Molecular Characterization of Vancomycin-resistant Enterococcus Clinical Isolates Recovered from Hospitalized Patients in China

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Abstract

Background: To characterize 101 unique vancomycin-resistant enterococci (VRE) recovered from (12) hospitals in China.

Methods: 96 *E. faecium* and five *E. faecalis* strains were collected from 2005 through 2011. Identification was performed by PCR. Susceptibility testing was performed by CLSI methods (M07-A9). Vancomycin resistance (*vanA*/*B*) and virulence genes (*esp*/*hyl*) were screened by PCR. All strains were subjected to PFGE and MLST. Conjugation was performed by filter mating. Resistance and virulence gene locations were determined by Southern blot/hybridization.

Results: *E. faecalis* exhibited vancomycin and teicoplanin MIC results of ≥256 mg/L and harboured *vanA*, except for one strain (MIC, 32 and 1 mg/L, respectively) that had *vanB*. *E. faecium* displayed vancomycin MIC values of ≥64 mg/L with variable results for teicoplanin (1-256 mg/L). One *E. faecium* had a teicoplanin MIC value of 1 mg/L and carried a *vanB*, while all other strains carried *vanA* and had teicoplanin MICs ≥16 mg/L, except for two strains (teicoplanin MIC, 4 - 8 mg/L). *vanA* plasmids were transferred by conjugation and similar glycopeptide resistance profiles were noted for transconjugants, except for one strain that had a confirmed teicoplanin MIC (32 mg/L) higher than the donor strain (4 mg/L). 78.4 and 28.4% of all strains carried *esp* and *hyl*, respectively. Overall, *esp* was chromosomally-located, while *hyl* was mostly carried by non-conjugative plasmids. *E. faecalis* strains belonged to clonal complex [CC]4, whereas the majority of *E. faecium* strains had STs associated to CC17.

Conclusions: *vanA*-carrying *E. faecium* predominated, which were mostly associated with a common and human-adapted lineage (CC17). Plasmids carrying *vanA* and *hyl* may play an important role in the dissemination of these genes, while the *esp* virulence gene seems to be associated to the chromosome of a specific enterococcal lineage.

Introduction

Enterococcus faecalis and *E. faecium* have become important organisms due to their increased incidence among Gram-positive pathogens causing nosocomial infections. While *E. faecalis* isolates are more susceptible to several antimicrobial agents, the majority of *E. faecium* currently recovered from hospitals worldwide are vancomycin-resistant (VRE), and many exhibit a multidrug-resistance (MDR) phenotype, including resistance to ampicillin and aminoglycosides.

There is growing evidence demonstrating that enterococcal isolates can rapidly adapt to the hospital environment and colonize mucosal surfaces. Among enterococcal isolates, a specific clonal complex (CC) of *E. faecium* strains, CC17, has been responsible for numerous hospital outbreaks. Usually exhibiting ampicillin, aminoglycoside and glycopeptide resistance phenotypes and carrying virulence genes, such as *esp* and *hyl*, CC17 strains seem to have become adapted to the hospital environment. This study assessed the phenotypic and genotypic characteristics of 101 unique VRE recovered from 12 hospitals in China.

Methods

Bacterial strain collection. A total of 96 and five vancomycin-resistant *E. faecium* and *E. faecalis* strains were included in this study, respectively. These strains were recovered from 101 patients hospitalized in 12 hospitals in China between March 2005 and November 2011. Species identification was confirmed by using species-specific primers in a multiplex PCR format.

Antimicrobial susceptibility testing. Isolates were tested for susceptibility by the broth microdilution method using customized 96-well frozen-form panels with cation-adjusted Mueller-Hinton broth (fresh frozen media) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Antimicrobial agents tested were: vancomycin, teicoplanin, daptomycin, ampicillin, gentamicin and streptomycin (high-level), levofloxacin, nitrofurantoin, tetracycline, quinupristin/dalfopristin, chloramphenicol, tigecycline, and linezolid. Validation of the minimum inhibitory concentration (MIC) values was performed by concurrent testing of CLSI-recommended quality control reference strains (*Staphylococcus aureus* ATCC 29213, *E. faecalis* ATCC 29212 and ATCC 51299). MIC interpretations were based on CLSI (M100-S23) and EUCAST (2013) breakpoint criteria, when available.

Screening for virulence and glycopeptide resistance genes. The presence of five virulence genes *asa1*, *gelE*, *cylA*, *esp,* and *hyl* were determined by a multiplex PCR approach. Detection of glycopeptide resistance genes was also performed by using a multiplex PCR assay.

Molecular typing. Pulsed-field gel electrophoresis (PFGE) was performed using standard protocols. Interpretation of banding patterns was initially performed by visual inspection. Subsequently, cluster analysis was performed using the GelCompar II software (Applied Math, Kortrijk, Belgium) applying the method of Dice and the unweighted-pair group method using average linkages (UPGMA). The band tolerance was set at 1.2%, and the threshold cutoff value was set at 82%. All isolates were further characterized by multilocus sequence typing (MLST) using a standard protocol available at the MLST website (http://efaecium.mlst.net/misc/info.asp and http://efaecalis.mlst.net/misc/info.asp). Sequence types (ST) were designated using the MLST website (http://efaecium.mlst.net and http://efaecalis.mlst.net) and eBURST analysis was utilized for determining CCs.

Conjugation and gene location. Conjugative transfer of *van* genes was conducted by filter mating using representatives of each PFGE type as donors and rifampicin-resistant *E. faecium* ATCC 35667 as a recipient strain. Selection of transconjugants was performed using brain heart infusion (BHI) agar plates containing rifampicin (100 mg/L) and vancomycin (8 mg/L). Purified single colonies of transconjugants were also plated on the same selective BHI agar media. PFGE was performed in all transconjugants using the restriction enzyme SmaI in order to confirm that they were derivatives of the recipient strain. Enterococcal clinical isolates total DNA were subjected to S1 nuclease digests coupled with PFGE in order to determine the location of *van* and *esp*/*hyl* virulence genes. S1 nuclease-treated linearized plasmids were transferred to a nylon membrane and hybridized using the respective gene probes.

Results

- *E. faecalis* exhibited vancomycin and teicoplanin MIC results of ≥256 mg/L and harboured *vanA*, except for one strain (MIC, 32 and 1 mg/L, respectively) that had *vanB* (**Table 1**). *E. faecium* displayed vancomycin and teicoplanin MIC values of ≥64 and ≥16 mg/L, respectively. Exceptions were observed among three *E. faecium* strains with teicoplanin MIC value of 1 mg/L (*vanB*) and 4 - 8 mg/L (*vanA*; **Tables 2** and **3**).
- Overall, VRE *E. faecium* clinical isolates showed a MDR phenotype and only daptomycin ($MIC_{50/90}$, 2/4 mg/L; 100% susceptible), linezolid ($MIC_{50/90}$, 1/1 mg/L; 100% susceptible) and tigecycline ($MIC_{50/90}$, 0.03/0.12 mg/L; 100% susceptible) demonstrated *in vitro* coverage (**Table 2**).
- Among *E. faecium* strains included in the study, 67 isolates representative of each PFGE type were selected for further investigations. *vanA* plasmids (46- to 190-kb) were transferred by conjugation and similar vancomycin MIC distributions were observed between donor ($MIC_{50/90}$, 256/>256 mg/L) and transconjugant strains ($MIC_{50/90}$, 256/>256 mg/L; **Table 3**).
- *E. faecium* transconjugant strains had teicoplanin MIC results ($MIC_{50/90}$, 32/32 mg/L) two- to eight-fold lower when compared with the MIC results obtained against donor strains ($MIC_{50/90}$, 64/256 mg/L; **Table 3**). The teicoplanin modal MIC value against transconjugants (32 mg/L) was eight-fold lower than that of donor isolates (256 mg/L).
- One transconjugant strain containing a *vanA*-carrying plasmid exhibited confirmed vancomycin and teicoplanin MIC values of 256 and 32 mg/L, respectively; while the donor strain demonstrated MIC results of 128 and 4 mg/L, respectively, when carrying this *vanA* plasmid (data not shown).
- *E. faecalis* strains belonged to CC4, whereas all but three *E. faecium* strains had STs associated to CC17.
- Among all *E. faecium* strains, 78.4 and 28.4% carried *esp* and *hyl*, respectively. *esp* was chromosomally-located in CC17 strains, while *hyl* was usually carried by plasmid DNAs (194- to 325-kb). These plasmids were not transferred to the recipient strains using the conjugation filter mating experiments and established selecting criteria.

Conclusions

- Transconjugant *E. faecium* strains displayed similar vancomycin MIC results when compared to donor strains. However, the teicoplanin MIC values among recipient strains were consistently lower (two- to eight-fold) than selected clinical isolates.
- The lower teicoplanin MIC results observed for the transconjugant strains suggest that expression of the *van* gene operon and/or post-transcriptional regulations are subjected to the strain background and may affect the teicoplanin MIC results.
- Moreover, when the *vanA*-carrying plasmid detected in an *E. faecium* isolate exhibiting a VanB-phenotype (teicoplanin MIC, 4 mg/L) was transferred to the recipient ATCC 35667 strain, the teicoplanin MIC value increased four-fold (32 mg/L). These results also suggest that the genetic background may affect susceptibility for teicoplanin.
- Plasmids carrying *vanA* and *hyl* appear to play important roles in the dissemination of these genes. The enterococcal surface protein *esp* gene was dominantly detected in the chromosome of *E. faecium* strains, likely within a pathogenicity island. Therefore, the dissemination of this gene seems to be associated to the spread of the CC17 lineage.

Table 1. Antimicrobial susceptibility and virulence profiles of five *E. faecalis* clinical isolates recovered from urine specimens of patients in a single hospital in Beijing.

Isolate	MIC (mg/L)[a]												Virulence gene					Epidemiology typing[b]		
	VAN	TEI	AMP	GEN	STR	TET	CHL	LVX	NIT	Q/D	TGC	DAP	LZD	*esp*	*hyl*	*asaI*	*gelE*	*cylA*	PFGE	ST	CC
10831	>256	256	2	>1024	≤1000	32	8	>8	≤16	8	0.03	2	1	+	-	-	+	+	A	471	4
10832	>256	256	1	>1024	≤1000	32	8	8	≤16	8	0.03	2	1	+	-	+	+	+	A1	471	4
10830	>256	256	2	>1024	≤1000	32	8	>8	≤16	8	0.03	2	1	+	-	-	+	+	A2	471	4
10828	>256	256	2	>1024	≤1000	32	4	8	≤16	8	0.03	2	1	+	-	+	+	+	A2	470	4
10827	32	1	4	≤500	≤1000	0.5	8	64	≤16	8	0.03	2	0.5	+	-	-	+	+	B	410	4

a. VAN, vancomycin; TEI, teicoplanin; AMP, ampicillin; GEN and STR, gentamicin and streptomycin high-level; TET, tetracycline; CHL, chloramphenicol; LVX, levofloxacin; NIT, nitrofurantoin; Q/D, quinupristin/dalfopristin; TGC, tigecycline; DAP, daptomycin; LZD, linezolid.
b. PFGE, pulsed-field gel electrophoresis; ST, sequence type; CC, clonal complex.

Table 2. Activity of several antimicrobial agents when tested against 96 clinical isolates of *E. faecium* included in this study.

Antimicrobial agents	MIC (mg/L)		Range	% susceptible / % resistant[a]		Antimicrobial agents	MIC (mg/L)		Range	% susceptible / % resistant[a]	
	MIC_{50}	MIC_{90}		CLSI	EUCAST		MIC_{50}	MIC_{90}		CLSI	EUCAST
Vancomycin	>256	>256	128->256	0.0 / 100.0	0.0 / 100.0	Chloramphenicol	8	16	1-32	82.3 / 1.0	- / -
Teicoplanin	64	256	1-256	3.1 / 79.2	2.1 / 97.9	Levofloxacin	>8	>8	>8	0.0 / 100.0	- / -
Daptomycin	2	4	1-4	100.0 / 0.0	- / -	Nitrofurantoin	128	128	≤16->128	20.8 / 56.2	43.8 / 56.2
Linezolid	1	1	0.5-2	100.0 / 0.0	100.0 / 0.0	Quinupristin/dalfopristin	1	2	≤0.5-4	81.3/ 7.3	81.3 / 0.0
Ampicillin	>32	>32	32->32	0.0 / 100.0	0.0 / 100.0	Tetracycline	≤1	>32	≤1->32	57.3 / 42.7	- / -
Gentamicin (HL)[b]	>1000	>1000	≤500->1000	25.0 / 75.0	NA	Tigecycline	0.03	0.12	0.015-0.12	- / -	100.0 / 0.0
Streptomycin (HL)[b]	≤1000	>2000	≤1000->2000	76.0 / 24.0	NA						

a. Criteria as published by the CLSI (2013) and EUCAST (2013).
b. HL, high-level.

Table 3. Vancomycin and teicoplanin MIC distributions when tested against *E. faecium* clinical isolates (donor) and transconjugant strains.

E. faecium (No. tested)	MIC (mg/L)		Number (cumulative %) of isolates inhibited at each MIC (mg/L) of:[a]							
Antimicrobial agents	50%	90%	≤4	8	16	32	64	128	256	>256
All (96)										
Vancomycin	>256	>256	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(1.0)	8(9.4)	34(44.8)	53(100.0)
Teicoplanin	64	128	2(2.0)[b]	1(3.0)	19(22.9)	9(32.3)	**35(68.8)**	22(91.7)	8(100.0)	
Donor (67)										
Vancomycin	256	>256	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	7(10.4)	28(52.2)	**32(100.0)**
Teicoplanin	64	256	1(1.5)	1(3.0)	15(25.4)	6(34.3)	**25(71.6)**	10(86.6)	9(100.0)	
Transconjugant (67)										
Vancomycin	256	>256	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	24(35.8)	**42(90.0)**	1(100.0)
Teicoplanin	32	32	0(0.0)	15(22.4)	14(43.3)	**34(94.0)**	2(97.0)	2(100.0)		

a. Modal MIC values are in bold.
b. One VanB-type strain with a teicoplanin MIC value of 1 mg/L.

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