12.50%)	5 (13.89%)	1.00
Diabetes mellitus	7 (43.75%)	3 (8.33%)	0.006
Postoperative	4 (25.00%)	19 (82.78%)	0.06
Days of hospitalization			
Mean ± SD	8.94±3.64	8.14±2.63	0.50
Median (range)	8.5 (4:18)	7.5 (4:14)	
Antibiotic	16 (100%)	30 (83.33%)	0.16
Central venous catheter	16 (100%)	10 (27.78%)	<0.0001
Urinary catheter	16 (100%)	18 (50.00%)	<0.0001

Invasive infections include sepsis and endocarditis *

*Non-invasive infcetions include pneumonia, surgical site infection, and urinary tract infection

Table 4. Distribution and frequency of virulence genes among enterococcal species (*E. faecalis* and *E.faecium*).

Virulence genes	*E. faecalis* N=30	*E.faecium* N=22	*p-value*
gel E	20 (66.67%)	18 (81.82%)	0.22
asa1	24 (80%)	20(90.91%)	0.44
hyl	16 (53.33%)	4(18.18%)	0.01*
esp	24(80%)	18(81.82%)	1.00
cyl A	10(33.33%)	6 (27.27%)	0.64

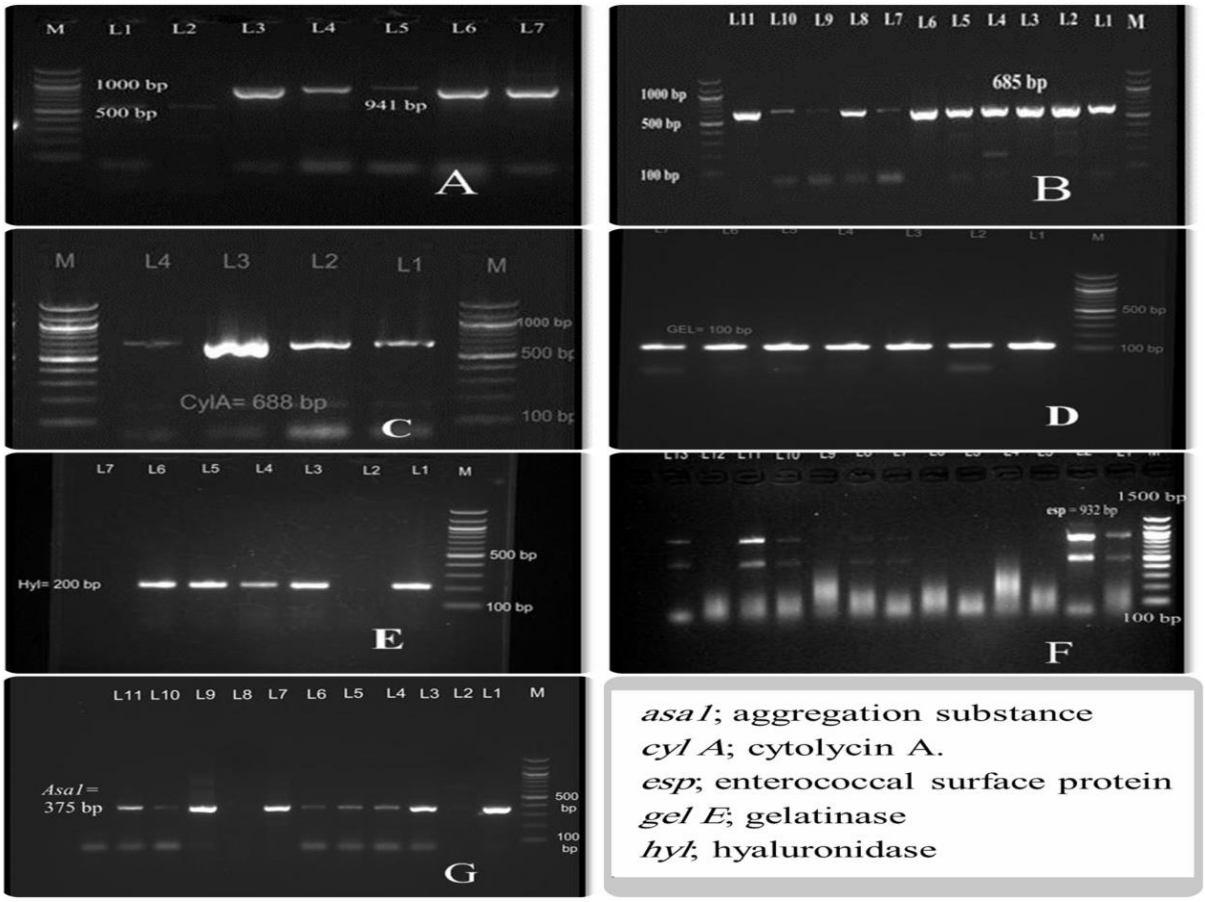
Table 5. Distribution of virulence genes according to the type of infection.

Virulence genes	Invasive infections		Non Invasive infections			*p* value
	Endocarditis N=4	Sepsis N=12	Pneumonia N=4	SSI N=18	UTI N=14	
gel E	2(50%)	12(100%)	4(100%)	18(100%)	2(14.29%)	<0.0001
asa 1	4(100%)	10(83.33)	4(100%)	18(100%)	8(57.14%)	0.01
hyl	0 (0%)	8(66.67%)	0(0%)	6 (33.33%)	6(42.86%)	0.053
esp	2(50%)	12(100%)	4(100%)	16(88.89%)	8(57.14%)	0.02
cyl A	0(0%)	4(33.33%)	0(0%)	10(55.56%)	2(14.29%)	0.03

Table 6. Relation between *esp*, *asa 1* and *gel E* genes with biofilm formation.

Biofilm N	*esp* gene		*p* value
	Negative N=10	Positive N=42	
Non biofilm forming	10 (100%)	4 (9.52%)	<0.0001
Biofilm forming	0	38 (90.48%)	
Biofilm N	***asa 1* gene**		***p* value**
	Negative N=8	**Positive N=44**	
Non biofilm forming	8 (100%)	6 (13.64%)	<0.0001
Biofilm forming	0	38 (86.36%)	
Biofilm N	***gel E* gene**		***p* value**
	Negative N=14	**Positive N=38**	
Non biofilm forming	4 (28.57%)	10 (26.32%)	0.59
Biofilm forming	10 (71.43%)	28 (73.68%)	

Figure 1 . Agarose gel electrophoresis of PCR products.



A;showing *E. faecalis* gene = 941 bp, M: DNA marker 100- 1500 bp. B; showing *E. faecium* gene = 685 bp, C; showing *cyl A* gene = 688 bp, D: showing *gel E* gene = 100 bp , E: showing *hyl* gene = 200 bp, F: showing *esp* gene= 932bp, G; showing *asa1* gene= 375 bp.

Discussion

Enterococcus faecalis is considered as the most pathogenic and virulent species, it contains highly efficient mechanisms for horizontal gene transfer like highly transmissible plasmids which can disseminate virulence genes to the less virulent species as *E. faecium* [15]. In this study, *E. faecalis* was identified as the major cause of enterococcal infections, also the frequency of vancomycin resistant *E. faecalis* was higher than vancomycin resistant *E. faecium*, which is consistent with **Sabouni et al.** [17].

In this study, diabetes mellitus, urinary catheterization and central venous catheters were the main predisposing factors for infection with enterococci in invasive diseases, but there was no significant association of any risk factor with infection by VRE. Inconsistence to our results, previous study documented that chronic diseases as diabetes mellitus, obstructive pulmonary disease, chronic renal failure and chronic heart disease, in addition to antibiotic and corticosteroid usage were the main predisposing factors for infection with VRE in ICU patients [18].

Enterococcal virulence factors mediate adhesion, colonization and invasion. Many virulence factors are involved in adherence of enterococci to host cells and biofilm formation such *esp*, aggregation substance and gelatinase. Invasion is mediated by hyaluronidase and gelatinase in presence of damaged tissues. Enterococci which have virulence factors are potentially capable of inducing an infection with a more severe course than strains deprived of them [19].

Study was performed by **Nasaj et al.** [20] showed an association between the expression of virulence markers and emergence of nosocomial infections caused by enterococci. However, **Shokoohizadeh et al.** [21] did not find association between the presence of vancomycin resistance, virulence markers and the type of infection. While, in a research conducted by **Beceiro et al.** [22] he concluded that increased antibiotic resistance and

virulence finally proceed together to confer the bacteria with a selected advantage.

In this study, there was no significant difference in the distribution of virulence markers between *E. faecalis* and *E. faecium*, so *E. faecium* is virulent as *E. faecalis*.

Gelatinase is a metalloprotease that can hydrolyze gelatin and collagen, It is encoded by *gel E* operon, Some studies suggest the involvement of gelatinase in adhesion and biofilm formation [23]. In this study, gelatinase was the least frequently detected virulence factor, we detected gelatinase in 34.6% of enterococci, our results are consistent with **Kadhem** [24]. While, the *gel E* was detected in 73% of enterococci, so the presence of the *gel E* gene in enterococci was not strictly associated with its expression. This is explained by the presence of silent *gel E* genes in some isolates of enterococci that are expressed only under certain conditions in vivo, or due to the presence of gene mutations or involvement of other genes that control expression of *gel E*.

In this study, *esp* was detected in 80.7% of the isolates of enterococci, *esp* gene is coding for *esp* protein that mediates interaction with primary surfaces, contributes to colonization and participates in biofilm formation which enhance bacterial survival and involved in resistance to antimicrobial drugs [25].

There are divergent findings concerning the relation of *esp* with biofilm production. We suggest that there is a relation between the presence of *esp* and the ability of enterococci to form biofilms, because none of the *esp* negative isolates were capable of forming biofilms. However, **Toledo-Arana et al.** [26] found that insertional inactivation of *esp* did not resulting in loss of the ability of enterococci to form biofilm in vitro. While **Di Rosa et al.** [27] found that *esp* and biofilm were present together only in enterococci isolated from clinical infections, suggesting their important role in the successful establishment of the infectious process.

Asa1 gene is coding for aggregating substance that increases bacterial adherence to cells of endocardium, intestinal epithelial and renal tubules facilitating colonization of gastrointestinal and urinary tract and poses a risk for patients with cardiac surgery [28]. Asa1 gene was the most common virulence marker detected in this study and it was associated with biofilm formation.

In contrast, *cyl A* gene is coding for toxin which has a cytolytic activity, affecting the integrity of the cell membrane (as erythrocytes) and attacks the cells of the immune system disturbing their functions. Enterococcal isolates contain *cyl A* are dangerous for haematological patients. *Hyl* is coding for hyaluronidase which promotes invasion by destruction of the connective tissue [29]. *Cyl A* and *hyl* were the least frequently detected virulence genes in this study and detected only in cases of SSI, UTI and sepsis.

In this study, we found that there was no relation between the distribution of virulence markers and the type of infection either for invasive or non-invasive infections. An important finding in this study was the high dissemination rates of genes coding for some virulence markers among enterococci isolated from SSI and pneumonia as *gel E* and *asa1* in comparison to enterococci isolated from UTI. Also, among invasive infections, enterococci isolated from sepsis carried many virulence genes with high frequency in comparison to enterococci isolated from endocarditis cases. Inconsistence to our results, **Strateva et al.** [30] detected high prevalence of virulence genes in enterococci isolated from UTIs.

Multi-drug resistance is defined as non-susceptibility to at least one agent in three or more antimicrobial classes, according to this definition, we detected MDR in 57.6% of enterococcal isolates which is consistent with **Ghaziasgar et al.** [31] who detected MDR in 42% of all isolates. In this study we detected higher level of multi drug resistance in *E. faecium* than *E. faecalis* (72.73%, 46.67% respectively). Our findings are consistent with **Fallah et al.** [32] who detected that 100% of *E. faecium* were MDR while 74.6% of *E. faecalis* were MDR. Horizontal gene transfer is responsible for the increased resistance levels to different antimicrobials, because plasmids and transposons carry resistance genes for more than one antibiotic class. In contrast to our results, **Durgesh et al.** [33] detected low antibiotic resistance rates among the isolated enterococci as following (teichoplanin 0%, vancomycin 4% and amoxicillin 6%) .

Biofilm formation is important in pathogenesis of enterococci, as it plays an important role in colonization and development of drug resistance. Bacterial biofilms can develop on tissue epithelia as well as on body implant devices which is a significant risk factor in addition to extensive

use of antibiotics in hospitals promoting the development of biofilm in enterococci [34].

In this study, MDR strains of *E. faecium* were more biofilm producers than MDR strains of *E. faecalis* , **Ghaziasgar et al.** [31] found the prevalence of MDR strains didn't differ between the two enterococci species and there was no significant relationship between biofilm formation and MDR in enterococci.

This study has some limitations. Firstly, the study has only involved clinical samples from our hospital, thus, the findings cannot be generalized to all hospitals. Secondly, environmental samples should be included to study the genetic evolution of enterococci, because any significant findings may be useful to relevant authorities in managing enterococcal infections by a One Health Concept accordingly. Thirdly, the small sample size could have compromised the statistical analysis. Further studies with a higher number of isolates and more antibiotic agents are recommended.

Conclusion

Enterococcus faecalis was identified as the major cause of enterococcal infections in ICU. There was no considerable difference in the distribution of virulence markers between *E. faecalis* and *E. faecium***.** Multi-drug resistance was detected in 57.6% of enterococci. An association was noted between the esp & *asa1* genes and biofilm formation, VRE was detected in 38.4 % of enterococci, vancomycin resistance was higher in *gel E* positive and *asa1* positive *E. faecalis*. Enterococci isolated from SSI, pneumonia and sepsis possessed multiple virulence genes with high percentage.

Conflicts of interest

The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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