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Virulence Factor and Biofilm Formation in Clinical Enterococcal Isolates of the West of Iran

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Abstract

Background: *Enterococcus* spp., a part of the normal flora of the human intestine, possess several virulence factors that can develop biofilms to endure harsh environments. Their ability to cause nosocomial infections makes them as critical opportunistic pathogens in hospital settings.
Objectives: The current study aimed at determining the occurrence of 6 genes coding virulence factors and their ability to develop biofilms, and conducting phenotypical assessments of haemolysin and gelatinase in clinical enterococci isolated from the West of Iran.
Methods: A total of 126 isolates were screened for harbouring the following genes: aggregation substance (*asa1*), cytolysin (*cylABM*), enterococcal surface protein (*esp*), and gelatinase (*gelE*). Isolates were tested for haemolysin and gelatinase expression phenotypically and for biofilm production quantitatively, using the microtiter method.
Results: Of the 126 tested isolates, 95 (73%) were *Enterococcus faecalis* and 28 (21%) were *E. faecium*. The total frequency of virulence gene was *cylA* 92 (73%), *cylB* 85 (67%), *cylM* 57 (45%), *asa1* 26 (21%), *gelE* 64 (51%), and *esp* 66 (53%); while 98 (75%) of the isolates were able to form biofilm. A total of 74 (58%) and 46 (35%) isolates could secret haemolysin and gelatinase.
Conclusions: There was a significant difference between the frequency of virulence gene in *E. faecalis* and *E. faecium*. *Enterococcus faecium* isolates lacked the *gelE* and asa1 genes and the frequency of *cylABM* genes were lower than that of *E. faecalis* isolates. *Enterococcus faecalis* isolates were relatively rich in virulence factors; no association was observed between biofilm formation and the presence of specific virulence genes.

Keywords: Virulence Factor, Biofilm, *Enterococcus Faecalis*, *Enterococcus Faecium*

1. Background

Enterococci spp. are Gram-positive and catalase negative cocci, able to grow in the temperature range of 10°C to 45°C and media containing 6.5% NaCl (1). Enterococci are the second or third most prevalent organism responsible for nosocomial infections (2). Among the 50 identified species, *Enterococcus faecium* and *E. faecalis* are the most medically significant ones. *Enterococcus faecalis* is the most predominant species in hospital settings and accounts for 80% to 90% of nosocomial infections, compared to *E. faecium*, which causes 1% to 5% of such infections (3, 4). Enterococcal infections commonly occur in patients hospitalised for long periods and patients with severe chronic diseases such as renal failure, neutropenia, transplantation, and catheterisation. Important infections caused by enterococci are urinary tract infections (UTIs), bacteraemia, endocarditis, intra-abdominal and pelvic disease, and wound infections (2).

Enterococci are equipped with many genes encoding virulence factors that enable them to survive in harsh environments and sustain infection in vulnerable hosts. Some virulence factors such as cytolysin (*cylA*, *cylB* and *cylM*), gelatinase (gel-E), and aggregation substances (*asa1*) might increase the severity of the infections (5). Cytolysin is the main virulence factor of *E. faecalis*. The toxin is associated with increased pathogenicity of enterococcal infections in bacteraemia, endocarditis, and intraperitoneal infections (6). Cytolysin, as a lantibiotic, can target and lyse bacterial and mammalian cells (7-10). Nucleotide sequence determination for the cytolysin operon revealed a complex determinant encoding 5 genetic markers, of which *cylA*, *cylB*, and *cylM* are the most important ones (11, 12).

Observation of enterococcal biofilms on endodontic surfaces, biliary duct stents, urinary catheters, heart valves, and tissue surfaces suggested a correlation between the lifestyle and virulence (13). Biofilm formation is reportedly less common in *E. faecium* compared to *E. faecalis*, although the clinical outcome of infections caused by *E. faecium* may be worsening as a result of biofilm formation (14, 15). Biofilm production has profound effects on the devel-

opment of endocarditis, periodontitis, and various device-related infections, and also causes resistance to antibiotics (16, 17).

Enterococcal surface protein (Esp) may induce persistent UTIs and increase the ability of microorganisms to colonise in hospitalised patients (18, 19). The corresponding gene, *esp*, is more frequent in clinical *E. faecalis* and *E. faecium* isolates, compared to environmental or food product ones (20, 21). The *esp* expression is related to the primary bacterial adherence and biofilm formation (22, 23). Gelatinase encoded by *gelE* gene is an extracellular zinc metalloprotease that hydrolyses gelatine, collagen, casein, haemoglobin, and antimicrobial peptides of the innate immune system (24, 25). The *asa1* is encoded by pheromone-responsive plasmids, which often harbour antibiotic resistance genes (26, 27). The protein causes clumping of *E. faecalis* cells and survival inside polymorphonuclear leucocytes, internalisation by intestinal cells, and increases in bacterial binding to cultured renal epithelial cells (28, 29).

2. Objectives

Considering the importance of bacterial virulence factors in the outcome of infections and lack of any comprehensive information about the prevalence of such factors in clinical isolates of enterococci in Iran, the current study aimed at investigating a possible relationship between the biofilm formation ability and virulence factors of enterococci isolated from clinical samples of an educational hospital in Kermanshah province, West of Iran, and also the role of virulence genes in biofilm development, and their prevalence especially as high-biofilm-producing isolates.

3. Methods

3.1. Bacterial Isolation and Identification

Among 130 suspicious clinical samples collected from January 2012 to April 2013, a total of 126 isolates were selected for the current study; samples were taken mostly from urine, blood, and body fluids samples. All samples were cultured on bile esculin agar (Himedia, India); positive cultures (colonies with a black halo around them) were confirmed at the species level using the standard biochemical methods.

3.2. Hemolysin Production

Haemolysin activity was determined by an overnight incubation of the isolates cultured on blood agar (Himedia, India) supplemented with 5% defibrinated human blood. A clear zone around the colonies indicated haemolysis.

3.3. Gelatinase Production

A pure 24-hour culture was stabbed into tubes containing a 0.8% nutrient broth (Himedia, India) supplemented with 12% gelatine. After 24 to 72 hours of incubation, tubes were refrigerated for 30 minutes. Sufficient gelatinase production by the isolates leads to liquefaction of cultured media, even following the refrigeration; but the presence of intact gelatine after refrigeration means a lack of ability to produce gelatinase.

3.4. Biofilm Formation Assay

The test was conducted using the previously described method (30). An overnight culture was diluted 1:100 into a fresh tryptic soy broth (TSB) medium (Himedia, India) supplemented with 1% glucose and inoculated into 96 polystyrene microplate wells. Following 18 hours of incubation at 37°C, the plates were gently washed 3 times with phosphate-buffered saline (PBS), air dried, and stained with crystal violet. Biofilm bounded to crystal violet was diluted with 200 μL of an 80:20 mixture of ethanol and acetone and, then, optical density (OD) of the suspension was measured at 570 nm, using an automatic spectrophotometer (Stat Fax ® 4200). The ability to form biofilm was scored as follows: OD $\leq$ 0.120: non-producers, 0.120 $\leq$ OD $\geq$ 0.240: weak producers, and OD $\geq$ 0.240: strong producers. As a control, the level of crystal violet binding to wells was measured for the wells exposed only to the medium without bacteria. All biofilm assays were performed in triplicate (31).

3.5. DNA Extraction

A loopful of pure enterococci culture on brain-heart infusion (BHI) agar (Himedia, India) was suspended in microtubes containing 0.5 mL sterile deionised water. The microtubes were placed in a boiling water bath for 5 minutes, centrifuged for 10 minutes at 10000 rpm, and the supernatant were transferred to new tubes and used as DNA templates for the polymerase chain reaction (PCR).

3.6. Primer Design and PCR

The whole sequence of the enterococcus genome ID number: 427183854, retrieved from the NCBI database, was used as the template. On the basis of the retrieved sequence, 3 sets of primers were designed for *E. faecalis* and *E. faeciumddl* genes, which were strain-specific genes and enterococcus *tuf* gene, a genus-specific gene. Then, 6 sets of primers were designed for *asa1*, *esp*, *gelE*, and *cylABM*. To optimise the results, the multiplex PCR reaction for 2 separate sets of genes was designed and performed. Enterococcal Tuf gene, *E. faecalis* and *E. faecium ddl* genes, *gelE*, and *esp* genes were placed in a set; *cylMBA* and *asa1* were placed

in another set, separately. The primer sequences were summarized in Table 1.

Multiplex PCR reactions were performed with the thermal cycler (Biorad, USA). The oligonucleotides for PCR amplification were purchased from Takapouzist Biotech Company (Iran). Reactions were performed in a total volume of 25 μL, using 0.75 pmol of each of the 4 primer sets, 7.5 μL 5X buffer, 50 mM $MgCl_2$, 0.50 μL dNTP 10 mM, and 1 U HStaq DNA polymerase (Kappa Biosystem USA). The amplification conditions were as follows: an initial denaturation step at 95°C for 1 minute; 34 cycles of denaturation at 95°C for 10 seconds; annealing at 55°C for 30 seconds for *gelE*, *esp*, enterococcus *tuf*, *E. faecalis ddl*, and *E. faecium ddl*; annealing at 52°C for 30 seconds for *cylA*, *cylB*, *cylM*, and *asa1*, and extension at 72°C for 1 minute, followed by the final extension at 72°C for 30 seconds. DNA from *E. faecalis* FI9190 was used as a positive control in the corresponding PCR reactions.

The PCR products were subjected to electrophoresis through 1% agarose gel, stained with ethidium bromide solution, and visualised under the UV light in the Gel Documentation system (Biorad, USA). Images were analysed using Image Lab software version 2.0.

3.7. Statistical Analysis

All statistical analyses were performed using SPSS version 21. Crosstabs, Chi-square, and the Fischer exact test were performed based on the data. A P value of < 0.05 was considered statistically significant.

4. Results

4.1. Bacterial Isolates Origin

Of the 126 clinical samples, 78 (60%) were isolated from urine, 30 (23%) and 22 (17%) from blood and body fluids samples, respectively; 95 (73%) isolates were *E. faecalis* and 28 (21%) *E. faecium*.

4.2. Frequency of Extracellular Enzyme Secretion

In the current study, 74 (58%) isolates were haemolysin producers, of which 51(68%) of *E. faecalis* and 20 (71%) of *E. faecium* isolates showed haemolytic activity. Alpha haemolytic strains were 26% of *E. faecalis* and 32% of *E. faecium* isolates, while beta haemolytic strains were 27% of *E. faecalis* and 39% of *E. faecium*. The results showed that 46 (35%) *E. faecalis* isolates could produce gelatinase. None of the *E. faecium* could liquefy gelatine; therefore, 49.3% of the *E. faecalis* isolates harbouring *gelE* did not show gelatinase activity.

4.3. Capacity for Biofilm Formation

Overall, 98 (75%) of the isolates could form biofilm, of which 75 (76%) and 23 (24%) were *E. faecalis* and *E. faecium*, respectively.

4.4. Distribution of Biofilm Production Capacity Among Virulence Marker

As many as 61 (92%) of all esp^+ and 64 (87%) of isolates that carried *gelE* gene could produce biofilm. Biofilm producers were 55 out of 64 $gelE^+$, and 51 out of 64 $gelE^-$ isolates; the mean biofilm ODs for $gelE^+$ was more than that of $gelE^-$ isolates (2.033 versus 1.746) (Table 2). This trend was observed in the esp-lacking isolates, the mean biofilm OD of $gelE^+$ isolates was higher than that of $gelE^-$ ones, but the different was not significant (mean OD, 1.747 for 24 $esp^-/gelE^+$ isolates versus 1.392 for 31 $esp^-/gelE^-$ isolates), which suggested a possible contribution of the *gelE* in biofilm formation in the esp-lacking isolates (Table 2). It was also noted that biofilm ODs were higher in 40 $esp^+/gelE^+$ isolates (mean OD = 2.319). Moreover, the lowest mean of OD for the 31 esp^-/gelE$^-$ isolates (mean OD = 1.392) showed that esp^+ isolates produced more biofilm than the esp^- ones. A total of 26 $esp^+/gelE^-$ isolates (mean OD= 2.103) also produced high amounts of biofilm, but a little less than $esp^+/gelE^+$ (mean OD = 2.319). This could constitute strong evidence for the role of *esp* in biofilm formation in the absence of the *gelE* (Table 2).

However, even among the esp-lacking/$gelE^-$ isolates, 14 isolates produced strong biofilm and 8 isolates produced medium biofilm. Further, no significant difference was observed in the mean value of biofilm between the esp^+ and esp^- isolates (ODs 1.569 vs. 2.211), indicating that neither *esp* nor *gelE* was essential to biofilm production. The relationship between biofilm formation capacities, OD values, *esp*, and *gelE* are summarised in Table 2.

4.5. Frequency of Virulence Genes

The number of virulence marker genes in *E. faecalis* isolates varied from 1 to 6. The majority of *E. faecalis* isolates were positive for 4 virulence genes, but most of *E. faecium* isolates were positive for 3 virulence genes (Table 3). Of the 95 *E. faecalis* isolates, 10 (9.7%) were positive for 6 virulence genes, whereas 90 (91.9%) were positive for 2 or more virulence genes. None of *E. faecium* isolates was positive for *cylABM*, while 46 (48%) of *E. faecalis* strains harboured a *cylABM* complete set of genes. Among *E. faecalis* and *E. faecium* isolates, the *cylM* gene had a significantly lower incidence (Table 3).

The amplification results of enterococcal *tuf* gene, *E. faecalis*, and *E. faecium ddl* genes, *gelE*, and *esp* genes are illustrated in Figure 1A and those of *cylMBA* and asa1 in Figure 1B.

Table 1. Designed Primers Sequences

Primer Name	Virulence Factor	Oligonucleotide Sequence (5'to 3')	Amplicon Size, bp	Reference
***E. faecium* F**	*Faecium* species	GAAGGACAATGGGTCAAAGG	596	The current study
***E. faecium* R**		ACT TCG CCA GGC AAA GTC		
***Enterococcus* spp. F**	*Enterococcus* genus	TAC TGA CAA ACC ATT CAT GAT G	109	The current study
***Enterococcus* spp. R**		TTC GTC ACC AAC GCG AAC		
***E. feacalis* ddl F**	*faecalis* species	CCA CAA GTA CCA TTC GTG C	305	The current study
***E. feacalis* ddl R**		GCG ACA TCT TTC ACC ACT TC		
***esp* F**	Enterococcal surface protein	GGT ATG GGT TCA ATC ACT GAC	714	The current study
***esp* R**		CTC TGC ATC TTC AGG TAA G		
***gelE* F**	Gelatinase	GAC CAG AAC AGA TTC ACT TG	409	The current study
***gelE* R**		GGA TAC TCA GAG TGT TGA CC		
***cylM* F**	Cytolysin	AGG GAG ACT CTC ATA GTC G	801	The current study
***cylM* R**		CAG CAT ATC TTT GAG TTG GTC		
***cylA* F**		GAT ATA ATA AAC GTG AGT CTT GG	238	The current study
***cylA* R**		AGT CAG CAA TAT CAC CAC TC		
***cylB* F**		CTG GCT GGA TTG TTA CAA CC	526	The current study
***cylB* R**		GAT CTT CAT GCT TTC CAA TAC		
***asa1* F**	Aggregation substance	CAA GAA CAA CCA GTA GTA AGC	960	The current study
***asa1* R**		TGA ATT TAA TGA AGC ACC TG		

Table 2. Biofilm Formation Quantity Based on the Presence of *esp*/*gelE* Gene

Virulence Factor	OD of Isolates with				
	Strong Biofilm Formation, (OD570 $\leq$ 2)	Medium Biofilm Formation, (OD570, 1 - 2)	Weak Biofilm Formation, (0.5 $\leq$ OD570 $\geq$ 1)	No Biofilm Formation, (OD570 $\leq$ 0.5)	Mean $\pm$ SD, P value
$Esp^{+}/gelE^{+}$	2.419 (2.783 - 2.132) (34)	1.284, (1.074 - 1.494) (2)	0.883 (2)	0.204 (1)	2.319 (2.783 - 0.204) (40) (0.57) 0.053
$Esp^{+}/gelE^{-}$	2.650 (2.947 - 2.048) (17)	1.618 (1.855 - 1.154) (6)	0.783 (0.652 - 0.914) (2)	0.209 (1)	2.103 (2.813 - 0.209) (26) (0.79) 0.263
$esp^{-}/gelE^{+}$	2.440 (2.674 - 2.190) (8)	1.516 (1.988 - 1.108) (12)	0.756 (0.658 - 0.854) (2)	0.115 (0.121 - 0.111) (2)	1.206 (2.718 - 0.111) (24) (0.76) 0.925
$esp^{-}/gelE^{-}$	2.611(2.993 - 2.000) (14)	1.461 (1.986 - 1.005) (8)	0.595 (0.661 - 0.579) (8)	0.028 (0.025 - 0.031) (7)	1.392 (2.993 - 0.025) (31) (0.94) 0.196
Total	73	28	14	11	126

Table 3. Frequency of Virulence Genes[a]

Variables	*cylA*	*cylB*	*CylM*	*cylABM*	*Asa1*	*cylABM, asa1*	*gelE*	*esp*	*gelE, esp*
E. faecalis	83 (87)	77 (81.1)	52 (54.4)	46 (59)	26 (27)	17 (36)	64 (67)	55 (59)	52 (41)
E. faecium	9 (32)	8 (28)	5 (17)	0	0	0	0	11 (40)	0
Total (126)	92 (73)	85 (67)	57 (45)	46 (37)	26 (21)	(14.38)	64 (51)	66 (53)	41 (33)

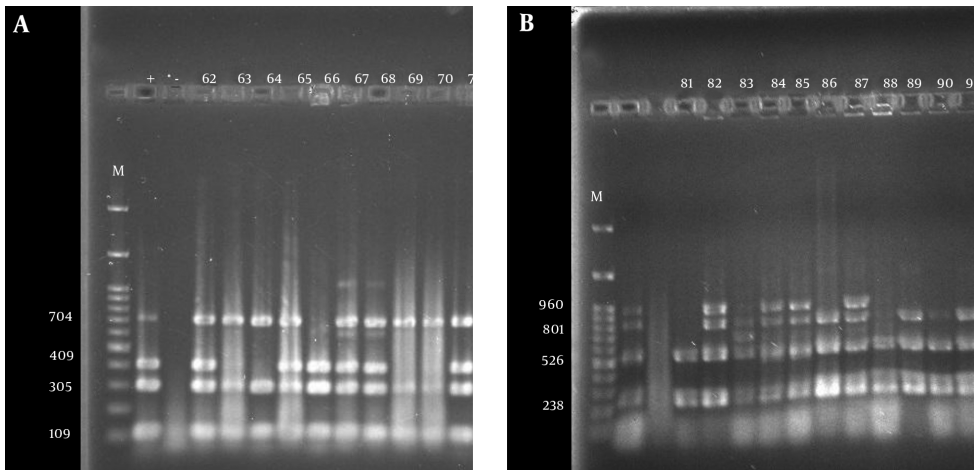
[a] Values are expressed as No. (%).

5. Discussion

Enterococcus is among the 4 most common causes of nosocomial infections worldwide. Due to its intrinsic resistance to antimicrobial agents and harsh environments, it can survive and spread in hostile niches, such as hospitals. In enterococcal infections, biofilm plays an essential role by providing a context to enhance microbial survival in the host (32-34). Several studies were conducted to identify the virulence factors of enterococci and their possible association with biofilm formation, but this issue is not yet well understood. To reduce the rate of nosocomial infections and implement the correct treatment strategies, it is vital to obtain reliable knowledge on bacterial capacities and their virulence factors (17, 32, 33, 35-39).

The current study aimed at evaluating the frequency of several virulence factors in clinical enterococcal isolates and their relationship with biofilm production. Therefore, a multiplex PCR was designed to simultaneously detect *tuf* (*Enterococcus* genus), *ddl* (*E. faecalis* species), *ddl* (*E. faecium* species), *gelE* (gelatinase), *esp*, *cylA*, *cylB*, *cylM* (cytolysin), and *asa1*. All 126 isolates were analysed for the presence of virulence genes by the researcher-designed primers. The

Figure 1. Figure 1



A, From left to right: Lane 1, Sinacolon 100 bp DNA marker; Lane 2, Positive control of 109, 305, 409, and 714 bp of targeted genes; Lane 3, Negative control; Lane 4 - 13, Electrophoresis of PCR products. B, From left to right: Lane 1, Sinaclon 100 bp DNA marker; Lane 2, Positive control of 238, 526, 801 and 960 bp of targeted gene; Lane 3, Negative control; Lane 4 - 12, Electrophoresis of PCR products.

results are presented in Table 3, which revealed a relatively high incidence of virulence factors among isolates. The frequencies of all 6 virulence factors were significantly high in *E. faecalis* than *E. faecium* (Table 3). Previous observations revealed a higher rate of clinical isolates harbouring *esp* compared to the isolates from other resources (19, 21).

In the present study, the prevalence of *esp* was consistent with those of other studies in Iran and other parts of the world, although some studies failed to find esp^{+}*E. faecium* (18, 36, 40, 41). The Esp has a role in colonisation and persistence of *E. faecalis* in the urinary tract (18). Since most isolates in the current study were isolated from UTIs, a high incidence of *esp* was not surprising. The Esp protein has a high sequence similarity with Bap (biofilm-associated protein of *Staphylococcus aureus*); thus, this protein may be important for biofilm formation (42). Di Rosa suggested that the synergy between *esp* and biofilm formation helps to establish a successful infection (43). The current study also detected the ability of enterococcal isolates to form biofilms and their possible relationship with *esp*.

According to the literature, all enterococcal strains isolated from urinary and blood stream sources can produce biofilm, with a rate typically higher than that of the current study (30, 42, 44). Despite the lack of a significant association between Esp and biofilm formation in the current study, other researches reported conflicting results about the role of *E. faecalis* Esp in biofilm formation (23, 42, 43, 45-48). Although biofilm formation in esp-deficient mutants of *E. faecalis* was not reported in all studies, many others report no correlation between the presence of *esp* and biofilm formation, which is a multifactorial process (49, 50).

Gelatinase, another virulence factor of *E. faecalis*, was also detected in the current study. The frequency of *gelE* was the same as those of previous studies (5, 44, 51), which also could not detect this gene in *E. faecium* isolates, although many *E. faecalis* isolates harboured it (52). Furthermore, phenotypical expression of *gelE*, which leads to the secretion of gelatinase, was assessed and many $gelE^{+}$*E. faecalis* isolates failed to secrete gelatinase. A reason for the phenomenon could be that the presence of a specific gene does not automatically mean phenotypic expression of that gene. This could explain the presence of the $gelE^{+}$ isolates, which were unable to liquefy gelatine (53-56).

Despite demonstrations about the necessity of gelatinase enzymatic activity to establish biofilm (45), the current study found no difference in the *in vitro* biofilm production between $gelE^{+}$ and $gelE^{-}$ isolates; which indicated that, similar to *esp*, neither *gelE*, nor gelatinase was required for biofilm formation. In summary, although biofilm production was higher in $esp^{+}/gelE^{+}$ compared to $esp^{-}/gelE^{-}$ isolates, no significant difference was observed in biofilm production ability between $gelE^{+}$ and $gelE^{-}$ isolates. The *asa1* was not found in any of the *E. faecium* strains investigated in the current study. In other studies, *asa1* was detected only in *E. faecalis* strains (28, 44, 51, 54, 57). Udo reported a relatively similar frequency of *asa1*; Moniri and Seno reported a higher frequency (41, 58, 59).

Cytolysin can induce tissue damage through the lysis of erythrocytes and polymorphonuclears (PMNs) (60).

Cytolysin production could also significantly deteriorate the severity of endocarditis (7). Investigation of cytolysin genes revealed the presence of *cylA*, *cylB*, and *cylM* in a high proportion of *E. faecalis* and a low proportion of *E. faecium* isolates, but in the studies by Udo, Moniri, and Cosentino, the prevalence of *cylA*, *cylB*, and *cylM* were lower than those of the current study (41, 59, 61). However, Abriouel reported a higher frequency of *cyl* operon in *E. faecalis*, although with lower haemolytic activity (62).

Enterococcus faecalis isolates harboured 1 to 6 virulence markers, similar to a report by a Brazilian study in which *E. faecalis* isolates harboured 1 to 8 virulence-associated genes (63). The current study observed the dominance of some genes or gene combinations. The most common combinations of virulence genes were *cylA* and *cylB* (76%) for *E. faecalis*, and *cylM* and *cylA* 1 (12%) for *E. faecium* isolates. Among all E. faecalis isolates, 9 isolates had 6 virulence markers, all of which were strong biofilm producers.

All *E. faecalis* isolates harboured more than 1 virulence-associated gene, suggesting that *E. faecalis* infection depended upon the transaction of several genes bound to the secretion and regulation of the expression of the virulence factors. The small number of *E. faecium* isolates was the limitation of the current study. However, the current study reinforced the well-known characteristics of *E. faecalis* species in terms of its virulence, and confirmed that biofilm formation was a multifactorial process requiring different genes and their products.

5.1. Conclusion

In conclusion, *E. faecalis* strains isolated from UTIs were characterised by higher-virulence strains from other clinical sources. *Enterococcus faecium* was involved in important processes such as initiating colonisation, infection in the host, and biofilm formation. Overall, *esp* was not required for biofilm formation, but its presence was significantly associated with a higher proportion of biofilm production in clinical isolates. Therefore, the genotypic and phenotypic assays appear necessary for a better characterisation of the strains, due to silent virulence genes (*cyl* and *gelE*). The current study results may serve as a basis for the additional surveillance studies of infections caused by this microorganism.

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