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ORIGINAL ARTICLE

Enterococcal Biofilm in Colonization and Disease, Correlation with Virulence Determinants and Vancomycine Resistance

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ABSTRACT

Background: *Enterococci possess many virulence genes implicated in their pathogenesis. Biofilm help the organism to colonize and cause infections.* ***Objectives:*** *This study was set out to investigate and compare biofilm formation ability and presence of asa1 and esp genes among E. faecium and E. faecalis isolates from diverse sources. Furthermore, association between biofilm formation, esp, asa1 genes and vancomycin resistance was analyzed.* ***Methodology:*** *76 pathogenic enterococcal isolates and 36 enterococcal isolates from healthy individuals were collected. All isolates were investigated for biofilm using microtitre plate, asa1and esp genes were detected by primer-specific PCR, vancomycin resistance were screened using agar method and confirmed by PCR.* ***Results:*** *the majority of clinical isolates (80.5%) were biofilm producers, however biofilm was detected only in 36.4% of colonizing isolates. E. faecalis (82.6%) produced biofilm more than E. faecium (36.1%), esp gene (48.5%) was presented more than asa1gene (15.2%). Virulence genes were detected in high rates among biofilm producers isolates, low vancomycin resistance rate was seen among isolates which produced slim layers.* ***Conclusion:*** *biofilm was detected in high rate in E. faecalis harbored esp gene. Biofilm, asa1 and esp genes were more presented among isolates from non invasive sites than invasive sites, thus aids enterococci to provoke clinical infections, lower biofilm was seen in E.faecum. However, vancomycin resistant isolates produced less slim than vancomycin sensitive.*

INTRODUCTION

Enterococci have evolved over the past century from being an intestinal commensal organism to becoming from the most prevalent pathogen causing hospital infections[1]. In 2017, the World Health Organization listed Enterococci in their "Global Priority list of antibiotic-resistant bacteria"[2].

Although about a dozen of enterococcus species were identified, *E. faecalis* and *E. faecium* are the most predominant pathogenic species and accounts for 90% of infections caused by enterococci .They are considered as a global cause of many serious nosocomial infections; urinary tract infections, endocarditis, intra-abdominal infections and septicemia with high mortality[3].

Apart from the trend of high antibiotic resistance seen in enterococci, they are equipped with many genes encoding virulence that enable them to adhere, colonize host tissue and develop biofilm[4].

Biofilm in enterococci is complex, multifactorial and may be attributable to adherence and spreading factors[5], including *Asa1* (aggregation substance) and *esp* (enterococcal surface protein), *gelE* (gelatinase) and *hyl* (hyaluronidase)[6].

Esp is a cell wall associated protein increases adherence and production of biofilm in enterococci, which lead to resistance to surrounding toxins and antimicrobial agents[7]. *Asa1* increases bacterial adherence[8].

Biofilm has a vital role in pathogenesis of infections, it can promote and sustain infection due to restricted penetration of antimicrobials and also expression of possible resistance genes as they are not easily eradicated by bactericidal antibiotics, around 80% of chronic diseases are related to biofilms[9]. *E. faecium* and *E. faecalis* are now well recognized as multidrug-resistant pathogens, and about 30 years ago, both species acquired resistance to the important last-line bactericidal drug, vancomycin[10]. The current study was set out to investigate and compare biofilm formation ability and presence of *asa*1 and *esp* genes among *E. faecium* and *E. faecalis* isolates from diverse sources. Furthermore, possible association between the occurrence of biofilm with presence of virulence genes and vancomycin resistance was analyzed.

METHODOLOGY

Collection of samples:

A total of 72 clinical enterococcus isolates were collected from different samples (urine, pus, blood , endotracheal aspirates and sputum samples) from patients admitted to Menoufia University hospitals (MUH) and having nosocomial infections. Additionally, 33 enterococci strains were collected from healthy people (stool samples) as colonizing isolates. All samples were processed according to conventional methods[11].

Bacterial Isolation and Identification:

All clinical and stool samples obtained were cultured and identified according to standard microbiological methods[11]. Identification of all enterococcus isolates by API system **(**bioMèrieux) was done. A total of **72** clinical *enterococcus* isolates, consisting of (24) *E. faecium and* (48) *E. faecalis* and **33** fecal isolates, consisting of (12) *E. faecium* and (21) *E. faecalis* were enrolled in this study.

Vancomycin Resistance:

E. faecium and *E. faecalis* isolates were tested for vancomycin susceptibility using the agar screen method and broth dilution method[12,13].

Biofilm detection by Microtiter Plate method (MTP):

E. faecium and E. faecalis isolates were assayed for their ability to form biofilms on microtiter plates and interpreted as described previously[14]. Bacteria subcultured onto trypticase soy agar (Oxoid) plus 5% glucose then transferred to trypticase soy broth plus 5% glucose, then were inoculated in wells of polystyrene plate. After incubation for 48h., the plates were shaken then fixed with methanol for 10 min. The attached bacterial material was stained by adding 150 ml crystal violet for 20 min. The optical density was measured and interpreted with an ELISA reader at a wavelength 570nm[14].

Detection of vanA and vanB genes in VRE:

Detection of *vanA* and *vanB* genes in VRE using multiplex PCR. Primers chosen for amplification are shown in table (1). Rapid DNA extraction method was performed, PCR amplification was done as described by Co *et al.* [15] .

Detection of Virulence genes:

All primer sequences are listed in table (1) .PCR amplification was performed in a total volume of 50 ml, containing 2 PCR Master Mix, 0.5mMof each primer, and 1 ml template DNA. The cycling conditions were as follows:95°C for 3 min; followed by 30 cycles at 95°C for 30 s, 52°C for 30 s, 72°C for 60 s; and a final 10 min extension step at 72°C[8].

Table 1: Target genes and primers used in this study.

Target gene	Primers (5′ to 3′)	Product (bp)	References
vanA	**CAT GAA TAG AAT AAA AGT TGC AAT A CCC CTT TAA CGC TAA TAC GAT CAA**	**1030**	**Co *et al.* [15]**
vanB	**GTG ACA AAC CGG AGG CGA GGA CCG CCA TCC TCC TGC AAA AAA**	**433**	**Co *et al.* [15]**
asa1	**GCACGCTATTACGAACTATATGA asa2: TAAGAAAGAACATCACCACGA**	**375**	**Vankerckhoven *et al.* [8]**
esp	**GGAACGCCTTGGTATGCTAAC GCCACTTTATCAGCCTGAACC**	**510**	**Vankerckhoven *et al.* [8]**

RESULTS

In the current work, enterococcus biofilms and its possible correlation with virulence determinants were investigated. A total of 72 enterococcus isolates were recovered from clinical sites, out of them, 24 (33.3%) isolates were identified as *E. faecium* and 48 (66.7%) isolates as *E. faecalis*), moreover, 33 enterococcal fecal isolates (colonizing) were included, 12 *E. faecium* and 21 *E faecalis* isolates.

In this study, *E. faecium* were commonly seen with invasive sites infection (71.4%), while *E. faecalis* were significantly recovered from noninvasive sites (82.3 %). *E. faecium* clinical isolates were 100% associated with blood stream infection, on the other hand, 87.5% of urine pathogens were *E. faecalis*; other results are presented in table (3).

In our study, biofilm was observed in 66.6% of all isolates .Quantitative evaluation of biofilm among studied isolates revealed its role in pathogenesis of infection as the majority (80.5%) of clinical isolates showed slim layers compared to colonizers (36.4%) with statistically significant difference ($P<0.001$). Also, most of *E. faecalis* isolates 57/69 (82.6%) had biofilms on contrary to *E. faecium* (36.1%) ($P<0.001$). A remarkable finding was that non of colonizing *E. faecium* isolates expressed biofilm [table 2 and fig. 1].

Table 2: Biofilm formation among clinical and colonizing *E. faecalis* and *E. faecium.* isolates

	Total isolates	Biofilm-producer enterococci	Non-biofilm-producer enterococci	P
Total enterococcl isolates	**N=105**	**70(66.6%)**	**35(33.4%)**	
Clinical enterococcal isolates	***E. faecalis* =48/ 72 (66.7%)**	**45/48 (93.8%)**	**3(6.2%)**	***P* <0.001**
	***E. faecium* =24/ 72 (33.3 %)**	**13/ 24(54.2%)**	**11(45.8%)**	***P* > 0.05**
	Total clinical isolates (n=72)	**58/ 72(80.5%)**	**14/ 72(19.5%)**	***P* <0.001**
Colonizing enterococcal isolates	***E. faecalis*=21/33 (63.6%)**	**12/21 (57.1%)**	**9/ 21(42.9%)**	***P* > 0.05**
	***E. faecium* =12/ 33 (36.4%)**	**0**	**12/ 12(100%)**	***P* <0.001**
	Total Colonizing (n=33)	**12/ 33(36.4%)**	**21/ 33(63.6%)**	***P* < 0.05**
All E. faecalis n(%) / E. faecium n(%)	**69(65.7%)/ 36(34.3%)**	**57/ 69(82.6%) / 13/ 36(36.1%)**	**12/ 69(17.4%) / 23/ 36(63.6%)**	***P* <0.001**

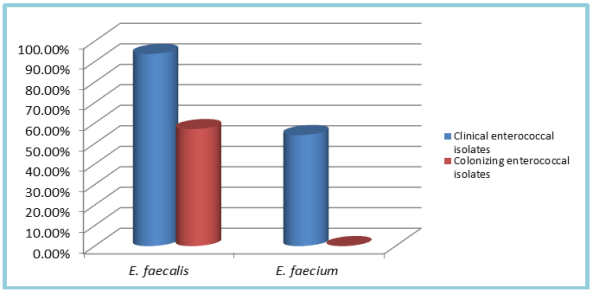

Fig. 1: Frequency (%) of Biofilm formation among clinical and colonizing *E. faecalis* and *E. faecium* isolates.

With respect to isolation sites table (3), All isolates detected in urine samples formed biofilms, whether in *E. faecalis* or *E. faecium* (100%), followed by wound swab (88.8%) and burn swab (80%); the likelihood of biofilm percentage was similar across the remaining sources. Moreover, most of isolates from noninvasive sites (94.1%) showed more biofilm than isolates from invasive infection sites (47.6%).

Table 3: Biofilms formation with regard to isolation sites , invasive and non invasive sites of infection among *E. faecalis* and *E. faecium* clinical isolates.

	***E. faecalis*(48)**		***E. faecium*** (24)		
Site of isolation	***E. faecalis* isolates (n=48)**	***E. faecalis* Biofilm Producer (n=45)**	***E. faecium* isolates (n=24)**	***E. faecium* Biofilm Producer (n=13)**	**All positive biofilms**
Endotracheal aspirates(14)	6(42.8%)	5/ 6(83.3%)	8 (%57.2)	3 /**8** (37.5%)	**8 (57.1%)**
Blood culture(7)	0	0	7 (100%)	2/**7** (28.5%)	**2 (28.5%)**
Invasive sites infection (21)	**6(28.6%)**	**5/ 6 (83.3%)**	**15 (71.4%)**	**5/ 15(33.3%)**	**10 (47.6%)**
Burn swab (10)	8 (80%)	6/ 8(75%)	2(20%)	2/ 2(100%)	**8 (80%)**
Wound swab (9)	6 (66.6%)	6/**6** (100%)	3 (33.4%)	2/ 3(66.6%)	**8 (88.9%)**
Urine (32)	28(87.5%)	28/ 28 (100%)	4(12.5%)	4/ 4(100%)	**32 (100%)**
Noninvasive sites infection (51)	**42 (82.3 %)**	**40/42 (95%)**	**9/ (17.7%)**	**8/ 9(89%)**	**48 (94.1%)**
Total n=72	**(n=48)**	**45/48 (93.8%)**	**(n=24)**	**13/ 24 (54.2%)**	58(80.5%)

In terms of genes encoding for potential virulence , in the current study the distribution of virulence genes among either *E. faecalis/E. faecium* and clinical/colonizing isolates were compared in table (4) and figure (2a and 2b) . *Esp* gene 51/105(48.5%) was significantly presented more than *asa1*gene 16 /105 (15.2%), (P<0.05), among total enterococci. Virulence determinants were more prevalent in *E. faecalis* compared to *E. faecium* isolates, *esp* gene was significantly associated with *E. faecalis* (56.7%) more than *E. faecium*(33.3%) (P<0.05), and in clinical isolates 38/72(52.8%) compared to colonizing isolates (39.3%).

However, *asa1* gene detected in small number of isolates of both *E. faecalis* (21.7%) and *E. faecium* (2.7%) strains, moreover, non of colonizing isolates harbored this gene. A remarkable finding in this study, the majority of isolates had either only *esp* or *asa1* gene , but fewer clinical isolates 7 (15.2%) having both genes and 29.5% of total isolates lacking both genes.

Table 4: Distribution of virulence determinants among clinical and colonizing *E. faecalis* and *E. faecium* isolates

Isolates genotypes	Total isolates	Clinical Isolates(72)		Total clinical isolates (n=72)	Colonizing isolates(33)		Total colonizing (n=33)	*E. faecalis* n (%) /*E. faecium* n (%)	P
		E. faecalis	*E. faecium*		*E. faecalis*	*E. faecium*			
***esp*+ isolates**	51/105 (48.5 %)	30/48 (62.5%)	8/ 24 (33.3%)	38 (52.8%)	9 / 21 (42.8%)	4/ 12 (33.3%)	13 (39.3%)	39 (56.5%) / 12(33.4 %)	P<0.05
***asa1*+isolates**	16/105 (15.3 %)	15/48 (31.3 %)	1/24 (4%)	16 (22.2%)	0	0	0	15 (21.7%)/ 1(2.7%)	P>0.05
***esp*+ and *asa1*+ isolates**	7/105 (6.7%)	2/48 (4.2%)	5/24 (20.8%)	7 (9.7%)	0	0	0	2 (2.9%)/ 5 (13.9%)	P>0.05
***Esp*¯, *asa1*¯ isolates**	31/105 (29.5%)	1/48 (2%)	10/24 (41.6%)	11 (15.3%)	12/ 21 (57.2%)	8/ 12 (66.7%)	20 (60.7%)	13(18.9%)/ 18(50%)	P<0.05
Total n=105	105	48	24	72	21	12	33		

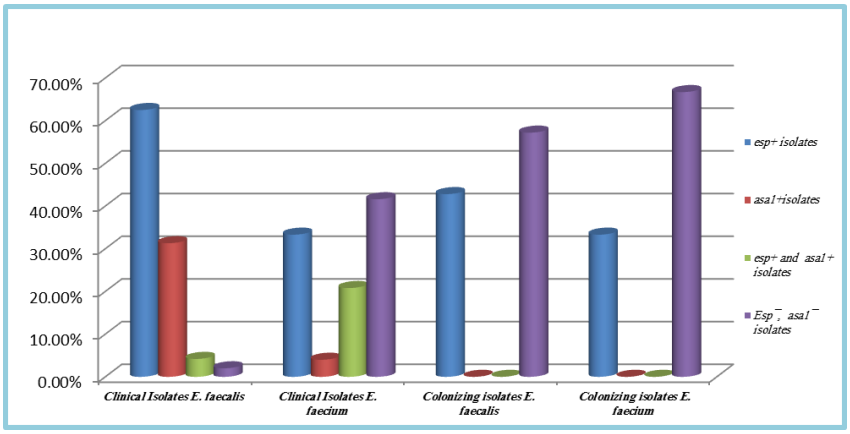

Fig. 2a: Distribution of virulence determinants among clinical and colonizing *E. faecalis* and *E. faecium* isolates.

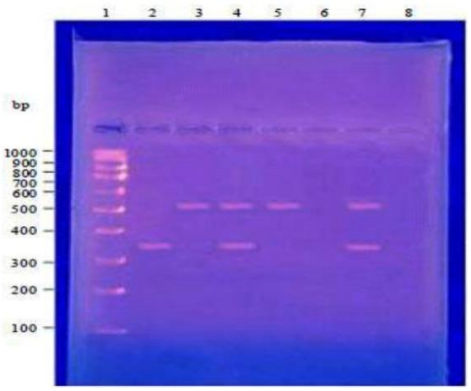

Fig. 2b: Virulence genes e*sp* and a*sa*1 is 510 bp and 375 bp respectively.(L1): molecular size marker 100

In the present work, a strong association between the presence of virulence determinants and biofilm occurrence were detected and delineated in table (5) and figure (3). Majority of biofilm producers possessed either *asa1* , *esp* gene or both 70/74 (94.5%). It should be pointed out that 7 isolates that expressed both genes showed (100%) biofilm production, even though among 31 isolates lacking both *esp* and *asa1* genes, 4/ 31(12.9%) isolates were bioflm producers. A strong relation were observed whether in clinical or colonizing isolates harbored *esp* gene and biofilm formation ,47 /51(92%). Interestingly all clinical esp positive isolates (38/ 38; 100%) showed biofilm formation ability. On the other hand, no significant difference was detected concerning *asa1* gene association with biofilm , as 20.7% of the biofilm producing clinical isolates and 28.5% of the nonbiofilm producing isolates carried it .

Table 5: Association between biofilms formation and presence of virulence genes among Enterococcus isolates of diverse origin.

	Clinical Isolates		Colonizing isolates		
Biofilm genotype	**Biofilms producer**	**Non-biofilms producer**	**Biofilms producer**	**Non-biofilms producer**	**Total Biofilm+ / total genotype**
***esp*+ gene (51)**	**38/58 (65.5%)**	**0**	**9/12 (75%)**	**4/21 (19%)**	**47 /51(92%)**
***asa1*+gene (16)**	**12/58 (20.7 %)**	**4/14 (28.5%)**	**0**	**0**	**12/16 (75%)**
***esp*+gene and *asa1*+gene (7)**	**7/58 (12.1%)**	**0**	**0**	**0**	**7/ 7(100%)**
***Esp*¯gene, *asa1*¯ gene (31)**	**1/58 (1.7%)**	**10/14 (71.5%)**	**3/12 (25%)**	**17/21 (81%)**	**4 / 31(12.9%)**
Total	**n=58**	**n=14**	**n=12**	**n=21**	**70/74(94.5%)**

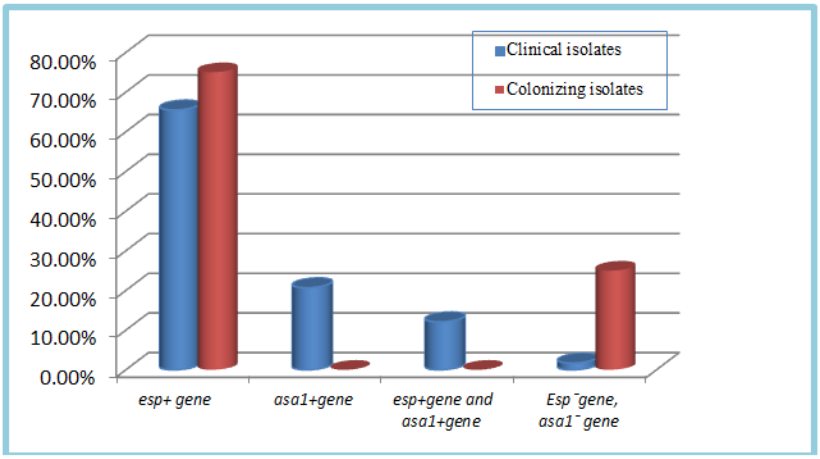

Fig. 3: Frequency (%) of virulence genes among positive biofilm producers enterococcus isolates of diverse origin.

In this study, among the 105 e*nterococcus* isolates, 24 (22.9%) and 81(70.1%) were VRE and VSE isolates, respectively. VRE detected by MIC tests were also confirmed by PCR methods, all the VRE isolates harbored the *vanA* gene. Furthermore, *E. faecium* (44.4%) showed higher vancomycin resistance compared to *E. faecalis* (11.5%) , only 12.9% (9/70) of all detected biofilm producers enterococci were vancomycin resistance and all were clinical isolates. Biofilms formation were lower among VRE, 9/24 (37.5%) compared to VSE 61/ 81(75.3%), none of colonizing VRE showed slim layer. Slime-producing strains 9/24 (37.5%) showed low vancomycin resistance compared to non slime-producing strains 15(62.5%), table (6).

Table 6: Relation between vancomycin resistance and presence of biofilms among enterococcal isolates.

Total (n=105)	Biofilm-producer enterococci n=70 (%)	Non-biofilm-producer enterococci n=35 (%)
Total VRE n=24/105 (22.9%)	9**(37.5%)**	15**(62.5%)**
Clinical VRE n=22/72 (30.5 %)	9**(40.9%)**	13**(59.1%)**
Colonizing VRE n=2/ 33(6%)	0	2**(100%**
Total VSE n=81/ 105(70.1%)	61**(75.3%)**	20**(24.7%)**
Clinical VSE n=50/72 (69.5%)	49**(98%)**	1**(2%)**
Colonizing VSE n=31/ 33(94%)	**12(38.7%)**	19**(61.3%)**
Total VR *E. faecalis n*=8/ 69 (11.5%)	5**(62.5%)**	3**(37.5%)**
Total VR *E. faeciumn*=16/ 36 / (44.4%)	4**(25%)**	14**(75%)**

DISCUSSION

Enterococci have recently emerged as a global threat in hospital sittings, being the third most frequently reported nosocomial pathogen that increasingly associated with antibiotic resistance and hospital mortality[16]. *E. faecalis* is known to be the predominant species involved in enterococcal infections, a finding detected in this study and was in agreement with previous studies[17,18]. However, recently increasing vancomycin resistance among enterococci was explained by predominance of *E. faecium* in hspitals[19].

Enterococci have tendency to be incased in slim layers , which is a vital strategy allowing them to presist in bad environmental conditions[17]. In this work, pathogenic enterococci were significantly exihibted biofilm more than colonizing isolates (80.5% vs 36.4%) ,(P <0.001). This finding was comparable to that reported previously by Mohamed *et al.,*[20] Al mohamad *et al.*[21] and Goudarzi *et al.*[5], moreover, Hashem *et al.*[22] in Egypt observed higher percentages of biofilm among his isolates compared to other developing countries[23]. In contrast to our result, Johansson and Rasmussen[24], reported that colonizer isolates showed high percentage of biofilm compared to pathogenic isolates and assumed that pathogenic enterococci needs virulence factors to help invasion not adherence as biofilm**.**

Regarding biofilm production among *E. faecalis* and *E. faecium*, in our study, biofilm production was significantly associated (P<0.001) with *E. faecalis* (82.6% vs 36.1% in *E. faecium*). Moreover, most of *E. faecalis* biofilm producer were from clinical isolates (93.8%) and non of colonizing *E. faecium* strains exhibited slim layer. This result was in agreement with Kashef *et al.*[1], Hashem *et al.,* [22] and Soares *et al.,* [25], they declared higher frequency of biofilm among *E. faecalis* (93%, 76% and 80% respectively) vs *E. faecium*. Biofilm is extremely common among *E. faecalis* isolates, it is therefore possible that the few non-biofilm producing *E. faecalis* strains may carry nonfunctional biofilm genes[26].

Enterococci have variable ability to make biofilm, worldwide. In Italy, 80% of *E. faecalis* and 48% of *E. faecium* clinical isolates were able to form biofilms. In Japan, Poland, and Spain (90%, 59% and 57% respectively) of *E. faecalis* isolates were more biofilm producer compared to *E. faecium*[27]. Additionally, *E. faecalis* isolates may be representative of hospital-adapted strains as biofilms enable it to better survive in adverse conditions, including antibiotics and disinfectants [28,29].

With respect to correlation between enterococcal isolation sites and biofilm formation, our study confirmed that most strains (94.1%) isolated from noninvasive site (urine) exhibited biofilm compared to invasive infections (BSI) (47.6%) isolates. Comparable to our results, Soares *et al.,*[25] founded that 85.3% of isolates were from noninvasive site (urine). In this study, 100% biofilm production was reported for both *E. faecalis* and *E. faecium* pathogens isolated from urine samples. Enterococcal strains isolated from urine samples can produce biofilm with higher rates[1,7,26], as biofilm may help in persistence of infections, especially on indwelling catheters[30].

Regarding association of virulence determinants with infection or colonization, the present work screened the two well-defined genes, *esp* and *asa1* among 105 enterococcal isolates. *Esp* gene 51/105(48.5%) was significantly presented than *asa1*gene 16 /105 (15.2%) among total isolates , additionally *esp* gene was more common in clinical 38/72(52.8%) as compared with colonizing 13/33 (39.3%) isolates. Our data concerning e*sp* gene was comparable with that reported by Gozalan *et al.,* [3], Comerlato *et al.*,[32] and Upadhyaya *et al.*[33] The previous findings may highlights the essential role of *esp* trait in provoking infection however, it is not essential for colonization or translocation in enterococci[34].

Even though in this work, colonizer isolates of both species didn't carry *asa1* gene, which is in agreement with a multicenter study showed absence of *asa1* and *gelE* in colonizer strains[35].

Additionally, both *esp* gene (56.7% vs 33.3%) and *asa1*gene (33.3% vs 2.7%) were encountered more frequently in *E. faecalis* than in *E. faecium* isolates, this result confirmed previously by *Strateva et al.,* [35] and Papadimitriou *et al.,*[36]. However, this is in contrast with Shankar *et al.* who failed to find *esp* gene or any virulence determinant in *E. faecium.*[37]

The role of *esp* gene in biofilm formation had conflicting results, many authors were in agreement with our finding and reported an association between biofilm and *esp*[17,36]. Notwithstanding, other studies have failed to find evidence of such a link .[4,32]

In this study, significant association linked *esp* gene to biofilm was detected. Interesting finding was that the seven clinical isolates harbored both *esp* and *asa1* genes, showed biofilm , *esp* gene was expressed in 65.5% (38/58) of clinical biofilm-producing isolates , all clinical esp positive isolates (38/ 38; 100%) showed biofilm formation ability , which is in consistence with most of previous literatures, (Tsikrikonis *et al.,*[34] and Papadimitriou *et al.*[36]). The synergy noticed with *esp gene* and biofilm may help to establish infection[30]. The striking finding in our study was observation of 4 *esp*+ isolates - biofilm negative isolates , moreover among isolates that lack *esp*- and *asa1*- genes, 3 colonizing and one clinical strains were biofilms positive, this remarkable finding suggested that even though *esp* is important in biofilm formation but still many other factors may affect its production[38,39].

In this work, *asa*1 gene didn't predict the occurrence of biofilm, as 20.7% of biofilm producing clinical isolates and 28.5% of the nonbiofilm producing isolates harbored that gene with no statistical difference , this finding is in consistent with a prior study by Zheng *et al.*[27] showed that negative association.

In this study, clinical isolates (30.5%) displayed higher vancomycin resistance compared with colonizing isolates (6.5%) . This result was comparable to Goudarz *et al.*[5]. Biofilm formation was lower among VRE, 9/24 (37.5%) compared to VSE 61/ 81(75.3%), non of colonizing VRE showed slim layer.

Biofilm positive strains when compared with non biofilm producers, as regard vancomycin resistance, were more sensitive to vancomycin (low resistance). Antibiotic resistance and biofilm are two different aspects of bacterial pathogenesis, therefore increased antimicrobial resistance might not always be associated with increased virulence , yet no conclusions on the exact association[9]. In a study to determine the difference in virulence expressed by VRE and VSE, it was clearly found that biofilm formation was more in VSE than VRE isolates[19]. Determinants in enterococci and virulence genes are plasmid borne with immense ability for genetic exchange both intragenically and intergenically. Consequently acquisition of one plasmid may lead to loss of the other either due to incompatibility or due to fitness cost benefits[19].

CONCLUSION

A positive association between esp gene and biofilm formation especially in E. faecalis clinical isolates was detected. Biofilm formation and asa1, esp genes were more presented among E. faecalis isolates especially from non invasive sites, thus aid enterococci to provoke clinical infections , compared to E. faecium isolates from invasive infection sites. However, the acquisition of vancomycin resistance may decrease the ability of biofilm formation

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