DETERMINATION OF PRESENCE OF RESISTANCE TO ERYTHROMYCIN AND TETRACYCLINE ANTIBIOTICS IN ISOLATED STREPTOCOCCUS SPECIES FROM GROUP C AND GROUP G

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The purpose of this study was to determine the presence of resistance to macrolide and tetracycline in β-haemolytic streptococci which belong to group C (GCS) and group G (GGS), isolated from various clinical specimens collected at the Institute of Public Health of Serbia during the period 2006-2008. After determination of resistance in isolated streptococci to tested antibiotics, their phenotypic and genotypic characteristics were investigated. Resistance to erythromycin and tetracycline were evaluated in a total of 112 GGS and 29 GCS isolates. Resistance to erythromycin was determined in 6 (6.9%) GGS isolates and 4 of them were also resistant to tetracycline. Resistance to erythromycin was determined in 2 (5.4%) GCS isolates, but both isolates were sensitive to tetracycline. The erythromycin-resistance phenotypes were determined by the double-disk test with erythromycin and clindamycin disks. All 8 isolates showed the MLSB macrolide resistance phenotype leading to macrolide, lincosamide and streptogramin B resistance. These 8 isolates were genotyped for the presence of the erm(TR), erm(B), mef(A) and tet(M) genes and transposon of the Tn916-Tn1545 family by polymerase chain reaction. The presence of erm(TR) gene was detected in 3 GGS isolates and in both GCS isolates, while the presence of erm(B) gene was detected in other 3 GGS isolates. The presence of tet(M) gene with transposon of the Tn916-Tn1545 family was detected in all 4 tetracycline-resistant GGS isolates. The results of this study indicate that continued monitoring of macrolide- and tetracycline- resistance in tested groups of streptococci in Belgrade and in Republic of Serbia is necessary.

Key words: groups C and G, macrolide resistance, Streptococcus, tetracycline resistance
INTRODUCTION

There is an increasing interest in the role of group C streptococci (GCS) and group G streptococci (GGS) in the development of nosocomial and opportunistic infections in humans, as well as in animals, which are more frequent nowadays. The spectrum of human infections caused by these organisms includes primary and secondary bacteremia in healthy and immunocompromised hosts, as well as inflammations manifested as cellulitis, endocarditis, pericarditis, skin and wound infections, meningitis, arthritis, osteomyelitis, pneumonia, abscesses, puerperal infections and pharyngitis (Zaoutis et al., 2001; Luyx et al., 2001; Kim et al., 2002; Tong et al., 2003; Malini et al., 2004). Group C streptococci is frequently isolated from clinical cases of equine and canine pneumonia and pleuropneumonia (Harrington et al., 2002; Chalker et al., 2003; Taylor et al., 2006; Newton et al., 2008; Barquero et al., 2009). Outbreaks of a fatal haemorrhagic pneumonia have recently been reported in dogs associated with GCS (Kim et al., 2007; Pesavento et al., 2008).

In the absence of a β-lacatam allergy, the treatment of choice for infections caused by beta-haemolytic streptococci is penicillin, while the first-line alternative treatments are macrolides or lincosamides when indicated. During the past 2 decades an increased incidence of macrolide resistance among beta-haemolytic streptococci isolated from both humans and animals has been reported in several countries (Martel et al., 2003; Ergin et al., 2003; Luthje et al., 2006; Grivea et al., 2006; Littauer et al., 2006; Ashley Robinson et al., 2006; Uh et al., 2007; Perez-Trallero et al., 2007; Hung et al., 2008), while the resistance to other antibiotics, like tetracyclines, occurs in some strains (Ayer et al., 2007; Brenciani et al., 2007). Moreover, a concomitant increase in resistance to both these antibiotics has been observed among pathogenic and commensal streptococci, mostly because their major resistance determinants are carried on the same mobile element (Agerso et al., 2006).

There are two main acquired macrolide resistance mechanisms in streptococci: posttranscriptional target site modification and macrolide efflux (Perez-Trallero et al., 2007). Target site modification is due to the presence of an rRNA methylase that modifies an adenine residue in 23S rRNA. Methylation results in a conformational change in the ribosome leading to reduced binding of macrolide, lincosamide and streptogramin B (MLSb) antibiotics to their target site in the 50S ribosomal subunit. MLSb resistance can be expressed constitutively or inducibly, and is mediated by the erm(B) and erm(TR) genes. Active efflux results in M resistance phenotype (erythromycin resistance and clindamycin susceptibility). The efflux mechanism selectively pumps 14- and 15-membered macrolides out of the cell, but not 16-membered macrolides or lincosamides. This kind of resistance is mediated by the mef(A) gene.

The genus *Streptococcus* can carry tet(K), tet(L), tet(M), tet(O), tet(Q) and tet(T) genes (Chopra et al., 2001). Mobile genetic elements carrying tet(M) sometimes harbor genes encoding macrolide resistance (Brenciani et al., 2007). Selection for resistance to one antibiotic can influence the evolution of resistance to another drug in multiply resistant bacteria (Nielsen et al., 2004).
The purpose of this study was to determine the prevalence, as well as the phenotypic and genotypic characterization of macrolide- and tetracycline-resistance in Group C (GCS) and Group G (GGS) β-haemolytic streptococci isolated from various clinical specimens (pharynx, wound, cervix, skin, tracheostoma) collected at Institute of Public Health of Serbia "Dr Milan Jovanovic Batut" during the period from 2006 to 2008. A total of 112 GGS and 29 GCS nonduplicate isolates were evaluated for resistance to erythromycin and tetracycline. Resistance to erythromycin was determined in 6 (6.9%) GGS isolates and 4 of them were also resistant to tetracycline. Resistance to erythromycin was determined in 2 (5.4%) GCS isolates, but both isolates were sensitive to tetracycline. The erythromycin-resistance phenotypes were determined by the double-disk test with erythromycin and clindamycin disks. These 8 isolates were genotyped for the presence of the \( \text{erm}(\text{TR}) \), \( \text{erm}(\text{B}) \), \( \text{mef}(\text{A}) \) and \( \text{tet}(\text{M}) \) genes and transposon of the \( \text{Tn}916-\text{Tn}1545 \) family by polymerase chain reaction (PCR).

Six GGS isolates (6.9%) showed resistance to erythromycin and 4 of them were also resistant to tetracycline. Two GCS isolates (5.4%) showed resistance to erythromycin, but they were sensitive to tetracycline. The erythromycin-resistance phenotypes were determined by the double-disk test. These 8 isolates were genotyped for the presence of the \( \text{erm}(\text{TR}) \), \( \text{erm}(\text{B}) \), \( \text{mef}(\text{A}) \) and \( \text{tet}(\text{M}) \) genes and transposon of the \( \text{Tn}916-\text{Tn}1545 \) family by polymerase chain reaction (PCR).

MATERIAL AND METHODS

**Bacterial isolates**

Group C (GCS) and Group G (GGS) β-haemolytic streptococci isolates were identified using standard procedures: colony morphology, catalase test, beta-haemolysis on blood agar, bacitracin test and latex agglutination test (Slidex StreptoKit A, B, C, D, F, G, bioMerieux). The isolates were stored at -20°C in trypticase-soya broth with 10% glycerol until testing.

**Susceptibility testing**

Susceptibility testing was performed for penicillin, cefotaxime, vancomycin, erythromycin, clindamycin, chloramphenicol and tetracycline (antibiotic disks Biorad, France) using a disk diffusion method on Mueller-Hinton agar supplemented with 5% sheep blood according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008). The plates were incubated for 24 h at 37°C in the presence of 5% CO₂.

The erythromycin-resistance phenotypes were determined by the double-disk test with erythromycin (15 μg) and clindamycin (2 μg) disks, separated by 12 mm. After 24 h incubation at 37°C, blunting of the clindamycin inhibition zone near to the erythromycin disk indicates an inducible type of MLS\(_B\) resistance and resistance to both erythromycin and clindamycin indicated a constitutive type of MLS\(_B\) resistance.
Detection of erythromycin and tetracycline resistance genes and 
Tn916-Tn1545 by PCR

The MLS resistance mechanism was determined by PCR using \textit{erm} primers, 
while the efflux-pump mechanism was tested by PCR using primers and specific 
conditions for amplification of \textit{mef}(A) gene. The following primers were used: 5'- 
CGA GTG AAA AAG TAC TCA ACC-3' and 5'-GGC GTG TTT CAT TGC TTG ATG-3' 
to detect \textit{erm}(B); 5'-GCA TGA CAT AAA CCT TCA-3' and 5'-AGG TTA TAA TGA 
AAC AGA-3' to detect \textit{erm}(TR); and 5'-AGT ATC ATT AAT CAC TAG TGC-3' and 5'- 
TTC TTC TGG TAC TAA AAG TGG-3' to detect \textit{mef}(A).

The presence of \textit{tet}(M) gene and transposon of the \textit{Tn916-Tn1545} family 
was tested by PCR.

RESULTS AND DISCUSSION

All 112 GGS and 29 GCS tested isolates were susceptible to penicillin, 
cefotaxime, vancomycin and chloramphenicol. Resistance to erythromycin was 
determined in 6 (6.9\%) GGS isolates and 4 of them were also resistant to 
tetracycline. Resistance to erythromycin was determined in 2 (5.4\%) GCS 
isolates, but both isolates were sensitive to tetracycline.

According to the double disc test, all 8 tested isolates showed the MLS\textsubscript{B} 
macrolide resistance phenotype leading to macrolide, lincosamide and 
streptogramin B resistance. Five GGS studied isolates showed the constitutive 
MLS\textsubscript{B} (cMLS\textsubscript{B}) resistance phenotype, while one GGS and both GCS studied 
isolates showed the inducible MLS\textsubscript{B} (iMLS\textsubscript{B}) resistance phenotype. The presence 
of \textit{erm}(TR) gene was detected in 3 GGS isolates (one with the iMLS\textsubscript{B} phenotype 
and two with the cMLS\textsubscript{B} phenotype) and in both GCS isolates, while the presence 
of \textit{erm}(B) gene was detected in other 3 GGS isolates. The presence of \textit{tet}(M)gene 
(Figure 1) and transposon of the \textit{Tn916-Tn1545} family (Figure 2) was detected in 
all 4 tetracycline-resistant GGS isolates.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Analysis by agarose gel electrophoresis of the PCR product: the 1862 bp DNA 
corresponds to the \textit{tet}(M) gene. L - gene ruler-Fermentas}
\end{figure}
Table 1. Distribution of macrolide resistance phenotypes, macrolide resistance genes and tetracycline resistance determinates among 2 GCS and 6 GGS isolates collected during the period from 2006-2008.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Macrolide resistance phenotype</th>
<th>Macrolide resistance gene</th>
<th>Tetracycline resistance determinates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \text{erm}(B) )</td>
<td>( \text{erm}(\text{TR}) )</td>
</tr>
<tr>
<td>GGS 1</td>
<td>cMLS(_B)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GGS 2</td>
<td>iMLS(_B)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GGS 3</td>
<td>cMLS(_B)</td>
<td>+</td>
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<td>GGS 4</td>
<td>cMLS(_B)</td>
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<tr>
<td>GGS 5</td>
<td>cMLS(_B)</td>
<td>+</td>
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</tr>
<tr>
<td>GGS 6</td>
<td>cMLS(_B)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GCS 1</td>
<td>iMLS(_B)</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>GCS 2</td>
<td>iMLS(_B)</td>
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</table>

The incidence of erythromycin resistance is highly variable so local statistics are of crucial value for guiding therapy. Surveillance of both the prevalence of macrolide resistance and the various resistance mechanisms is justified by the rapid emergence of erythromycin resistance amongst streptococci. Tetracycline resistance has been considered to be an important cofactor in the selection of erythromycin resistance (Nielsen et al., 2004). Tetracycline is widely used as a treatment for a variety of human and veterinary infections, as well as animal
growth promoters (Chopra et al., 2001). In Serbia, as well as in other countries, a number of different antibiotics and chemotherapeutics are used in the prevention and therapy of infections in farm animals (Krnjać et al., 2005). There is a practice of adding low concentrations of antibiotics to animals to improve growth and feed efficiency. An obvious outcome of this practice is that animals need less food to reach marketable weight. The mechanisms responsible for growth promotion have not been fully elucidated, but appear to include enhancement of vitamin production by gastrointestinal microorganisms, elimination of subclinical populations of pathogenic microorganisms and increased intestinal absorption of nutrients. Increasing concern about contribution of growth promoters to the development of resistant human isolates led to a ban of the use of tetracyclines for growth promotion in European Union and many other countries.

The spread of tetracycline resistance in streptococci may be particularly relevant from an ecological point of view, both in human and in animal populations. It is conceivable, however, that resistance genes have been horizontally transferred to pathogenic streptococci from other species more directly exposed to tetracycline selective pressure, e.g., viridans group streptococci and other human and animal comensal organisms (Malhotra-Kumar et al., 2004; Ioannidou et al., 2003).

The bacteria from genus Streptococcus can carry tet(K), tet(L), tet(M), tet(O), tet(Q) and tet(T) genes (Chopra et al., 2001). Efflux tet(K) and tet(L) genes code for membrane-associated proteins which export tetracycline from the cell. Export of tetracycline reduces the intracellular drug concentration and thus protect the ribosomes within the cell. Ribosome protection tet(M) and tet(O) genes code for ribosomal protection proteins which protect the ribosomes from the action of tetracycline and confer resistance to doxycycline and minocycline. They confer a wider spectrum of resistance to tetracyclines than is seen with bacteria that carry tetracycline efflux proteins. The tet(M) gene is often associated with a conjugative element of the Tn916-Tn1545 family. This group of elements form nonreplicating circular intermediates, which are essential for both intracellular transposition and intercellular conjugative transfer. Conjugative transposons of the Tn916-Tn1545 family are found in different species of the genus Streptococcus (Perez-Trallero et al., 2007). These elements can carry several determinants of resistance and contribute to horizontal dissemination of multidrug resistance.

CONCLUSION

This study showed low rates of erythromycin and tetracycline resistance in streptococci strains which belong to group C and group G isolated from various human specimens. Since changes of resistance rates and their prevailing mechanisms can occur rapidly, continual monitoring of antimicrobial resistance among human streptococci from group C and G, as well as animal streptococci from group C, in Republic of Serbia is needed. This monitoring could provide current data regarding the resistance mechanisms that are most common in tested bacteria at the local and regional level. This important issue needs to be
addressed to relevant scientific and expert institutions financed by the government in which are teams of medical and veterinarian clinicians, microbiologists and epidemiologists.

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Utvrdivanje prisustva rezistencije na makrolidne i tetraciklinske antibiotike kod izolovanih vrsta streptokoka iz grupe C i G

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SADRŽAJ

Cilj ovog ispitivanja je bio da se utvrdi prisustvo rezistencije na makrolide i tetracikline kod β-hemolitičkih streptokoka koje pripadaju grupi C (GCS) i grupi G (GGS), a izolovane su iz različitih kliničkih uzoraka prikupljenih u periodu od 2006-2008. godine. Nakon utvrđivanja prisustva rezistencije kod izolovanih vrsta streptokoka na navedene antibiotike ispitivane su njihove fenotipske i genotipske karakteristike. Rezistencija na eritromicin i tetraciklin ispitana je kod ukupno 112 izolata streptokoka iz grupe G i 29 izolata streptokoka iz grupe C. Rezistencija na eritromicin je ustanovljena kod 6 (6,9%) izolata streptokoka iz grupe G, od kojih su 4 izolata bila rezistentna i na tetraciklin. Rezistencija na eritromicin je ustanovljena kod 2 (5,4%) izolata streptokoka grupe C, uz napomenu da su oba izolata bila osetljiva na tetraciklin. Fenotipovi rezistencije na eritromicin su determinisani primenom duplog disk testa pomoću diskova eritromicina i klindamicina. Kod svih 8 izolata je ustanovljen MLSB fenotip rezistencije koji ukazuje na rezistenciju na makrolide, linkozamide i streptogramin B. Primenom reakcije lančane polimerizacije kod 8 izolata je izvršena genotypizacija na prisustvo erm(TR), erm(B), mef(A) i tet(M) gena i transpozona koji pripada familiji Tn916-Tn1545. Prisustvo erm(TR) gena je detektovano kod 3 izolata streptokoka iz grupe G i dva izolata iz grupe C, dok je prisustvo erm(B)gena detektovano kod druge 3 izolata streptokoka iz grupe G. Prisustvo tet(M) gena sa transpozonom koji pripada familiji Tn916-Tn1545 je detektovano kod sva 4 tetraciklin rezistentna izolata streptokoka iz grupe G. Rezultati ovih ispitivanja ukazuju na neophodnost kontinuiranog monitoringa rezistencije na makrolide i tetracikline streptokoka koje su izolovane kako od ljudi, tako i od životinja u Republici Srbiji.