Original Article

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Molecular Characterization and Resistant Spectrum of *Enterococci* Isolated from a Haematology Unit in China

Microbiology Section

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ABSTRACT

Objectives: The present study screened clinical isolates of *E. faecalis* and *E. faecium* to determine resistant spectrum and the potential virulence genes characterization among them of haematology patients.

Methods: Clinical *Enterococci* isolates were obtained from a haematology unit in a tertiary care hospital in China.

Results: Among 125 isolates available for the investigation, 46 were identified as *E. faecium*, and 79 were *E. faecalis*. Urine was the most common source (82, 65.6%). *E. faecium* isolates were more resistant than *E. faecalis*. Among *E. faecium*, maximum resistance was seen against PEN 93.5% and AMP 93.5% followed by CIP 87%. Eight vancomycin-resistant *E. faecium* (*VREfm*) isolates were obtained, positive for *vanA* genotype. Of 125 *Enterococci* isolates, 67(53.6%) were *acm*, and 42.4%, 25.6%, 25.6%, 24.8%, 23.2%, 20.8%, 10.4% and 7.2% of isolates were positive for *esp*, *cylL-A*, *asa 1*, *cylL-S*, *cpd*, *cylL-L*, *gel-E* and *ace*, respectively. *E. faecalis* isolates have more virulence genes (VGs) than *E. faecium*. MLST analysis of *VREfm* identified three different STs (ST17, ST78 and ST203).

Conclusion: The study provides the molecular characterization and resistant spectrum of *Enterococci* isolated from a haematology unit in China. Molecular analysis showed that all *VREfm* isolates belonged to pandemic clonal complex-17(CC17), associated with hospital-related isolates. Therefore, determining resistant spectrum and virulence characterization is crucial for the prevention and control of the spread of nosocomial infections caused by *Enterococci* in the haematology unit.

Keywords: Molecular analysis, Nosocomial infections, Vancomycin-resistant *Enterococci*

INTRODUCTION

Haematological malignancies are immunocompromised patients who have undergone chemotherapy and received haematopoietic stem cells transplantation for treatment. Neutropenia, prolonged hospitalization, the frequent use of multiple broad-spectrum antibiotics and the use of invasive procedures all increase the risk of the nosocomial infections [1-3].

Enterococci are the most common species of nosocomial infections, associated with hospital-acquired infections such as UTIs (urinary tract infections), wounds, bacteremia, endocarditis and meningitis [4,5]. Infections with *Enterococci* in critically ill patients, with severe underlying diseases or immunologically suppressed, are often severe when they are exposed to vancomycin-resistant *Enterococci* (VRE) [6]. In addition, the ability of VRE to colonize patients and hospital's environment, has labeled them as a major hospital-associated pathogen [7]. In one study [8] VRE intestinal colonization was reported in 40% of 92 neutropenic patients, of which 34% developed bacteremia with *Enterococci*, following mortality rate of 36%. Extensive use of vancomycin in hospitals has contributed to the emergence and unusual increase of VRE over the past 20 years [9,10].

Acquisition of potential virulence factors by *Enterococci* strains might increase their fitness in the hospital environment. Some virulence factors may change the severity of infections, such as cytolysin (*cylL-L*, *cylL-S*, *cylL-A*), gelatinase (*gel-E*), aggregation substances (*asa1*); collagen adhesine (*ace*), and sex pheromones (*cpd*). Gelatinase (Gel-E) plays a role in modulating the surface display of *E. faecalis* Ace [11]. Cytolysin increases the toxicity of enterococcal infections in Human bacteremia, Rabbit endocarditis and Mouse intraperitoneal infection [12-14]. Several other factors may increase the ability to colonize hospitalized patients, such as enterococcal surface protein (*esp*) [15]. *Esp* anchors to the cell wall and also affects biofilm formation [5].

The purpose of this study was to assess the molecular characterization and resistant spectrum of *Enterococci* isolated from a haematology unit in a tertiary care hospital in China.

MATERIALS AND METHODS

Selection of the Strains

One hundred and twenty five *Enterococci* strains were isolated from a haematology unit in the First Affiliated Hospital of Soochow University from September 2013 to September 2014. The haematology unit of the first affiliated hospital of soochow university has 200 beds. These strains were obtained from urine (82), blood (10), sputum (5), wound swabs (3) and others (25). The species identification of *Enterococci* (*E. faecalis* and *E. faecium*) was done by VITEK-2 COMPACT.

Susceptibility Testing

Antimicrobial susceptibility test for isolates of *Enterococci* was performed against gentamicin (GEM120, 120μg), penicillin (P, 10μg), ampicillin (AMP, 10μg), tetracycline (TCY, 30μg), ciprofloxacin (CIP, 25μg), vancomycin (VAN, 30μg), teicoplanin (TEC, 30μg) and linezolide (LZD, 30μg) (Oxoid, UK), by the disc diffusion method. MICs of vancomycin were determined by the E-test (Biomerieux, China) method on Mueller-Hinton agar. The results were interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI-2011). The vancomycin-resistant gene was identified with *vanA* and *vanB* primers. The resistant genes of *aac (6')-Ie-aph (2'')-Ia*, *tetM* and *tem* were identified. All the primer sequences [Table/Fig-1] have been reported in Kariyama's studies [16].

DNA Isolation

All isolates were cultured on blood agar and incubated overnight at 37°. Genomic DNA was isolated from all strains with Wizard Genomic DNA purification kit (Promega), according to the manufacturer's

instructions (http://cn.promega.com/~/media/files/resources/protocols/technical%20manuals/0/wizard%20genomic%20dna%20purification%20kit%20protocol.pdf), and used as template for PCR.

Multilocus Sequence Typing (MLST) Analysis of VRE Strains

MLST analysis was performed as described by Homan et al., [17]. The internal fragments of 7 housekeeping genes (*atpA*, *ddl*, *gdh*, *purk*, *gyd*, *pstS* and *ddk*) of the *E. faecium* isolates were amplified and sequenced. The sequence types (ST) were determined at the MLST database website (http://efaecium.mlst.net).

Detection of Virulence Genes

The genes encoding *Enterococci* virulence genes (*cylL-L*, *cylL-S*, *cylL-A*, *esp*, *acm*, *gel-E*, *asa 1*, *cpd*, *ace*), were performed by simplex PCR as reported by Sapri et al., [18]. The primers used in this study are listed in [Table/Fig-1].

RESULTS

Bacterial Isolates and Antibiotic Susceptibility Testing

A total number of 125 *Enterococci* were isolated from hospitalized haematological malignancy patients. Among the isolates, 79(63.2%) were identified as *E. faecalis*, 46(36.8%) as *E. faecium*. Urine was the most common source (82, 65.6%).

The antibacterial resistant profiles of *Enterococci* isolates are summarized in [Table/Fig-2]. The disk diffusion indicated that the majority of *Enterococci* isolates were resistant to CIP (63.2%), PEN (60.8%), AMP (57.6%), TCY (51.2%) and GEH (48.0%). Among *E.faecium*, maximum resistance was seen against PEN 93.5% and AMP 93.5% followed by CIP 87%. They were susceptible to linezolid except one isolate of *E. faecium. E. faecalis* were resistant to TCY (62.0%) and CIP (49.4%). *E. faecium* isolates were more resistant than *E. faecalis*. Vancomycin resistance were detected and 8(6.4%) *VREfm* were found.

The *vanA*, *vanB*, *aac(6')-Ie-aph(2'')-Ia*, *tetM* and *tem* were identified. 8 strains were positive for *vanA* genotype and a 732-bp PCR product was obtained in all the positive isolates (data in [Table/Fig-2,3]. Forty (50.6%) *E. faecalis* strains were positive for *tetM*, and 15(32.6%) *E. faecium* positive for *tetM* (data in [Table/Fig-2]). Twenty (25.3%) *E. faecalis* strains were positive for *aac(6')-Ie-aph(2'')-Ia*, and 21(45.7%) *E. faecium* positive for *aac(6')-Ie-aph(2'')-Ia* (data in [Table/Fig-2]). However, no *vanB* and *tem* products were detected in any of the isolates.

PCR Analysis of Virulence Genes

All *Enterococci* isolates for the presence of 9 virulence genes (VGs) was tested. The distribution of virulence gene numbers was as followed: 1 VGs (45, 36.0%), 2 VGs (32, 25.6%), 3 VGs (6, 4.8%), 4 VGs (6, 4.8%), 5 VGs (5.6%), 6 VGs (10, 8.0%), 7 VGs (3, 2.4%) and 8 VGs (3, 2.4%). 12 (9.6%) *Enterococci* strains did not harbor any of the tested genes. Main of these strains were isolated from non-UTI and *E. faecalis* (date not shown).

Of 125 *Enterococci* isolates, 67(53.6%) were *acm*, and 42.4%, 25.6%, 25.6%, 24.8%, 23.2%, 20.8%, 10.4% and 7.2% of isolates were positive for *esp*, *cylL-A*, *asa 1*, *cylL-S*, *cpd*, *cylL-L*, *gel-E* and *ace*, respectively (data in [Table/Fig-2]). The distribution of VGs among *E. faecalis* and *E. faecium* isolates entirely was shown in [Table/Fig-2].

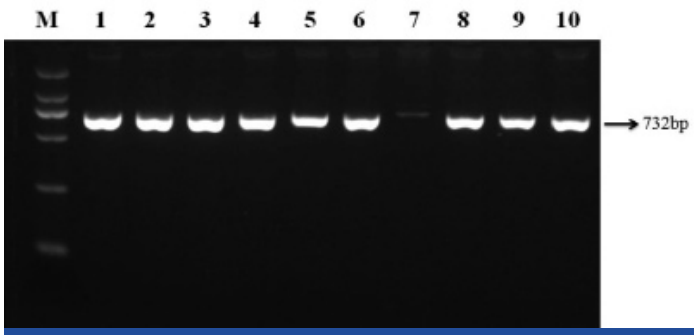
E. faecalis isolates have more VGs than *E. faecium* (2.63 vs. 1.83, $p<0.05$). However, the most prevalent virulence determinant among *E. faecium* was *acm* (91.3%). Correlation analysis of *E. faecium* showed that the number of virulence factors and the resistant spectrum were negatively correlated ($p<0.000$). But, the number of virulence factors and the resistance spectrum of *E. faecalis* were never correlated [Table/Fig-3].

Primers	Oligonucleotide sequence (5′–3′)	Sizes (bp)	T_m(°C)	Specificity	Reference
vanA-F	GGGAAAACGACAATTGC	732bp	56	*vanA*	[19]
vanA-R	GTACAATGCGGCCGTTA				
vanB-F	ATGGGAAGCCGATAGTC	635bp	56	*vanB*	[19]
vanB-R	GATTTCGTTCCTCGACC				
Aph-F	CCAAGAGCAATAAGGGCATA	220bp	56	*aac (6')-Ie-aph(2'')-Ia*	[20]
Aph-R	CACTATCATAACCACTACCG				
tetM-F	GTGTGACGAACTTTACCGAA	501bp	56	*tetM*	
tetM-R	GCTTTGTATCTCCAAGAACAC				
tem-F	AGGAAGAGTATGATTCAACA	535bp	56	*tem*	
tem-R	CTCGTCGTTTGGTATGGG				
cylL-L-F	AACTAAGTGTTGAGGAAATG	159bp	52	*cylL-L*	[21]
cylL-L-R	AAAGACACAACTACAGTTAC				
cylL-S-F	AGAACTTGTTGGTCCTTC	134bp	52	*cylL-S*	[21]
cylL-S-R	GCTGAAAATAATGCACCTAC				
cylL-A-F	ACAGGTTATGCATCAGATCT	507bp	52	*cylL-A*	[21]
cylL-A-R	AATTCACTCTTGGAGCAATC				
esp-F	AGATTTCATCTTTGATTCTTGG	500bp	50	*esp*	[21]
esp-R	AATTGATTCTTTAGCATCTGG				
acm-F	GGCCAGAAACGTAACCGATA	353bp	51	*acm*	[21]
acm-R	CGCTGGGGAAATCTTGTAAA				
gelE-F	AATTGCTTTACACGGAACGG	548bp	52	*gelE*	[21]
gelE-R	GAGCCATGGTTTCTGGTTGT				
asa1-F	GCACGCTATTACGAACTATGA	375bp	52	*asa1*	[22]
asa1-R	TAAGAAAGAACATCACCACGA				
cpd-F	TGGTGGGTTATTTTTCAATTC	782bp	52	*cpd*	[22]
cpd-R	TACGGCTCTGGCTTACTA				
ace-F	GGAATGACCGAGAACGATGGC	616bp	52	*ace*	[22]
ace-R	GCTTGATGTTGGCCTGCTTCCG				

[Table/Fig-1]: Primers used for amplification of Resistant and Virulence genes

	E. faecalis (n=79, 63.2%)	*E. faecium* (n=46, 36.8%)	p-value	Total (n=125, 100%)
Resistant spectrum				
PEN	33(41.8)	43(93.5)	<0.001	76(60.8)
AMP	29(36.7)	43(93.5)	<0.001	72(57.6)
VAN	0(0)	8(17.4)	<0.001	8(6.4)
GEH	37(46.8)	23(49.8)	0.552	60(48.0)
LNZ	0(0)	1(2.2)	0.191	1(0.08)
TCY	49(62.0)	15(32.6)	0.001	64(51.2)
CIP	39(49.4)	40(87.0)	<0.001	79(63.2)
TEC	0(0)	6(13.0)	0.001	6(4.8)
Resistant genes				
tetM	40(50.6)	15(32.6)	0.051	55(44.0)
Aph	20(25.3)	21(45.7)	0.019	41(32.8)
vanA	0(0)	8(17.4)	<0.001	8(6.4)
tem	0(0)	0(0)	-	0(0)
Virulence factors				
cylL-L	23(29.1)	3(6.5)	0.002	26(20.8)
cylL-S	29(36.7)	2(4.3)	<0.001	31(24.8)
cylL-A	30(38.0)	2(4.3)	<0.001	32(25.6)
esp	27(34.2)	26(56.5)	0.016	53(42.4)
acm	25(31.6)	42(91.3)	<0.001	67(53.6)
gel-E	11(13.9)	2(4.3)	0.092	13(10.4)
asa 1	30(38.0)	2(4.3)	<0.001	32(25.6)
cpd	26(32.9)	3(6.5)	0.001	29(23.2)
ace	7(8.9)	2(4.3)	0.310	9(7.2)

[Table/Fig-2]: Molecular characterization and resistant spectrum of *Enterococci*



[Table/Fig-3]: Agarose gel electrophoresis of amplified *vanA* gene. Lane M: D2000 DNA Marker(100, 250, 500, 750, 1000, and 2000bp); Lanes 1-6, 8-9: Isolates positive for the *vanA* genes; Lane 10: positive control.

Characterization of *VREfm*

The 8 *VREfm* isolates showed a 100% rate of resistance to PEN, AMP and CIP. The VAN MIC values for each *VREfm* isolate are presented in [Table/Fig-4] and only the *vanA* gene was detected in all the *VREfm*. The *esp* gene was detected in 87.5% (7/8) of the isolates, and the *acm* gene was present in 100% (8/8) of them.

In the study, 8 *VREfm* isolates were subjected to MLST genotyping. Five of the 8 *VREfm* clinical isolates 62.5% belonged to ST78, two to ST203 and one to ST17 (data in [Table/Fig-4]). eBURST analysis of the *VREfm* isolates revealed they belonged to clonal complex 17 (CC17).

DISCUSSION

Enterococci are important hospital-acquired pathogens, especially in the haematology unit. In the study, *E. faecalis* (63.2%) were predominant strains than *E. faecium* (36.8%). The species distribution is similar to that reported from different parts of the world [23,24]. The findings of multidrug resistance against the tested antibiotics were more obvious in *E. faecium* strains than *E. faecalis* strains. *E. faecium* strains displayed higher resistance to PEN, AMP, and CIP (≥87%). However, *E. faecalis* were more resistant to TCY than *E. faecium* (62.0% vs. 32.6%, *p*=0.001). The reason may be that *E. faecalis* is easier than *E. faecium* to obtain and carry genetic elements of the resistance to TCY. The result of *tetM* gene by PCR also confirmed this conclusion (50.6% vs. 32.6%, p=0.051).

The enterococcal surface protein (Esp) encoded by *esp* gene is thought to promote primary surface attachment, contributing to colonization and persistence of *Enterococci* in the urinary tract and biofilm formation [15]. Acm (encoded by *acm*), a predictor of collagen adherence, mediates *E. faecium* adherence to collagen [25]. Data from other paper show that Acm has contributed to the emergence of *E. faecium* and CC17 genotype in nosocomial infection [26]. The present study clearly revealed that *E. faecalis* strains carried significantly more virulence determinants than *E. faecium* strains (2.63 vs. 1.83, p<0.05). *Esp* and *acm* were two genes with a higher incidence in *E. faecium* isolates than in *E. faecalis* (*esp*: 56.5% vs. 34.2%, p=0.016; *acm*: 91.3% vs. 31.6%, p<0.001. The result indicates that *esp* and *acm* may make it easier for *E. faecium* isolates to adhere and contribute to long-term colonization.

Virulence and resistance play an important role in determining the outcome of a bacterial infection, and allow bacteria to avoid host defenses and antimicrobial treatment. The result of *E. faecalis* strains carrying more virulence determinants and lower resistance than *E. faecium* strains indicates *E. faecalis* isolates produce a high number of virulence factors, such as CylL, GelE, Asa1and Cpd, and introduce it in the tissues and multiply locally in spite of the host immune system. For long-term colonization and acquisition of resistance, *E. faecium* may reduce the introduction of the host defenses by less virulence. However, the mechanism is unclear and needs further study.

MLST is an important tool for studying the molecular epidemiology of outbreaks of *E. faecium* and microbial population biology [27]. In the study, MLST analysis of 8 *VREfm* isolates revealed three different STs: ST78, ST203 and ST17. ST17, ST78 and ST203 belonged to the clonal complex-17 (CC17) lineage, which is the cause of most of the nosocomial VRE outbreaks in Asia, Europe and Latin America, including in China [28-31]. The hospital-adapted CC17 has rapidly spread globally during the last two decades [4,28,32]. The *esp* gene was detected in 87.5% (7/8) of the isolates, and the *acm* gene was present in 100% (8/8) of them. The *esp* and the *acm* genes are associated with CC17 [33]. All *VREfm* patients were immunocompromised and associated with prolonged hospital stay (>60 days) and use of broad-spectrum antimicrobials. These results indicate that factors common to haematology populations (neutropenia, prolonged hospital stay and broad-spectrum antibiotic therapies) are risk factors for VRE acquisition. However, the *VREfm* patients had no infection symptoms, indicating that *VREfm* were just colonization strains. The prevalence and persistence of colonized VRE is a potential risk factor for immunocompromised patients.

CONCLUSION

Our data indicates that *E. faecalis* have different virulence factors and different resistant spectrum, compared with *E. faecium*, and *VREfm* belonged to an internationally disseminated CC17 lineage. *E. faecalis* isolates carry more virulence factors than *E. faecium,* but *E. faecium isolates* show more resistance than *E. faecalis.* The result indicates that virulence and resistance are two different mechanisms for infection or colonization of *Enterococci*. Molecular characterization and resistant spectrum among *E. faecalis* and *E. faecium* of haematology patients explored in this study enhanced our current knowledge of the pathogenicity and genetic characteristics of *Enterococci*. Moreover, determining resistant spectrum and virulence characterization is crucial for the prevention and control of the spread of nosocomial infections caused by *Enterococci* in the haematology unit.

Enterococcus strain	Clinical characteristics of hematologic patients	VAN (MIC, mg/L)	Vancomycin-resistance genes	Resistant phenotype	Other resistance genes	virulence genes	ST (CC)
VREfm 1	Chronic mylogenous leukaemia	>256	vanA	PEN-CIP-VAN-TEC-AMP-LNZ	tetM	esp-acm	ST78(CC17)
VREfm 2	Acute lymphocytic leukaemia	>128	vanA	PEN-CIP-VAN-TEC-AMP	tetM	esp-acm	ST78(CC17)
VREfm 3	Chronic mylogenous leukaemia	>256	vanA	PEN-CIP-VAN-TEC-AMP-GEH-TCY	tetM-aph	esp-acm	ST78(CC17)
VREfm 4	Acute lymphocytic leukaemia	>256	vanA	PEN-CIP-VAN-TEC-AMP-GEH-TCY	tetM-aph	esp-acm	ST17(CC17)
VREfm 5	Acute lymphocytic leukaemia	>128	vanA	PEN-CIP-VAN-AMP--TCY	tetM-aph	esp-acm	ST78(CC17)
VREfm 6	Mixed phenotype acute leukemia, Lung and skin infections, Broad-spectrum anti-infectious treatment	>256	vanA	PEN-CIP-VAN-TEC-AMP-GEH	tetM-aph	acm	ST203(CC17)
VREfm 7	Acute lymphocytic leukaemia	>256	vanA	PEN-CIP-VAN-AMP-GEH-TCY	aph	esp-acm	ST203(CC17)
VREfm 8	Chronic mylogenous leukaemia	>256	vanA	PEN-CIP-VAN-TEC-AMP-GEH-TCY	tetM-aph	esp-acm	ST78(CC17)

[Table/Fig-4]: Characteristics of *VREfm* isolates recovered from hematologic malignancy patients

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