Occurrence of Aminoglycoside-Modifying-Enzyme Genes \textit{aac(6')}–\textit{aph(2'')}, \textit{aph(3')}, \textit{ant(4')} and \textit{ant(6)} in Clinical Isolates of \textit{Enterococcus faecalis} Resistant to High-Level of Gentamicin and Amikacin

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\textbf{ABSTRACT.} The genes coding for 4 aminoglycoside-modifying enzymes \textit{AAC(6')}–\textit{APH(2'')}, \textit{APH(3')} and \textit{ANT(4')} and \textit{ANT(6)} were determined in 44 Slovak clinical isolates of \textit{Enterococcus faecalis} with high-level resistance to gentamicin (HLGR, collection 1) and 48 \textit{E. faecalis} isolates with resistance to amikacin (AR, collection 2). The occurrence of spotted genes was (collection 1 vs. collection 2): \textit{aac(6')}–\textit{aph(2'')} 81.8 vs. 8.3 %, \textit{ant(4')} 52.3 vs. 81.3 %, \textit{aph(3')} 50 vs. 56.3 % and \textit{ant(6)} 6.8 vs. 4.2 %, the most frequent combinations of genes in the HLGR collection were \textit{aac(6')}–\textit{aph(2'')} + \textit{ant(4')} and \textit{aac(6')}–\textit{aph(2'')} + \textit{aph(3')}.

In contrast, the \textit{aph(3')} + \textit{ant(4')} gene profile was predominant in AR isolates. None of the isolates contained all four AGME genes simultaneously.

\begin{center}
\textbf{Abbreviations}
\begin{tabular}{ll}
AR & resistance to amikacin \hfill \\
HLR & high-level resistance \hfill \\
MIC & minimum inhibitory concentration \hfill \\
AGME & aminoglycoside-modifying enzyme \hfill \\
AAC(6')–APH(2'') & 6'-acetyltransferase–2''-phosphotransferase (bifunctional enzyme) \hfill \\
ANT(6) & '6-nucleotidyldtransferase' \hfill \\
ANT(4') & '4'-nucleotidyltransferase' \hfill \\
APH(3') & '3'-phosphotransferase' \hfill \\
Ami & amikacin \hfill \\
Amp & ampicillin \hfill \\
Chl & chloramphenicol \hfill \\
Cip & ciprofloxacin \hfill \\
Ery & erythromycin \hfill \\
Gen & gentamicin \hfill \\
Kan & kanamycin \hfill \\
Net & netilmicin \hfill \\
Nit & nitrofurantoin \hfill \\
Qui/Dal & quinupristin/dalfopristin \hfill \\
Rif & rifampicin \hfill \\
Str & streptomycin \hfill \\
Tei & teicoplanin \hfill \\
Tob & tobramycin \hfill \\
Van & vancomycin \hfill \\
\end{tabular}
\end{center}

Enterococci, as facultative pathogens, have been associated with infections of urinary tract, postsurgical wounds and endocarditis (Udo et al. 2003; Karlowsky et al. 2004; de Fatima Silva Lopes et al. 2005). Two species, \textit{E. faecalis} and \textit{E. faecium}, were predominantly isolated from clinical material (Slobodníková et al. 2001; Maschietto et al. 2004; Zarrilli et al. 2005).

Enterococci have a remarkable ability to adapt to an antibiotic exposure, mainly due to their intrinsic characteristics. They are naturally resistant to low-level concentrations of \beta-lactams and aminoglycosides. In addition to this, they also demonstrate acquired HLR to antibiotics (Chow 2000; Kobayashi et al. 2003; Bujdáková et al. 2003).

Although the increasing number of aminoglycoside-resistant enterococci in hospitals has been already reported (Maschietto et al. 2004; Rodriguez-Bano et al. 2005), aminoglycosides have still belonged among the main effective agents in therapy of enterococcal infections used in combination with \beta-lactams and glycopeptides because of their synergistic effect (Murray 1990; del Campo et al. 2000). HLR to aminoglycosides is mostly caused by the production of many aminoglycoside-modifying enzymes with the prevalence of the bifunctional enzyme AAC(6')–APH(2'') (6'-acetyltransferase–2''-phosphotransferase) (Udo et al. 2004; Zar-
rilli et al. 2005), modifying all clinically available aminoglycosides, such as Gen, Ami, Net, Tob and Kan with the exception of Str, which is modified by ANT(6) (6-nucleotidyltransferase) (Chow 2000; Kobayashi et al. 2001). Other enzymes, APH(3′) (3′-phosphotransferase) and ANT(4′) (4′-nucleotidyltransferase), have been observed less frequently (del Campo et al. 2000; Kobayashi et al. 2001; Udo et al. 2004; Zarrilli et al. 2005). These AGMEs inactivate Ami and Kan but they are different in their ability to modify Tob (Chow 2000).

This study compares the incidence of 4 important AGME genes, aac(6′)-aph(2″), aph(3′), ant(4′), and ant(6) in HLGR E. faecalis strains with amikacin-resistant clinical isolates of E. faecalis.

MATERIALS AND METHODS

Clinical isolates of E. faecalis were collected from clinical samples (urine, stool, vaginal tampons, and wound swabs) in the Slovak medical centers in 2003–2004. Two different collections were created on the basis of resistance to high level of Gen (44 isolates) and of Ami (48 isolates). Isolates were identified by commercial biochemical test (ENCOCCUStest; Pliva-Lachema, Czechia) and polymerase chain reaction (PCR) with primers for the ddl gene (D-Ala-D-Ala-ligase; Dutka-Malen et al. 1995). E. faecium CCM 2123 and E. faecalis CCM 1875 (Czech Collection of Microorganisms, Brno, Czechia) were used as standard strains.

Antibiotic susceptibility to Amp, Str, Gen, Ery, Rif, Chl, Qui/Dal, Nit, Van (all from Pliva-Lachema, Czechia), and Tei and Cip (Oxoid, UK) was determined by the disk-diffusion test (NCCLS 2003a). Values of MICs for Str (Applichem, Germany), Gen (Pliva-Lachema, Czechia), and Ami (Schering-Plough, USA) were determined by the agar-dilution method according to the NCCLS (2003b).

Distribution of aac(6′)-aph(2″), aph(3′), ant(4′) and ant(6) genes was monitored by PCR assay according to Jos et al. (1993) and Swenson et al. (1995).

RESULTS AND DISCUSSION

Dissemination of HLGR enterococci in a hospital environment can be an important factor leading to the challenge of nosocomial infections. Hence, epidemiological survey of aminoglycoside resistance and the assessment of AGMEs distribution are significant for the control of the spread of resistant enterococci (Wagenlehner et al. 2002; Udo et al. 2004; Rodriguez-Bano et al. 2005).

We collected enterococcal isolates according to HLGR and AR; as the correct identification of enterococcal isolates has been problematic, the commercial biochemical set (ENCOCCUStest) was complemented with ddl-primer-specific PCR assay. For experimental work only isolates identified as E. faecalis were selected.

The spectrum of resistance was detected by the disk-diffusion method (Table I). The majority of clinical isolates expressed a phenomenon of multi-drug resistance which is characteristic for aminoglycoside-resistant enterococci (Critchley et al. 2003; Maschietto et al. 2004). Clinical isolates from the HLGR collection were highly resistant to Gen, Qui/Dal, Ery and Cip. The percentage of resistance detected in the AR collection was lower in comparison with HLGR enterococci. All isolates from the two collections were susceptible to Amp, Van, Tei, and Nit with the exception of one E. faecalis from the AR collection resistant to Nit. In contrast to other countries reporting the presence of resistance to Amp, Nit and glycopeptidase E. faecalis isolates (Barisić and Punda-Polić 2000; Wagenlehner et al. 2002; Critchley et al. 2003; Karlowsky et al. 2004) those from our hospitals remained susceptible. However, the worldwide trend of incidence of Ery and Cip resistance has also been observed (Turnidge et al. 2002; Karlowsky et al. 2004; de Fatima Silva Lopes et al. 2005).

All clinical isolates in the HLGR collection were resistant to Gen (MIC > 512 μg/mL), 3 were resistant to Str (MIC > 2000), and 44 isolates were shown to be resistant to Ami (21 isolates with MIC > 256, 14 with MIC > 128, 9 MIC < 64). In the group of AR isolates,

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>HLGR</th>
<th>AR</th>
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<tbody>
<tr>
<td>Amp</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Str</td>
<td>6.8</td>
<td>14.6</td>
</tr>
<tr>
<td>Gen</td>
<td>100</td>
<td>8.0</td>
</tr>
<tr>
<td>Ery</td>
<td>68.2</td>
<td>26.0</td>
</tr>
<tr>
<td>Chl</td>
<td>18.1</td>
<td>22.0</td>
</tr>
<tr>
<td>Rif</td>
<td>11.4</td>
<td>18.0</td>
</tr>
<tr>
<td>Qui/Dal</td>
<td>97.7</td>
<td>64.0</td>
</tr>
<tr>
<td>Tei</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Van</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cip</td>
<td>54.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Nit</td>
<td>0</td>
<td>2.0</td>
</tr>
</tbody>
</table>