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ORIGINAL RESEARCH

Association of CRISPR-Cas System with the Antibiotic Resistance and Virulence Genes in Nosocomial Isolates of *Enterococcus*

Shuan Tao[1,2], Huimin Chen[1], Na Li[3], Yewei Fang[2], Yao Xu[4], Wei Liang[2]

[1]School of Medicine, Jiangsu University, Zhenjiang, People's Republic of China; [2]Department of Clinical Laboratory, Ningbo First Hospital, Ningbo, People's Republic of China; [3]Bengbu Medical College, Bengbu, People's Republic of China; [4]School of Medicine, Ningbo University, Ningbo, People's Republic of China

Correspondence: Wei Liang, Department of Clinical Laboratory, Ningbo First Hospital, No. 59. Liuting Road, Haishu District, Ningbo, ZheJiang, 315010, People's Republic of China, Tel +86 15351883016, Fax +86-051885213100, Email hslwys@163.com

Purpose: This study aimed to investigate the prevalence of the CRISPR-Cas system in nosocomial isolates of *Enterococcus* and their possible association with antibiotic resistance and virulence genes.

Materials and Methods: Identification and antimicrobial susceptibility of the microorganism were performed by the automatized VITEK 2 Compact system (bioMerieux, France). A total of 100 *Enterococcus* isolates were collected and identified by VITEK 2 Compact automatic microbial identification drug susceptibility analyzer. The prevalence of various CRISPR-Cas systems, antibiotic resistance genes and virulence genes were investigated by polymerase chain reaction (PCR). The prevalence of CRISPR-Cas systems associated with antibiotic resistance and virulence genes was performed by appropriate statistical tests.

Results: A total of 100 isolates of *Enterococcus* were identified and there were 62/100(62.0%) *Enterococcus faecalis* isolates and 38/100(38.0%) *Enterococcus faecalis* isolates. In total, 46 (46.0%) of 100 isolates had at least one CRISPR-Cas locus. CRISPR elements were more prevalent in *Enterococcus faecalis* isolates. The results of PCR demonstrated that CRISPR1-Cas, orphan CRISPR2, and CRISPR3-Cas were present in 23 (23.0%), 42 (42.0%) and 5 (5.0%) *Enterococcus* isolates, respectively. Compared with CRISPR-Casnegative isolates, the CRISPR-Cas positive isolates showed significant lower resistance rates against ampicillin, erythromycin, levofloxacin, tetracycline, vancomycin, gentamicin, streptomycin, and rifampicin. Presumably consistent with drug susceptibility, fewer CRISPR loci were identified in *vanA, tetM, ermB, aac6'-aph(2")*, *aadE*, and *ant(6)* positive isolates. There was a significant negative correlation between the CRISPR-Cas locus and the enterococcal virulence factors enterococcal surface protein (*esp*) gene.

Conclusion: In conclusion, the results indicated that the absence of the CRISPR-Cas system was negatively associated with some antibiotic resistance in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. Also, there was a negative correlation with the carriage of antibiotic resistance genes. Furthermore, CRISPR-Cas may prevent some isolates from acquiring certain virulence factors.

Keywords: *Enterococcus*, CRISPR-Cas system, antimicrobial-antibiotic resistance gene, virulence genes

Introduction

Enterococci are a group of facultative anaerobic Gram-positive opportunistic pathogens.[1] *Enterococcus faecium* and *Enterococcus faecalis* are the dominant species of enterococci.[2] One of the most prominent features of enterococci is their high level of intrinsic and acquired resistance to many antibiotics.[3] *Enterococci* are inherently resistant to many first-line antimicrobial agents, such as clindamycin, cephalosporins, compound sulfonamide, and low-concentration aminoglycosides.[4,5] In addition, *Enterococci* can acquire antimicrobial resistance through horizontal genes transfer (HGT) mediated by mobile gene elements (MGEs) such as plasmids, phages, and transposons, which is one of the main mechanisms that contribute to the spread of resistance genes between bacteria.[6,7] Antibiotic resistance of enterococci and the ability to acquire and spread antibiotic resistance is a challenge in the clinical setting and increases the difficulty of treating enterococcal infectious diseases.[8]

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The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) system is an acquired immune defense system with immune memory in prokaryotic genomes, which can effectively resist the acquisition of foreign mobile gene elements such as plasmids or phages to maintain the stability of bacterial genomes.[9,10] CRISPR-Cas system is one of the factors limiting the development and evolution of bacterial antibiotic resistance.[11] CRISPR-Cas is considered as a natural barrier to horizontal gene transfer and transmission of antibiotic resistance.[12] The CRISPR-Cas system is widely present in the genomes of bacteria and archaea and is an integral part of their immune systems.[13] The CRISPR-Cas system consists of a CRISPR array and the Cas gene family, in which the CRISPR array is composed of highly conserved repeat sequences and spacer sequences, with upstream leader sequences responsible for their transcription.[14] The Cas gene family, the CRISPR-related protein genes, is highly conserved, which encodes the Cas protein with a domain capable of cleaving DNA double-strands.[15] In the CRISPR array, the palindromic structure contained in the repeat sequence can form a conserved RNA secondary structure, which can bind to the Cas protein to form a complex and work together.[16]

The mechanism of CRISPR-Cas mainly includes adaptation, expression, and interference.[17] When exogenous plasmids or bacteriophages invade bacteria/archaea, the internal CRISPR-Cas system of these prokaryotes is activated, and some gene segments of exogenous DNA (called pre-spacer sequences or protospacer sequences, proto-spacer) integration into the original CRISPR array, the process called adaptation.[18] During the expression stage, when the homologous foreign gene invades the bacterium again, the CRISPR array is transcribed into the precursor CRISPR RNA (pre-crRNA) long chain, and then processed by Cas proteins into mature RNA units (crRNA), which are combined to form the Cas-crRNA complex.[19,20] In the interference stage, crRNA recognizes, binds, and cleaves the homologous exogenous nucleic acid sequence, directing the Cas-crRNA complex to degrade the target molecule.[21,22] Antibiotic resistance is mediated by the HGT between bacteria.[23] HGT is mainly achieved through transformation, conjugation, and phage-mediated transduction, which is also one of the main ways for bacteria to obtain drug resistance genes beneficial to their survival.[24] CRISPR-Cas system can defend against mobile genetic elements such as phages or plasmids.[25] Recent studies have revealed that the presence of CRISPR-Cas system is associated with antibiotic sensitivity and lack of virulence traits. Studies have demonstrated a highly significant negative correlation between the presence of the CRISPR-Cas system in Enterococcus faecalis and acquired antibiotic resistance.[26] Several studies have proposed the potential of the CRISPR-Cas system to regulate bacterial virulence. However, some researchers have found no evidence by a statistical model that the CRISPR-Cas system can prevent the occurrence of HGT on the time scale of bacterial evolution.[27] Gholizadeh et al[11] demonstrated that there was no significant relationship between the CRISPR-Cas system and antibiotic resistance in *Escherichia coli*. The study of the function of the CRISPR-Cas system and its effect on HGT should be more comprehensive and in-depth.

The purpose of this study was to investigate the prevalence of CRISPR-Cas in clinical Enterococcus isolates and to analyze their association with antibiotic resistance genes and virulence factors. Understanding the relationship between CRISPR loci and antibiotic resistance phenotypes and genotypes as well as virulence genes may provide new insights into the fight against infections caused by enterococcal drug-resistant pathogens.

Methods and Materials

Bacterial Isolates

A total of 100 clinical enterococcal isolates, including 62 *Enterococcus faecalis* and 38 *Enterococcus faecalis*, were randomly collected in the Ningbo First Hospital from January to August 2022. All *Enterococcus* isolates were inoculated on Colombian blood agar plates and incubated at 37°C for 24 hours. And identified by VITEK 2 Compact automatic microbial identification drug susceptibility analyzer. Enterococcal drug susceptibility testing was performed with the automated broth microdilution methods (VITEK 2 Compact, BioMérieux, France) and matching cards for GP67. Operation and results interpretation was performed strictly according to the American Society for Clinical and Laboratory Standardization (CLSI) 2019 standard. All experiments were approved by the Medical Ethics Committee of Ningbo First Hospital.

Bacterial Nucleic Acid Extraction

The isolates were taken out from the −80 °C refrigerator and resuscitated, inoculated on blood plates, and cultured in 37 °C incubators for 18 h. One colony of each isolate was taken and inoculated in 1mL of Luria Bertani (LB) medium liquid

medium at 37°C and 200 r/min for 24 hours. The DNA was extracted using the bacterial genomic DNA extraction kit (TIANGEN) according to the instructions.

Genotypic Detection of antibiotic Resistance Genes and Virulence Genes

Genotype analysis of antibiotic resistance genes and virulence genes for each *Enterococcus* isolate was based on the multiplex polymerase chain reaction (PCR). The antibiotic resistance genes included those encoding resistance to macrolide (*ermA* and *ermB*), tetracycline (*tetM* and *tetO*), penicillin (*blaZ*), aminoglycosides (*aadE, aac(6')-aph(2''), ant(6)*), and glycopeptides (vancomycin/teicoplanin) (*vanA* and *vanB*) (Table 1). The investigated virulence genes were: the *cylA* (cytolysin activator), *esp* (enterococcal surface protein), *asal* (the sugar aggregation material), *hyl* (hyaluronidase), *gelE* (gelatinase), *agg* (aggregation substance) (Table 1). PCRs experiments were done in a final volume of 50 µL containing 25µL TaqPCR MasterMix (2X), 0.5 µL of each primer (10 pmol), 2 µL of template genomic DNA, and 22 µL of sterile water. The PCR cycle conditions consisted of 94°C pre-denaturation for 5 min; 30 cycles of 94°C for 30s, 60 °C for 30s, 72°C for 45s, followed by a final extension at 72°C for 5 min.

Detection of CRISPR-Cas Systems

The presence of the CRISPR locus was identified by PCR, including the three CRISPR sites and the Cas genes of CRISPR1 and CRISPR3 (Table 1). The 50 µL PCR reaction mixture for each locus contained 25 µL PCR master mix (2X), 1 µL of each primer (10pMol), 2 µL of template DNA, and 21 µL of sterile water. The amplification condition consisted of 94°C for 5 min, 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 45s, followed by final elongation at 72°C for 5 min. All PCR products were separated by electrophoresis on 1% agarose gel in 1xTBE buffer at 100V for 30 minutes. Then the analysis was performed using the gel imaging system to identify the amplification of the target gene.

Table 1 Primers Used for the Detection of Antibiotic Resistance Genes, Virulence Genes and CRISPR-Cas Associated Genes Among Enterococcus Isolates

Primers Name	Sequences	Amplified Size (bp)	Reference
tetM-F	GAACTCGAACAAGAGGAAAGC	740	[38]
tetM-R	ATGGAAGCCCAGAAAGGAT		
tetO-F	AACTTAGGCATTCTGGCTCAC	515	[38]
tetO-R	TCCCACTGTTCCATATCGTCA		
blaZ-F	ACTTCAACACCTGCTGCTTTC	173	[38]
blaZ-R	TGACCACTTTTATCAGCAACC		
ermA-F	TATCTTATCGTTGAGAAGGGATT	139	[38]
ermA-R	CTACACTTGGCTTAGGATGAAA		
ermB-F	CTATCTGATTGTTGAAGAAGGATT	142	[38]
ermB-R	GTTTACTCTTGGTTTAGGATGAAA		
aadE-F	GCCCTTGGAAGAGTTAGATAATT	198	[38]
aadE-R	CGGCACAATCCTTTAATAACA		
ant(6)-F	ACTGGCTTAATCAATTTGGG	597	[38]
ant(6)-R	GCCTTTCCGCCACCTCACCG		

(*Continued*)

Table 1 (Continued).

Primers Name	Sequences	Amplified Size (bp)	Reference
aac(6')-aph(2")-F	CCAAGAGCAATAAGGGCATA	220	[38]
aac(6')-aph(2")-R	CACTATCATAACCACTACCG		
vanA-F	CATGAATAGAATAAAAGTTGCAATA	1030	[38]
vanA-R	CCCCTTTAACGCTAATACGATCAA		
vanB-F	GTGACAAACCGGAGGCGAGGA	433	[38]
vanB-R	CCGCCATCCTCCTGCAAAAAA		
clyA-F	ACTCGGGGATTGATAGGC	688	[31]
clyA-R	GCTGCTAAAGCTGCGCTT		
esp-F	AATTGATTCTTTAGCATCTGG	510	[31]
esp-R	AGATTCATCTTTGATTCTTGG		
asaI-F	GCACGCTATTACGAACTATGA	375	[31]
asaI-R	TAAGAAAGAACATCACCACGA		
gelE-F	TATGACAATGCTTTTTGGGAT	213	[31]
gelE-R	AGATGCACCCGAAATAATATA		
agg-F	CCAGTAATCAGTCCAGAAACAACC	406	[31]
agg-R	TAGCTTTTTTCATTCTTGTGTTTGTT		
Hyl-F	ACAGAAGAGCTGCAGGAAATG	276	[31]
Hyl-R	GACTGACGTCCAAGTTTCCAA		
CRISPR1-*cas csn1*-F	CAGAAGACTATCAGTTGGTG	783	[29]
CRISPR1-*cas csn1*-R	CCTTCTAAATCTTCTTCATAG		
CRISPR1-*cas* loci-F	GCGATGTTAGCTGATACAAC	315	[29]
CRISPR1-*cas* loci-R	CGAATATGCCTGTGGTGAAA		
CRISPR2 loci-F	CTGGCTCGCTGTTACAGCT	Variable	[29]
CRISPR2 loci-R	GCCAATGTTACAATATCAAACA		
CRISPR3-*cas csn1*-F	GCTGAATCTGTGAAGTTACTC	258	[29]
CRISPR3-*cas csn1*-R	CTGTTTTGTTCACCGTTGGAT		
CRISPR3-*cas* loci-F	GATCACTAGGTTCAGTTATTTC	224	[29]
CRISPR3-*cas* loci-R	CATCGATTCATTATTCCTCCAA		

Statistical Analysis

SPSS version 17.0 was used for all descriptive statistical analyses. Chi-squared test and Fisher's exact test were used to compare the association of the presence of CRISPR-Cas with antimicrobial susceptibility, antibiotic resistance genes, and

virulence genes in both *Enterococcus faecalis* and *Enterococcus faecium,* and P < 0.05 was considered as statistical significance.

Results

Source of Isolates

A total of 100 *Enterococcus* isolates were collected (62 *Enterococcus faecalis* and 38 *Enterococcus faecium*). The main specimens of clinical *Enterococcus faecalis* isolates were urine (38,61.29%), pus (20, 28.99%), puncture fluid (3, 4.84%), and secretions (1, 1.61%). The main specimen sources of *Enterococcus faecium* isolates were urine (28, 73.68%), blood (4, 10.53%), secretion (3, 7.89%), and puncture fluid (2, 5.26%). The distribution of these isolates is shown in Table 2.

Drug Susceptibility Test of *Enterococcus* Isolates

The phenotypic drug susceptibility testing among *Enterococcus faecalis* and *Enterococcus faecium* are shown in Table 3. Enterococci showed the highest rates of resistance to tetracycline, followed by erythromycin, ciprofloxacin, and levofloxacin, and showed the lowest rates of resistance to linezolid, teicoplanin, and vancomycin. Overall, the resistance rate of *Enterococcus faecium* was higher than that of *Enterococcus faecalis*, and the prevalence rate of ampicillin, ciprofloxacin, nitrofurantoin, levofloxacin, vancomycin, streptomycin, teicoplanin, and rifampicin resistance of *Enterococcus faecium* was significantly higher than that of *Enterococcus faecalis* (P <0.05).

Distribution of Antibiotic Resistance Genes and Virulence Genes in *Enterococcus* Isolates

The distribution of resistance and virulence genes in *Enterococcus* faecalis and *Enterococcus faecium* isolates are shown in Table 4. Overall, the ermB, aac (6 ") -aph (2"), aadE, and tetM genes were present in most isolates, while the blaZ gene was present in only 5% of isolates. The tetO, ermA, and vanB genes were not detected in all isolates. The prevalence rate of tetM, aac (6")-aph (2"), and vanA genes were significantly different between E. faecalis and E. faecium isolates (p <0.05).

The distribution of virulence genes of *Enterococcus* isolates is shown in Table 4. The most presence of the virulence genes among *Enterococcus* isolates was the *esp* gene, followed by *asal* and *clyA*. The prevalence of *clyA, asal, gelE*, and *agg* genes in *Enterococcus faecalis* were significantly higher than that in *Enterococcus faecium*, while the prevalence of *esp* genes was lower than that in *Enterococcus faecium* (p <0.05).

Prevalence of CRISPR-Cas Systems in Enterococcus

The prevalence of CRISPR-Cas in *Enterococcus faecalis* isolates and *Enterococcus faecium* isolates are shown in Table 5. Overall, CRISPR-Cas were detected in 46/100 isolates, with 23(23%) isolates positive for CRISPR1-Cas, 42(42%) isolates for CRISPR2-positive and 5 (5%) isolates positive for CRISPR3-Cas. The occurrence of CRISPR-Cas was more common in *Enterococcus faecalis* (n = 32, 51.63%) than in *Enterococcus faecium* (n=14, 36.84%) isolates. Specifically, Among the *Enterococcus faecalis* isolates, 18(29.03%), 30(48.39%), and

Table 2 The Distribution of Enterococcus Isolates

Source	Number of Samples (%)		
	Enterococcus faecalis (N=62) n(%)	Enterococcus faecium (N=38) n(%)	Total (N=100) n(%)
Urine	38(61.29)	28(73.68)	66(66)
Pus	20(32.26)	0(0)	20(20)
Puncture fluid	3(4.84)	3(7.89)	6(6)
Blood	0(0)	4(10.53)	4(4)
Secretion	1(1.61)	3(7.89)	4(4)

Table 3 Phenotypic Characteristics and Resistance Rates of Different Enterococcus Isolates to the Antibiotics

Antibiotic	Total (N=100) n (%)	Enterococcus faecalis (N=62) n (%)	Enterococcus faecium (N=38) n(%)	P
Ampicillin	45 (45)	10 (16.13)	35 (92.11)	0.000*
Ciprofloxacin	54 (54)	18 (29.03)	36 (94.74)	0.000*
Erythromycin	68 (68)	38 (61.29)	30 (78.95)	0.08
Nitrofurantoin	27(27)	3 (4.84)	24 (63.16)	0.000*
Levofloxacin	54(54)	17 (27.42)	37 (97.37)	0.000*
Tetracycline	75(75)	47 (75.81)	28 (73.68)	0.816
Vancomycin	28(28)	4 (6.45)	24 (63.16)	0.000*
Gentamicin	40(40)	23 (37.10)	17 (44.74)	0.53
Streptomycin	30(30)	13 (20.97)	17 (44.74)	0.015*
Teicoplanin	20(20)	0 (0)	20 (52.63)	0.000*
Rifampicin	33(33)	10(16.13)	23 (60.53)	0.000*
Linezolid	0(0)	0(0)	0(0)	–

Note: The asterisk *Indicates a statistically significant difference (P < 0.05).

Table 4 The Distribution of Antibiotic Resistance-Related Genes and Virulence Genes Between *Enterococcus faecalis* and *Enterococcus faecium* Isolates

	Genes	Total (N=100) n (%)	Enterococcus faecalis (N=62) n (%)	Enterococcus faecium (N=38) n (%)	P
Antibiotic resistance genes	tetM	50(50)	39(62.90)	11(28.95)	0.002*
	tetO	0(0)	0(0)	0(0)	–
	blaZ	5(5)	4(6.45)	1(2.63)	0.647
	ermA	0(0)	0(0)	0(0)	–
	ermB	62(62)	36(58.06)	26(68.42)	0.152
	aadE	53(53)	33(53.23)	20(52.63)	1.000
	ant (6)	48(48)	28(45.16)	20(52.63)	0.538
	aac(6')-aph(2")	59(59)	29(46.77)	30(78.95)	0.002*
	vanA	24(24)	0(0)	24(63.16)	0.000*
	vanB	0(0)	0(0)	0(0)	–
Virulence genes	clyA	42(42)	39(62.90)	3(7.89)	0.000*
	esp	74(74)	39(62.90)	35(92.11)	0.001*
	asal	54(54)	46(74.19)	8(21.05)	0.000*
	gelE	24(24)	22(35.48)	2(5.26)	0.001*
	agg	12(12)	11(17.74)	1(2.63)	0.027*
	Hyl	0(0)	0(0)	0(0)	-

Note: The asterisk *Indicates a statistically significant difference (P < 0.05).

4(6.45%) were identified as CRISPR1-Cas, orphan CRISPR2, and CRISPR3-Cas, respectively. CRISPR1-Cas, orphan CRISPR2, and CRISPR3-Cas were observed in 5 (13.16%), 8(31.58%), and 1 (2.63%) *Enterococcus faecium* isolates, respectively.

Table 5 The Prevalence of CRISPR-Cas in *Enterococcus faecalis* Isolates and *Enterococcus faecium* Isolates

CRISPR-Cas	Total (N=100) n(%)	Enterococcus faecalis (N=62) n(%)	Enterococcus faecium (N=38) n(%)	P
CRISPR1	1(1)	1(1.61)	0(0)	1
CRISPR2	20(20)	10(16.1)	10(26.32)	0.303
CRISPR3	1(1)	0(0)	1(2.63)	0.38
CRISPR1+CRISPR2	20(20)	17(27.42)	3(7.89)	0.035
CRISPR1+CRISPR3	0(0)	0(0)	0(0)	–
CRISPR2+CRISPR3	4(4)	4(6.45)	0(0)	0.294
CRISPR1+CRISPR2+CRISPR3	0(0)	0(0)	0(0)	–
Total	46	32	14	0.215

Association Between Antibiotic Resistance Genes, Virulence Genes, and the CRISPR-Cas System

The occurrence of antibiotic resistance genes and virulence genes between CRISPR-Cas-positive and -negative isolates of *Enterococcus faecalis* and *Enterococcus faecium* are shown in Table 6. The occurrence of CRISPR-Cas elements in *Enterococcus* isolates negatively correlated with antibiotic resistance rates. The prevalence of these antibiotic-resistance-associated genes was lower in the CRISPR- Cas-positive isolates compared to the CRISPR-Cas-negative isolates. The distribution of *tetM, ermB, aadE, ant (6),* and *aac (6') -aph (2")* between the two groups was statistically significant ($p < 0.05$) (Table 6). In addition, compared with CRISPR-Cas-negative *Enterococcus faecium*, CRISPR-Cas-positive *Enterococcus faecium* isolates were significantly less resistant to vancomycin (Table 6).

In *Enterococcus faecalis* isolates, the presence of *asal* virulence genes was the highest (74.19%), followed by *esp* (62.90%). The number of virulence genes *esp* (92.11%) in *Enterococcus faecium* isolates was higher. Both

Table 6 The Correlation Between Drug-Resistance Genes and CRISPR-Cas of Enterococcus faecalis and Enterococcus faecium

Genes	Enterococcus faecalis (N=62) n (%)		P	Enterococcus faecium (n=38) n (%)		P
	CRISPR-Cas Positive (N=32)	CRISPR-Cas Negative (N=30)		CRISPR-Cas Positive (N=14)	CRISPR-Cas negative (N=24)	
tetM	16(50)	23(76.67)	0.038*	1(7.14)	10(41.67)	0.030*
tetO	0(0)	0(0)	–	0(0)	0(0)	–
blaZ	2(6.25)	2(6.67)	1	0(0)	1(4.17)	1.000
ermA	0(0)	0(0)	–	0(0)	0(0)	–
ermB	14(43.75)	22(73.33)	0.023*	6(42.86)	20(83.33)	0.014*
aadE	12(37.5)	21(70)	0.037*	3(21.43)	17(70.83)	0.006*
ant(6)	10(31.25)	18(60)	0.04*	3(21.43)	17(70.83)	0.006*
aac(6')-aph(2")	10(31.25)	19(63.33)	0.02*	8(57.14)	22(91.67)	0.019*
vanA	0(0)	0(0)	–	0(0)	24(100)	0.000*
vanB	0(0)	0(0)	–	0(0)	0(0)	–
clyA	22(68.75)	13(53.33)	0.072	2(14.29)	0(0)	0.129
esp	15(46.88)	24(80)	0.009*	11(78.57)	24(100)	0.043*
asal	27(84.38)	19(63.33)	0.083	2(14.29)	6(25)	0.684
gelE	6(18.75)	16(53.33)	0.004*	2(14.29)	0(0)	0.129
agg	9(28.13)	3(10)	0.108	1(7.14)	0(0)	0.368
Hyl	0(0)	0(0)	–	0(0)	0(0)	–

Note: The asterisk *Indicates a statistically significant difference ($P < 0.05$).

Enterococcus faecalis and *Enterococcus faecium* isolates have lower virulence genes *agg* (19.35%, 2.63%, respectively). None of the virulence gene *hyl* was found in all *Enterococcus faecalis* and *Enterococcus faecium* isolates. Compared with CRISPR-negative isolates, the *Enterococcus faecalis* virulence gene *esp* had a lower presence in CRISPR-positive isolates. In addition, the absence of *Enterococcus faecalis* virulence gene *gelE* was significantly associated with the presence of CRISPR-Cas ($p<0.05$).

Discussion

In this study, we assessed 62 clinical isolates of *Enterococcus faecalis* and 38 clinical isolates of *Enterococcus faecium* to determine the association between antibiotic resistance, virulence genes, and the CRISPR system. We found that the prevalence of antibiotic resistance of *Enterococcus faecium* is significantly higher than that of *Enterococcus faecalis*. In addition, the results demonstrated that the presence of the CRISPR-Cas system is associated with the absence of antibiotic resistance genes. The results also showed a significant correlation between the presence of the CRISPR-Cas system and fewer virulence factors.

In the current study, we found that the presence of orphan CRISPR2(42%) was more in *Enterococcus* than CRISPR1-Cas (23%) and CRISPR3-Cas (5%), which was similar to Alduhaidhawi.[28] In non-clinical *Enterococcus faecium* isolates, Huescas et al[29] demonstrated a higher prevalence rate of CRISPR2 and lower frequency of CRISPR1-Cas (5.6%) and CRISPR3-Cas (1.8%) system. In contrast to our study, Palmer et al[30] found that CRISPR2 was present in all *Enterococcus faecalis* isolates. In addition. Dos Santos et al[31] reported that CRISPR3 (67.4%) was the most prevalent type among *Enterococcus* isolates. Our results indicated that co-occurrence of CRISPR1 and CRISPR3, as well as CRISPR1, CRISPR2, and CRISPR3 were not found in the same isolates, which is similar to Linderstrauss et al.[32] Similarly, in a study by Gholizadeh et al[33] found that none of the isolates had the CRISPR1 and CRISPR3, as well as CRISPR1, CRISPR2, and CRISPR3 at the same time in the dental-root canal and hospital-acquired isolates of *Enterococcus faecalis*. However, Burley et al[34] found three simultaneous sites of CRISPR1 and CRISPR3 (0.03%) in 88 root canals, oral cavity, and hospital-acquired isolates. Orphan CRISPR2 is thought to be inactive due to the lack of associated Cas proteins. A study revealed most multidrug-resistant *Enterococcus faecalis* isolates lack functional CRISPR-Cas and possess only the orphan CRISPR2.[35] In *Enterococcus* isolates with only orphan CRISPR2, it was found to exist with some antibiotic resistance genes, such as *aadE* and *ant(6), aac6'-aph(2")*, indicating that CRISPR2 alone is not immune to *Enterococcus*. Studies have shown that the consensus repeats of the CRISPR1 and CRISPR2 loci are identical, which suggests that the two sites are functionally linked.[36] In a study by Price et al demonstrated that the orphan CRISPR2 locus requires the presence of CRISPR1-Cas from *Enterococcus faecalis* for genomic defense against MGE.[35]

Antibiotic resistance genes are mainly transmitted through HGT.[6] The acquired immune system CRISPR-Cas is considered as a barrier to HGT.[37] Based on the presence of CRISPR-Cas, we compared the resistance rates of enterococci to different antibacterial drugs. In our study, there was a significant correlation between phenotypic antibiotic resistance, antibiotic resistance-related genes, and the absence of the CRISPR locus. Similarly, several studies have reported that multi-drug resistance was associated with the absence of the CRISPR locus.[38–40] It has also been found that the loss of antibiotic resistance is associated with the presence of CRISPR3 in *Enterococcus faecalis*.[34] Studies have shown that the presence of CRISPR1 in *Enterococcus faecalis* is associated with the lower pro-phage content of isolates such as *Enterococcus faecalis* OG1RF.[41–43]Studies show that CRISPR3-Cas is active in genomic defense with sequence specificity, which can be observed when the CRISPR3-mutant of T11 acquired cas9 (Δcas9 + CRISPR3) and interfered with pAD1 acquisition. They also observed that the deletion of only two loci could cause a significant reduction in genomic defense against the mobile genomic element(MGE).[35]

The presence of CRISPR-Cas was found to be associated not only with antibiotic sensitivity but also with the lack of virulence genes.[33,44] The present results showed that the presence of the CRISPR locus was significantly associated with fewer virulence factors. *Enterococcal* infections and their pathogenicity are related to their carriage of virulence factors. *Enterococcus* virulence factors such as *esp, asal, hyl, gelE, clyA, agg*, etc. can play a role in anti-phagocytosis, adhesion, biofilm formation, exoenzyme, toxin, and so on.[45] Literature reported that the CRISPR-Cas system is inversely correlated with some virulence factors.[46] In this study, we found a correlation between the presence of CRISPR-Cas and the absence

of the *esp* and *gelE* gene in *Enterococcus faecalis* (P = 0.009, P=0.004, respectively) and a correlation between the presence of CRISPR-Cas and the absence of *esp* (P = 0.0043) in *Enterococcus faecium*. In addition, there was a correlation between the absence of CRISPR loci and the absence of some virulence factors (such as *clyA, agg*). In a study by Gholizadeh et al indicated that CRISPR-Cas may prevent the acquisition of some pathogenic factors in dental root canals and hospital-acquired isolates of *Enterococcus faecalis*.[33]

Several studies have shown that the presence of the CRISPR-Cas system is inversely associated with the incidence of bacterial resistance.[47] The negative impact of the CRISPR-Cas system on antibiotic resistance has also been demonstrated in K. pneumoniae.[48] In addition, Ren et al[49] revealed that point mutations in the cas1 and cas2 genes may be associated with multiple drug resistance in Shigella.

However, studies have also reported that there was no significant correlation between CRISPR-Cas and the acquisition of integron, plasmids, antibiotic resistance, and virulence genes in Escherichia coli.[50,51] Shabbir et al[52] claimed that the CRISPR-Cas system promotes antimicrobial resistance in Campylobacter jejuni. Therefore, further studies of CRISPR-Cas with mobile genetic elements associated with drug resistance are required.

Although the limited number of isolates included in the analysis in this study, reference can be provided for the prevention and control of enterococcal nosocomial infection. The relationship between CRISPR loci and antibiotic resistance phenotypes and genotypes may provide new insights into combating infections caused by resistant pathogens.

Conclusion

The study revealed that the CRISPR-Cas system of *Enterococcus faecalis* has a higher carriage rate compared to *Enterococcus faecium* and concluded that CRISPR-Cas system may hinder the transmission of antibiotic resistance genes and virulence genes, which provides a reference for the prevention and control of enterococcal nosocomial infection.

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Disclosure

The authors have no conflict of interest to disclose.

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