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Antimicrobial susceptibility and species identification for clinical isolates of enterococci

Sue-Er Hsieh

Department of Medical Technology, Chungtai Institute of Health Sciences and Technology, Taichung, Taiwan, ROC

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In order to describe the susceptibility patterns and determine the identities of *Enterococcus* spp. isolated at a regional teaching hospital in Taichung during the period from June through November 1998, 96 clinical isolates of enterococci were collected for further analysis. Major sources of these isolates included urine, wound, and pus. Species identification was performed using the API 20 Strep system and supplemental tests. The minimum inhibitory concentrations (MICs) of six antimicrobial agents were determined by E-test for each isolate. Disk diffusion tests were also performed and the results were compared with those reported by the clinical laboratory. Because gentamicin susceptibility tests showed inconsistent results in many isolates, MIC determinations by the micro-broth dilution method were also performed for these isolates. All isolates were tested for ß-lactamase production using the chromogenic method. The results showed that *Enterococcus faecalis* was the most frequently encountered species (86.5%), followed by *Enterococcus faecium* (7.3%), *Enterococcus avium* (5.2%) and *Enterococcus casseliflavus* (1.0%). The MIC_{90} of ampicillin, penicillin, vancomycin, teicoplanin, ciprofloxacin and gentamicin for total isolates were 1, 3, 2, 0.25, 1, and more than 1024 μg/mL, respectively. All isolates were susceptible to vancomycin and teicoplanin. The same rate of resistance (3.1%) was found to penicillin, ampicillin and ciprofloxacin in all isolates. There were 50 (52%) and 48 (50%) isolates with high level streptomycin and gentamicin resistance, respectively. The MIC_{90} of ampicillin and penicillin for *E. faecium* were significantly higher than those for other species (96 and >256 vs. ≤1 and ≤3 μg/mL, respectively); in fact, ampicillin or penicillin-resistance was only found in *E. faecium*. No organism was found to produce ß-lactamase. There were 29 isolates showing discrepant results between the study findings and clinical laboratory report for gentamicin susceptibility. Most isolates (27/29) reported as susceptible to gentamicin by the clinical laboratory showed a high level gentamicin resistance by MIC determinations. The inconsistent results may be due to discrepancy in interpretations for heterogeneous resistance, bias in selecting colonies for the disk diffusion test, or variation in the quality of Mueller-Hinton agar used. The results suggest that any pure isolates growing some colonies within the inhibition zone should be considered as gentamicin resistant even if the zone diameter is equal or greater than the susceptible breakpoint. In order to obtain accurate results, the gentamicin MIC should be determined by the dilution method for enterococcal isolates that yield intermediate inhibition zones or zones just above the susceptible limit.

Key words: Disk diffusion method, enterococci, minimum inhibitory concentration (MIC), species identification

Enterococci have emerged as important pathogens in a growing number of serious clinical infections, particularly in nosocomial infections [1,2]. Although these organisms do not possess strong virulent factors, they are intrinsically resistant to a large number of antimicrobial agents and have a remarkable ability to acquire new resistant characteristics [3]. The wide prevalence of acquired glycopeptide resistance in *Enterococcus faecalis* and *Enterococcus faecium* (VRE) [4], the presence of intrinsic glycopeptide resistance in *Enterococcus casseliflavus* and *Enterococcus gallinarum* [5], and the different intrinsic susceptibility profiles for *E. faecalis* and *E. faecium* [6] have resulted in microbiological laboratories having to identify enterococci to the species level and determine their *in vitro* susceptibility. These organisms have become increasingly resistant to a broad range of antimicrobial agents [7]. The increasing incidence of serious infections caused by multiple drug resistant enterococci has posed therapeutic dilemmas for clinicians,

Corresponding author: Dr. Sue-Er Hsieh, Department of Medical Technology, Chungtai Institute of Health Sciences and Technology, 11, Po-Tze Lane, Pei-Twen District, Taichung, Taiwan, ROC.

particularly when the strains cause outbreaks in intensive care units [8]. Although rapid and accurate detection of resistant strains is critical for effective control of enterococcal infections, previous reports have documented the failures of several automated susceptibility testing systems and the disk diffusion method in detecting some resistant strains of enterococci, such as ß-lactamase-producing and low-level vancomycin-resistant enterococci [9,10]. The purpose of this study was to determine the antimicrobial susceptibility patterns of enterococci isolated from clinical specimens of patients treated at Taichung Cheng Ching Hospital, and to compare the results with those reported by the clinical laboratory. All isolates were identified to the species level by biochemical tests.

Materials and Methods

Bacteria strains and species identification

From June to November 1998, 96 isolates of *Enterococcus* species were collected from the Cheng Ching Hospital in Taichung. All strains were identified to the species level using the API-20 Strep system (BioMerieux, S.A., Marcy I'Etoile, France). All procedures were performed according to the manufacturer's instructions and the results were interpreted using the APILAB software provided by the manufacturer. Supplemental tests were also included when the identification probability was lower than 85%. All strains were stored at -60 °C in tryptic soy broth containing 15% glycerol. Prior to testing, all strains were subcultured twice on sheep blood agar to ensure a pure culture. *E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and 29213 were used as control strains.

Antimicrobial agents

E-strips (AB Biodisk, Sweden) of ampicillin, penicillin, vancomycin, ciprofloxacin, teicoplanin and high level gentamicin were used for minimum inhibitory concentration (MIC) determinations. Antimicrobial disks (Becton Dickinson, Cockeysville, MD, USA) of ampicillin, penicillin, vancomycin, ciprofloxacin, gentamicin (120 µg) and streptomycin (300 µg) were used in the disk diffusion method. Gentamicin powder was obtained from Sigma Chemicals (St. Louis, MO, USA).

Antimicrobial susceptibility testing

Disk diffusion tests were performed and interpreted according to the guidelines provided by the National Committee of Clinical Laboratory Standards (NCCLS) [11,12]. The MICs were determined by the E-test according to the manufacturer's instructions. MIC determinations by the broth microdilution method were also performed for isolates with inconsistent results between the study and clinical microbiology laboratory findings.

ß-lactamase detection

ß-lactamase production was detected by the chromogenic cephalosporin (nitrocefin) disk method (BBL Microbiology System).

Results

The results of species identification and the number of isolates from various sources of enterococci are shown in Table 1. *Enterococcus* spp. were isolated most frequently from urine and wounds. Among the isolates

Table 1. Species identification results of enterococci and number of isolates from various sources (n = 96)

Species	No. of isolates from various sources			
	Urine	Wound	Pus	Others
E. faecalis	28	30	21	4
E. faecium	3	1	3	0
E. avium	0	1	3	1
E. casseliflavus	1	0	0	0

Table 2. Susceptibility of enterococcal isolates to six antimicrobial agents as determined by E-test (n = 96)

Antimicrobial agent	MIC (µg/mL)[a]				No. of resistant isolates (%)
	Range	MIC_{50}	MIC_{90}	Breakpoint of resistance	
Ampicillin	0.023-96	0.5	1.0	≥16	3 (3.1)
Penicillin	0.094 - >256	2.0	3.0	≥16	3 (3.1)
Vancomycin	0.38-3.0	1.5	2.0	≥32	0
Gentamicin	1.0 - >1024	512	>1024	>500[b]	48 (50)[c]
Teicoplanin	0.0125-1.0	0.125	0.25	≥32	0
Ciprofloxacin	0.125 - >32	0.5	1.0	≥4	3 (3.1)

[a]MIC = minimum inhibitory concentration

[b]The interpretive criterion is recommended by the NCCLS for screening of high level gentamicin resistant enterococci (HLGR).

[c]There were 47 among 48 HLGR isolates expressing a gentamicin MIC over 1024 µg/mL.

Table 3. The MICs and resistance rate of *Enterococcus* species to six antimicrobial agents

Organism	Antimicrobial agent					
	Ampicillin	Penicillin	Vancomycin	Gentamicin	Teicoplanin	Ciprofloxacin
E. faecalis						
MIC range	0.38-1.5	1.5-3.0	1.0-3.0	1.5->1024	0.0125-1.0	0.19->32
MIC_{50}	0.5	2.0	1.5	512	0.125	0.5
MIC_{90}	1.0	3.0	2.0	>1024	0.19	0.75
No. of resistant isolates (%)	0	0	0	42 (50.6)	0	2
E. faecium						
MIC range	0.5-96	3.0->256	0.5-2.0	4->1024	0.064-0.5	0.125-0.5
MIC_{50}	0.75	3.0	0.75	>1024	0.094	0.125
MIC_{90}	96	>256	2.0	>1024	0.5	0.5
No. of resistant isolates	3	3	0	5	0	0
E. avium						
MIC range	0.023-0.75	0.094-2.5	0.38-2.0	1->1024	0.125-0.19	0.38-0.5
MIC_{50}	0.064	0.75	0.38	1.5	0.125	0.38
MIC_{90}	0.75	2.5	2.0	>1024	0.19	0.5
No. of resistant isolates	0	0	0	1	0	0
E. casseliflavus						
MIC	0.19	0.38	3.0	6.0	0.5	1.5
No. of resistant isolates	0	0	0	0	0	1

tested, *E. faecalis* was found most frequently, followed by *E. faecium* and then *E. avium*.

The MICs of six antimicrobial agents as determined by E-test are shown in Table 2. Various species showed different susceptibilities (Table 3). *E. faecium* showed a higher incidence of resistance to all drugs except vancomycin and ciprofloxacin. Although only a minor proportion of *E. faecium* [seven isolates (7.3%)] was identified among the isolates tested, three of them demonstrated a high-level resistance to penicillin with MICs more than 256 μg/mL. By contrast, none of the other species had MICs more than 64 μg/mL for penicillin, a concentration not achievable in human serum.

The results of the disk diffusion test are shown in Table 4. A gentamicin susceptible result predicted susceptibility to all other aminoglycosides except streptomycin; therefore, high level streptomycin disks were also included. It was found that 50% of isolates were resistant to gentamicin, 52% resistant to streptomycin, and 33.3% resistant to both agents.

All strains were tested for ß-lactamase production, but none of them was a ß-lactamase-producer.

Table 4. Susceptibility of enterococcal isolates to six antimicrobial agents by the disk diffusion method (n=96)

Antimicrobial agent	No. of isolates (%)		
	Susceptible	Intermediate	Resistant
Ampicillin (10 μg)	93 (96.9)	0	3 (3.1)
Penicillin (10 μg)	93 (96.9)	0	3 (3.1)
Vancomycin (30 μg)	96 (100)	0	0
Gentamicin (120 μg)	48 (50.0)	0	48 (50.0)
Streptomycin (300 μg)	46 (48.0)	0	50 (52.0)
Ciprofloxacin (5 μg)	81 (84.4)	12 (12.5)	3 (3.1)

Discussion

Because of different susceptibility profiles, *Enterococcus* species identification can direct antimicrobial therapy in addition to allowing epidemiological surveillance. Although commercial kits or automatic systems are commonly used for species identification of enterococci, they have been reported to be unreliable for the identification of less commonly encountered species, particularly *E. gallinarum* and *E. casseliflavus* [13]. The difficulty may arise in the differentiation of these species from the *E. faecium* group. Motility test and colony pigmentation test (detected by swabbing sheep blood agar culture following a 48-h incubation) have been reported to be useful for their differentiation [14]. Therefore, these tests were also performed in this study for isolates identified as *E. faecium* by the API 20 Strep system.

The results of species identities in this study are similar to previous reports showing that *E. faecalis* accounts for the majority of enterococcal infections, followed by *E. faecium* and then other species [15].

Infections caused by enterococci with antimicrobial resistance are being reported with increasing frequency throughout the world. Rapid and correct detection of antimicrobial resistance is necessary for control of nosocomial infections. In the present study, comparing the results of MIC determinations with those of the disk diffusion method reported by the clinical laboratory revealed that 29 isolates had discrepant results in gentamicin susceptibility. Two of these isolates, reported as resistant by the clinical laboratory, showed susceptibility to gentamicin in repeated disk diffusion test and MIC determinations. Among the other 27 isolates reported as susceptible by the clinical laboratory, nine yielded no inhibition zone in repeated disk diffusion test and showed MICs more than 1024 µg/mL by the E-test. The other 18 isolates yielded a zone of 7 to 12 mm around the gentamicin disk and an elliptic zone intersecting at 192 to 384 µg/mL on the gentamicin E-test strip, but all grew some colonies within the inhibition zone. This phenomenon is similar to the expression of heterogeneous resistance in methicillin-resistant *S. aureus*. In the micro-broth dilution method, all of these isolates showed gentamicin-MICs greater than 1024 µg/mL with one exception of 512 µg/mL. Therefore, they were considered as resistant strains in the present report. The discrepancy may have been due to bias in selecting colonies for disk diffusion test, because the most common source of enterococcal infections is intestinal flora and therefore mixed clonal infection in the same patient is not impossible. Another possible cause may have been the different expression of resistance genes that are found to be heterogeneous in clinical isolates of enterococcus with high-level gentamicin resistance [16]. In addition, the quality of Mueller-Hinton agar used could possibly have played a role since gentamicin susceptibility is easily affected by pH alteration. To elucidate the heterogeneous expression of gentamicin resistance, further evaluation by DNA-based analysis is necessary.

Enterococcus resistance to penicillin or ampicillin results mainly from the production of a low affinity penicillin-binding protein (PBP), and rarely from ß-lactamase-production [17,18]. The detection of resistance due to ß-lactamase-production cannot be performed by routine disk diffusion test [12]. It has been recommended that ß-lactamase test should be performed for enterococci isolated from blood or cerebral spinal fluid [12]. Results in the present study, similar to previous reports from Taiwan [19-23], suggest that ß-lactamase-producing enterococci are still not prevalent in Taiwan. In this study, all isolates of *E. faecalis* were susceptible to ampicillin and penicillin, however, 42.9% of *E. faecium* isolates showed high-level resistance to both agents and therefore they would also be resistant to synergy with an aminoglycoside. Indeed, all isolates of penicillin/ampicillin-resistant *E. faecium* were also resistant to high-level gentamicin.

Enterococcus resistant to glycopeptide (GRE or VRE) has become an important pathogen worldwide. In Taiwan, VRE have been found to cause severe or deadly infections [20,22]. According to a recent report, VRE are present throughout Taiwan, with a rate of 6% in Middle Taiwan [24]. In the present study, no VRE isolates were found. However, among 120 enterococcal isolates obtained from another teaching hospital during the period from April through August 1999, six strains of VRE were found (data not shown). The variation in prevalence of VRE isolates may be due to bias in collecting isolates and differences in hospital setting. According to this study and previous reports from Taiwan [19,22,24], VRE-associated infections have only been found sporadically in the Taichung area.

With respect to ciprofloxacin susceptibility, there were 12 isolates (12.5%) in this study which yielded intermediate results in the disk diffusion method, but only two of these strains showed intermediate MICs. These intermediate results were considered as indicating susceptibility because ciprofloxacin is one of the quinolones that would be physiologically concentrated in the urine and is therefore used only for urinary tract infection in cases of enterococcal infection [12]. These results suggest that the disk diffusion test for ciprofloxacin to enterococci should be interpreted carefully, and MIC-determinations may be necessary for strains with intermediate results in the disk diffusion method.

In conclusion, the results of this study, similar to previous reports in Taiwan [21-25], indicate that most isolates of enterococci were highly susceptible to penicillin, ampicillin, vancomycin and teicoplanin. To date, vancomycin-resistant enterococci have only been encountered sporadically and ß-lactamase-producing enterococci are not a problem in Taiwan. The incidence of infections caused by high-level aminoglycoside-resistant strains are continuously increasing. The disk diffusion method for gentamicin susceptibility must be performed and interpreted carefully according to the NCCLS guidelines to ensure reliable results. Any pure isolates growing some colonies within the inhibition

zone should be considered as gentamicin resistant although the zone diameter is within the susceptible range. In order to obtain accurate results, the gentamicin MIC should be determined by the dilution method for enterococcal isolates that yield intermediate inhibition zones or zones just above the susceptible limit.

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