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The prevalence of aminoglycoside-modifying enzyme and virulence genes among enterococci with high-level aminoglycoside resistance in Inner Mongolia, China

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ABSTRACT

This study highlights the prevalence of aminoglycoside-modifying enzyme genes and virulence determinants among clinical enterococci with high-level aminoglycoside resistance in Inner Mongolia, China. Screening for high-level aminoglycoside resistance against 117 enterococcal clinical isolates was performed using the agar-screening method. Out of the 117 enterococcal isolates, 46 were selected for further detection and determination of the distribution of aminoglycoside-modifying enzyme-encoding genes and virulence determinants using polymerase chain reaction -based methods. *Enterococcus faecium* and *Enterococcus faecalis* were identified as the species of greatest clinical importance. The *aac(6′)-Ie-aph(2′′)-Ia* and *ant(6′)-Ia genes* were found to be the most common aminoglycoside-modifying enzyme genes among high-level gentamicin resistance and high-level streptomycin resistance isolates, respectively. Moreover, *gelE* was the most common virulence gene among high-level aminoglycoside resistance isolates. Compared to *Enterococcus faecium*, *Enterococcus faecalis* harbored multiple virulence determinants. The results further indicated no correlation between aminoglycoside-modifying enzyme gene profiles and the distribution of virulence genes among the enterococcal isolates with high-level gentamicin resistance or high-level streptomycin resistance evaluated in our study.

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Introduction

Enterococci have emerged as an important source of hospital-acquired infections, including those related to the surgical site, respiratory tract, urinary tract, skin and soft tissue, and bacteremia.[1] Several studies have indicated increasing enterococci resistance to a broad range of antimicrobial agents via intrinsic and acquired mechanisms.[2] High-level aminoglycoside resistance (HLAR) has been recognized for several decades. Gentamicin and streptomycin are the two main aminoglycosides used in clinical practice. Recently, high-level gentamicin resistance (HLGR) (MIC $\geq$500 μg/ml) and high-level streptomycin resistance (HLSR) (MIC $\geq$2000 μg/ml) have been reported worldwide.[3–8] Clinical experience supports the use of aminoglycosides along with cell-wall inhibitors for treating serious enterococcal infections.[9] However, high-level resistance of clinical isolates of *Enterococcus* species to aminoglycosides negates the synergism between cell-wall inhibitors and aminoglycosides, making the treatment of serious enterococcal infections difficult.[10]

In general, enterococci are intrinsically resistant to clinically achievable concentrations of aminoglycosides. However, high-level resistance to aminoglycosides is primarily due to acquisition of genes encoding aminoglycoside-modifying enzymes (AMEs).[11] Over past decades, a great deal of research has been devoted to understanding the mechanisms behind the high-level resistance of enterococci to aminoglycosides. Until now, the following three classes of AMEs have been identified: acetyltransferase (AAC), aminoglycoside phosphotransferase (APH), and aminoglycoside nucleotidyltransferase (ANT).[12] The high-level resistance of enterococci to gentamicin is predominantly mediated by *aac(6′)-Ie-aph(2″)-Ia*, which encodes the bifunctional AME, AAC(6′)-APH(2″).[13] Ten years ago, *aph(2″)-Ib*, *aph(2″)-Ic*, and *aph(2″)-Id* were detected as newer AME genes conferring HLGR among enterococci.[14–16] Furthermore, HLSR among enterococci is mediated by *aph(3′)-IIIa* and *ant(6′)-Ia* encoding for APH(3′) and ANT(6′)-Ia, respectively.[3,11]

Additionally, the rise of drug-resistant virulent strains of enterococci is a serious problem in the treatment and control of enterococcal infections. The pathogenicity of enterococci is due to the presence of virulence determinants, such as the *Enterococcus faecalis* (*E. faecalis*) antigen A (*efaA*), adhesion of collagen from *E. faecalis* (*ace*) and products involved in aggregation (*agg*), biosynthesis of an extracellular metalloendopeptidase (*gelE*), biosynthesis of cytolysin (*cylA*) and immune evasion (*esp*).[17] Previous research has shown that clinical isolates of the *Enterococcus* species possess distinctive patterns of virulence factors.[18]

The difficulty in treating enterococcal infections is associated with determinants of virulence and antimicrobial resistance. Therefore, accurate identification of antibiotic susceptibility patterns and virulence determinants is essential for choosing appropriate therapies and means of infection control. The goal of the present study was to investigate the occurrence of HLAR among enterococci in Inner Mongolia, China. Additionally, the prevalence of six AME encoding genes, *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Id*, *aph(3′)-IIIa* and *ant(6′)-Ia*, among enterococcal isolates collected from different specimen sources were investigated. Moreover, the presence of virulence determinants, such as *efaA*, *ace, agg, gelE*, *cylA* and *esp*, was detected.

Experimental

Bacterial strains and identification

A total of 117 clinical isolates of enterococci were collected from four hospitals in Inner Mongolia, China, between May 2012 and May 2014. Duplicate isolates were excluded from the study. Institutional ethical clearance was obtained. The isolates were identified as enterococci by conventional biochemical tests and VITEK 2 Compact (BioMérieux, France). Identifications of *E. faecalis*, *E. faecium* and the other strains were further confirmed via PCR analysis using $ddl_{E.\ faecium}$, $ddl_{E.\ faecalis}$ and *16S rDNA* genes, respectively.[19] The isolates were then stored at $-80\,^{\circ}$C. Table 1 shows the primers and the product sizes of all the genes analyzed.[19–23]

Detection of HLAR in enterococci

Screening for HLAR was performed using the agar-screening method according to the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute (CLSI).[24] Briefly, brain heart infusion agar containing gentamicin (500 μg/ml) and streptomycin (2000 μg/ml) was used. Plates were incubated for 24 h at 37 $^{\circ}$C. The aminoglycoside-susceptible strain *E. faecalis* ATCC 29212 and the aminoglycoside-resistant strain *E. fecalis* ATCC 51299 were utilized as controls for HLAR detection.

Amplification of AMEs and virulence genes

The total DNA template was extracted from enterococci according to the instruction manuals of commercial DNA extraction kits (Hangzhou BioSci Biotech Co., Ltd, China). A PCR method was used to detect the presence of AME and virulence genes.[20–23] The primer couples, product sizes of the genes and annealing temperatures are shown in Table 1. PCR amplification was performed using 1 μg of the template DNA, 1 μl of each primer (100 pmol), and 25 μl of 2$\times$ PCR master mix (Hangzhou BioSci Biotech Co., Ltd, China) in a total volume of 50 μl. A C1000 Touch thermocycler (Bio-Rad, USA) was also employed. PCR conditions were as follows: initial denaturation at 94 $^{\circ}$C for 3 min; 35 cycles of denaturation at 94 $^{\circ}$C for 30 s, annealing for 30 s and elongation at 72 $^{\circ}$C for 1 min, and final extension at 72 $^{\circ}$C for 5 min. PCR products were analyzed by electrophoresis on 1% agarose gel following staining with ethidium bromide.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software (version 11.5). A *p* value <0.05 was considered statistically significant.

Table 1 – PCR protocols used in this study.				
Gene	Primers (5′–3′)	Size (bp)	Annealing temperature (°C)	Reference
$ddl_{E.\ faecium}$	F-TTGAGGCAGACCAGATTGACG R-TATGACAGCGACTCCGATTCC	658	54	Macovei and Zurek[19]
$ddl_{E.\ faecalis}$	F-TCAAGTACAGTTAGTCTTTATTAG R-ACGATTCAAAGCTAACTGAATCAGT	941	54	Macovei et al.[19]
16S rDNA	F-GGATTAGATACCCTGGTAGTCC R-TCGTTGCGGGACTTAACCCAAC	320	54	Macovei et al.[19]
aac(6′)-Ie-aph(2″)-Ia	F-CAGGAATTTATCGAAAATGGTAGAAAAG R-CACAATCGACTAAAGAGTACCAATC	369	56	Emaneini et al.[23]
aph(2″)-Ib	F-CTTGGACGCTGAGATATATGAGCAC R-GTTTGTAGCAATTCAGAAACACCCTT	867	56	Emaneini et al.[23]
aph(2″)-Ic	F-CCACAATGATAATGACTCAGTTCCC R-CCACAGCTTCCGATAGCAAGAG	444	56	Emaneini et al.[23]
aph(2″)-Id	F-GTGGTTTTTACAGGAATGCCATC R-CCCTCTTCATACCAATCCATATAACC	641	56	Emaneini et al.[23]
aph(3′)-IIIa	F-GGCTAAAATGAGAATATCACCGG R-CTTTAAAAAATCATACAGCTCGCG	523	56	Emaneini et al.[23]
ant(6′)-Ia	F-ACTGGCTTAATCAATTTGGG R-GCCTTTCCGCCACCTCACCG	577	56	Sepúlveda et al.[22]
cylA	F-TGGATGATAGTGATAGGAAGT R-TCTACAGTAAATCTTTCGTCA	517	54	Reviriego et al.[21]
gelE	F-AGTTCATGTCTATTTTCTTCAC R-CTTCATTATTTACACGTTTG	402	56	Duprè et al.[20]
esp	F-TTACCAAGATGGTTCTGTAGGCAC R-CCAAGTATACTTAGCATCTTTTGG	936	56	Duprè et al.[20]
ace	F-AAAGTAGAATTAGATCCACAC R-TCTATCACATTCGGTTGCG	320	56	Duprè et al.[20]
agg	F-CCAGTAATCAGTCCAGAAACAACC R-TAGCTTTTTTCATTCTTGTGTTTGTT	406	53	Duprè et al.[20]
efaA	F-CGTGAGAAAGAAATGGAGGA R-CTACTAACACGTCACGAATG	499	56	Duprè et al.[20]

Results and discussion

Results

Identification of Enterococcus species
A total of 117 strains of enterococci were isolated from various clinical samples, including urine 48/117 (41.0%), wounds 28/117 (23.9%), bile 18/117 (15.4%), vaginal/semen swabs 6/117 (5.1%), pus 6/117 (5.1%), blood 3/117 (2.6%), sputum 2/117 (1.7%), and others 6/117 (5.1%). The predominant species observed in our study were *E. faecium* 63/117 (53.8%) and *E. faecalis* 33/117 (28.2%). We also detected *E. avium* 11/117 (9.4%), *E. gallinarum* 6/117 (5.1%), *E. casseliflavus* 3/117 (2.6%), and *E. durans* 1/117 (0.9%).

Detection of HLAR in enterococci
Resistance rates were determined using the agar-screening method. Among the 117 isolates tested, 50/117 (42.7%) were HLGR, and 32/117 (27.4%) were HLSR. A total of 12/117 (10.3%) of our study isolates were both HLGR and HLSR. The highest resistance was observed among *E. faecium*, followed by *E. faecalis* and *E. avium*.

PCR amplification of AME genes in gentamicin-resistant or streptomycin-resistant isolates
Of the 117 enterococcal isolates evaluated, 46 were selected for further detection and determination of distributions of AME encoding genes (Table 2). Of 28 HLGR isolates (Table 2),

25 (89.3%) carried *aac(6′)-Ie-aph(2″)-Ia*, 2 (7.1%) carried *aph(2″)-Ic*, 3 (10.7%) carried *aph(2″)-Id*, and 7 (25.0%) carried *aph(3′)-IIIa*. However, *aph(2″)-Ib* was not detected among the isolates evaluated in this study. Some HLGR isolates carried multiple AME genes. Two isolates (7.1%) carried *aac(6′)-Ie-aph(2″)-Ia* and *aph(2″)-Ic*; 3 isolates (10.7%) carried *aac(6′)-Ie-aph(2″)-Ia* and *aph(2″)-Id*; and 6 isolates (21.4%) carried *aac(6′)-Ie-aph(2″)-Ia* and *aph(3′)-IIIa*.

Similarly, of the 14 HLSR isolates identified in our study (Table 3), 10 (71.4%) carried *ant(6′)-Ia*; 8 (57.1%) carried *aph(3′)-IIIa*; and 7 (50.0%) carried both *ant(6′)-Ia* and *aph(3′)-IIIa*. PCR analysis revealed the absence of resistance genes in susceptible isolates.

PCR amplification of AME genes in both gentamicin- and streptomycin-resistant isolates
Table 4 shows the AME gene profiles of both HLGR and HLSR isolates. Of the 12 isolates analyzed, all 12 (100%) carried *aac(6′)-Ie-aph(2″)-Ia*; 9 (75.0%) carried *ant(6′)-Ia*; 7 (58.3%) carried *aph(3′)-IIIa*; 2 (16.7%) carried *aph(2″)-Id*; and 1 (8.3%) carried *aph(2″)-Ic*. Moreover, 11 isolates (91.7%) carried multiple (two to four) AME genes. Five isolates (41.7%) carried two genes; 4 isolates (33.3%) carried three genes; and 2 isolates (16.7%) carried four genes.

PCR amplification of virulence genes in enterococci
Table 5 shows the presence of virulence genes among HLAR isolates. Of the 30 isolates evaluated, 10 (33.3%) carried *gelE*; 8 (26.7%) carried *efaA*; 7 (23.3%) carried *esp*; 7 (23.3%) carried *cylA*;

Table 2 – Distributions of aminoglycoside modifying enzyme-encoding genes in enterococci with high-level gentamicin resistance. Some isolates carried multiple aminoglycoside modifying enzyme-encoding genes.

AME gene	Distribution of high-level gentamicin resistance in enterococci ($n = 28$)			Total no. (%) of isolates
	E. faecium ($n = 19$)	E. faecalis ($n = 7$)	E. avium ($n = 2$)	
aac(6′)-Ie-aph(2″)-Ia	18	5	2	25 (89.3%)
aph(2″)-Ib	–	–	–	–
aph(2″)-Ic	–	2	–	2 (7.1%)
aph(2″)-Id	2	–	1	3 (10.7%)
aph(3′)-IIIa	6	1	–	7 (25.0%)
aac(6′)-Ie-aph(2″)-Ia + aph(2″)-Ic	–	2	–	2 (7.1%)
aac(6′)-Ie-aph(2″)-Ia + aph(2″)-Id	2	–	1	3 (10.7%)
aac(6′)-Ie-aph(2″)-Ia + aph(3′)-IIIa	6	–	–	6 (21.4%)

Table 3 – Distributions of aminoglycoside modifying enzyme-encoding genes in enterococci with high-level streptomycin resistance. Some isolates carried multiple aminoglycoside modifying enzyme-encoding genes.

AME gene	Distribution of high-level streptomycin resistance in enterococci ($n = 14$)		Total no. (%) of isolates
	E. faecium ($n = 12$)	E. faecalis ($n = 2$)	
ant(6′)-Ia	9	1	10 (71.4%)
aph(3′)-IIIa	7	1	8 (57.1%)
ant(6′)-Ia + aph(3′)-IIIa	6	1	7 (50.0%)

Table 4 – Distributions of aminoglycoside modifying enzyme-encoding genes in enterococci with high-level resistance to both streptomycin and gentamicin.

Enterococcal species	Strain	AME gene profile
E. faecium	6	aac(6′)-Ie-aph(2″)-Ia
	13	aac(6′)-Ie-aph(2″)-Ia + ant(6′)-Ia
	15	aac(6′)-Ie-aph(2″)-Ia + aph(2″)-Id
	20	aac(6′)-Ie-aph(2″)-Ia + ant(6′)-Ia
	21	aac(6′)-Ie-aph(2″)-Ia + aph(3′)-IIIa + ant(6′)-Ia
	29	aac(6′)-Ie-aph(2″)-Ia + ant(6′)-Ia
	30	aac(6′)-Ie-aph(2″)-Ia + aph(3′)-IIIa + ant(6′)-Ia
	34	aac(6′)-Ie-aph(2″)-Ia + aph(3′)-IIIa + ant(6′)-Ia
	43	aac(6′)-Ie-aph(2″)-Ia + aph(3′)-IIIa
	49	aac(6′)-Ie-aph(2″)-Ia + aph(3′)-IIIa + ant(6′)-Ia
	51	aac(6′)-Ie-aph(2″)-Ia + aph(2″)-Id + aph(3′)-IIIa + ant(6′)-Ia
E. faecalis	44	aac(6′)-Ie-aph(2″)-Ia + aph(2″)-Ic + aph(3′)-IIIa + ant(6′)-Ia

Table 5 – Distributions of virulence genes in enterococci with high-level aminoglycoside resistance.

Virulence gene	Distribution of high-level aminoglycoside resistance in enterococci ($n = 30$)			Total no. (%) of isolates
	E. faecium ($n = 20$)	E. faecalis ($n = 8$)	E. avium ($n = 2$)	
gelE	4	6	0	10 (33.3%)
esp	2	4	1	7 (23.3%)
cylA	3	4	0	7 (23.3%)
ace	0	1	0	1 (3.3%)
agg	0	0	0	0 (0%)
efaA	0	8	0	8 (26.7%)

and 1 (3.3%) carried ace. The agg gene was not detected in any of isolates. Moreover, AME gene profiles and distributions of virulence determinants were compared among HLGR or HLSR isolates, and no significant correlations were found.

Discussion

Enterococci have long been considered one of the most common sources of nosocomial pathogens. The rise of drug-resistant virulent strains of enterococci presents a serious problem with regard to controlling enterococcal infections. Aminoglycoside antibiotics provide efficient therapies for severe enterococcal infections. However, the introduction of two aminoglycoside antibiotics (gentamicin and streptomycin) was followed by the emergence of resistance in enterococci.

To date, several *Enterococcus* species have been reported, including *E. faecium*, *E. faecalis*, *E. avium*, *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. raffinosus*, *E. mundtii*, *E. malodoratus*, and *E. hirae*.[25] Similar to previous findings in China,[26]

E. faecium and *E. faecalis* comprise the predominant conditionally pathogenic bacteria in the present report. Additionally, our antibiotic susceptibility test results identified 42.7% HLGR and 27.4% HLSR clinical isolates, similar to other reports.[9] Moreover, in agreement with previous studies,[9] HLGR was found to be more common than HLSR in enterococci species.

HLAR is primarily due to the presence of AME genes. HLGR has reportedly been associated with *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Id*, and *aph(3′)-IIIa*. However, HLSR is mainly mediated by *aph(3′)-IIIa* and *ant(6′)-Ia*.[3,27] To understand HLAR in enterococci in our region, we investigated the distribution of AME genes among enterococci. As reported in other studies,[22,27] our analyses identified *aac(6′)-Ie-aph(2″)-Ia* and *ant(6′)-Ia* as the most common AME genes among HLGR and HLSR isolates, respectively. It is noteworthy that some HLAR isolates did not harbor any AME genes. We presume that this phenomenon may be due to the action of other resistance mechanisms. On the other hand, we detected some isolates that co-harbored two, three, or four AME genes simultaneously, consistent with previous studies.[28]

The distributions of virulence genes among enterococci were further investigated. As reported in other studies,[29] *gelE* was identified as the most frequent virulence factor and was more common in *E. faecalis* than *E. faecium*. *Esp*, *cylA* and *ace* genes were found in 23.3%, 23.3% and 3.3% of the HLAR isolates, respectively. Recently, Sharifi et al. reported that 52.1% of enterococci from hospitalized patients with urinary tract infections tested positive for the *esp* gene,[30] this value is higher than that determined by the present study. Like previous reports, our findings found that all HLAR *E. faecalis* isolates harbored *efaA*.[31] However, *agg* was not detected in any of the enterococci isolates studied herein. Similar to previous studies,[32] *E. faecium* and *E. faecalis* strains showed significantly different patterns with regard to the incidence of virulence determinants. Contrary to *E. faecium*, *E. faecalis* harbored multiple virulence genes.

Conclusions

This study indicates that enterococci recovered from clinical samples in Inner Mongolia, China, contain a variety of AME genes and virulence determinants. The *aac(6′)-Ie-aph(2″)-Ia* and *ant(6′)-Ia* genes were the most common AME genes among HLGR and HLSR enterococci, respectively, and *gelE* was the most frequent virulence determinant.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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