The prevalence of genotypes that determine resistance to macrolides, lincosamides, and streptogramins B compared with spiramycin susceptibility among erythromycin-resistant *Staphylococcus epidermidis*

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Coagulase-negative staphylococci, particularly *Staphylococcus epidermidis*, can be regarded as potential reservoirs of resistance genes for pathogenic strains, e.g., *Staphylococcus aureus*. The aim of this study was to assess the prevalence of different resistance phenotypes to macrolide, lincosamide, and streptogramins B (MLS\(_B\)) antibiotics among erythromycin-resistant *S. epidermidis*, together with the evaluation of genes promoting the following different types of MLS\(_B\) resistance: erm\(_A\), erm\(_B\), erm\(_C\), msr\(_A\), mph\(_C\), and lin\(_A/A'\). Susceptibility to spiramycin was also examined. Among 75 erythromycin-resistant *S. epidermidis* isolates, the most frequent phenotypes were macrolides and streptogramins B (MS\(_B\)) and constitutive MLS\(_B\) (cMLS\(_B\)). Moreover, all strains with the cMLS\(_B\) phenotype and the majority of inducible MLS\(_B\) (iMLS\(_B\)) isolates were resistant to spiramycin, whereas strains with the MS\(_B\) phenotype were sensitive to this antibiotic. The D-shape zone of inhibition around the clindamycin disc near the spiramycin disc was found for some spiramycin-resistant strains with the iMLS\(_B\) phenotype, suggesting an induction of resistance to clindamycin by this 16-membered macrolide. The most frequently isolated gene was erm\(_C\), irrespective of the MLS\(_B\) resistance phenotype, whereas the most often noted gene combination was erm\(_C\), mph\(_C\), lin\(_A/A'\). The results obtained showed that the genes responsible for different mechanisms of MLS\(_B\) resistance in *S. epidermidis* generally coexist, often without the phenotypic expression of each of them.

Key words: *Staphylococcus epidermidis* - MLS\(_B\) antibiotics - resistance - genotypes - spiramycin

Coagulase-negative staphylococci (CoNS), particularly *Staphylococcus epidermidis*, belong to the microbiota of human skin and the mucosal membrane of the upper respiratory tract, and they express low pathogenic potential as commensals in healthy people (Young & Otto 2002, Otto 2009). However, they can be responsible for several serious infections in immunocompromised patients, particularly those associated with biomaterials (e.g., catheters, prosthetics etc.), leading to bacteremia and sepsis (Ziebuhr et al. 2006, Caesy et al. 2007, Schoenfelder et al. 2010, Castro-Alarcón et al. 2011). On the other hand, as a natural part of the microflora, drug resistant strains may be selected during antibiotic therapy, which is a potential source of the resistance genes for pathogenic strains, e.g., *Staphylococcus aureus* (Reyes et al. 2007, Otto 2013, Vitali et al. 2014).

Resistance to macrolide, lincosamide, and streptogramins B (MLS\(_B\) antibiotics) in staphylococci is associated with the following three mechanisms: (i) target modification, (ii) efflux pumps, and (iii) enzymatic modification of antibiotics. The first macrolide-resistant staphylococcal strains were identified in the 1950s (Roberts 2004). Currently, a large number of strains exhibit resistance to these antibiotics via different mechanisms. It is known that macrolide-resistant strains often exhibit co-resistance to other MLS\(_B\) antibiotics. The most common mechanism is the modification of ribosomes as a result of methylation of adenine within 23S rRNA ribosomal subunits by a methylase encoded by the erm genes (predominantly erm\(_C\)). Conformational changes in the ribosome result in the reduced binding of all MLS\(_B\) antibiotics; these strains are resistant to all MLS\(_B\) antibiotics (the combination of quinupristin/dalfopristin loses bactericidal activity as the result of the development of resistance to quinupristin). The phenotypic expression of MLS\(_B\) resistance can be either inducible (iMLS\(_B\)) (generally induced by 14 and 15-membered macrolides) or constitutive (cMLS\(_B\)) (Weisblum 1995). The active efflux of antibiotics is mediated by msr genes (mainly msr\(_A\)) and is responsible for resistance only to 14 and 15-membered macrolides and streptogramins B (MS\(_B\)) phenotype (Reynolds et al. 2003). The third mechanism of resistance is based on the production of antibiotic-inactivating enzymes (e.g., phosphorylase encoded by mph or lin, the gene responsible for inactivation of lincosamides) (Chesneau et al. 2007, Achar et al. 2008).

The aim of this study was to assess the prevalence of different MLS\(_B\) resistance phenotypes among *S. epidermidis*, together with the evaluation of genes responsible for target modification (erm\(_A\), erm\(_B\), erm\(_C\)), antibiotic efflux (msr\(_A\)) or antibiotic inactivation (mph\(_C\), lin\(_A/A'\)). The evaluation of susceptibility to the 16-membered macrolide spiramycin was also performed.

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SUBJECTS, MATERIALS AND METHODS

Bacterial strains - A total of 197 strains of *S. epidermidis* were obtained from the mucosal membranes of the upper respiratory tracts of patients with nonsmall cell lung cancer who underwent hospitalisation. Nasal and pharyngeal swabs were obtained on the second day of the patients’ stays at the hospital. Among the strains, resistance to erythromycin was detected in 75 isolates.

Isolation and identification - Isolation and identification of bacterial strains were performed using routine microbiological tests. The following tests were used in the identification of CoNS: the coagulase test tube using rabbit plasma (Biomed, Poland) and API Staph strips (bioMérieux, France).

Identification of resistance to MLSb antibiotics - Susceptibility to MLSb antibiotics, including the detection of resistance mechanisms, was based on the D-test according to European Centre for Disease Prevention and Control (EUCAST) recommendations. In addition, disks containing lincomycin (15 mg) were used to identify the L-phenotype. Moreover, for detection of the effects of spiramycin on clindamycin susceptibility, discs containing spiramycin (100 mg) were applied next to clindamycin (2 mg).

Determination of minimal inhibitory concentrations (MICs) to spiramycin - Detection of MICs to spiramycin was based on EUCAST recommendations using the double broth dilution method. In the absence of breakpoints for spiramycin in EUCAST, only the MICs were evaluated without grouping the strains as susceptible or resistant.

Isolation of bacterial DNA - The DNA Genomic Mini Kit (A&A Biotechnology, Poland) was used to isolate *S. epidermidis* DNA according to the manufacturer’s guidelines.

Identification of genes by polymerase chain reaction (PCR) - The sequences of the primers and the conditions of the PCR reactions are presented in Table I. For the PCR reactions, PCR REDTaq® Ready Mix™ PCR Mix with MgCl2 (Sigma-Aldrich, USA) was used. The final volume of each PCR reaction was 25 μl and contained 12.5 μl of REDTaq Ready Mix, 1 μl of each forward and reverse primer (concentration between 0.1-1.0 mM), 1 μl of DNA (50-200 ng), and 9 μl of water. The reactions were performed using a Whatman Biometra thermocycler, whereas the PCR products were subjected to agarose gel electrophoresis (2% agarose, 1xTRIS-ace-tate-EDTA, 120 mV, 40 min). The gels were stained with ethidium bromide and the PCR products were visualised using a Wilbert Lambert transilluminator and compared with molecular size markers [Gene Ruler™ 100 bp DNA Ladder (Fermentas, Thermo Scientific, USA)].

Ethics - The study design and protocols were approved by the Ethical Committee of the Medical University of Lublin (KE-0254/75/2011).

RESULTS

The 75 *S. epidermidis* isolates expressed resistance to erythromycin with the following mechanisms of resistance: 27 (36%) strains exhibited cMLSb resistance, 14 (18.7%) strains exhibited iMLSb resistance, and 34 (45.3%) strains exhibited MSb resistance (Figure). Twenty-five isolates exhibited L-phenotypes and were determined to be either resistant to only lincomycin (24 strains) or resistant to lincomycin and clindamycin (1 strain).

The MICs of spiramycin among erythromycin-resistant *S. epidermidis* were evaluated as follows: > 128 mg/L for all cMLSb strains, from 4-> 128 mg/L for iMLSb strains, and from 1-4 mg/L for strains exhibiting the MSb resistance.

| Gene | Primers sequence | PCR conditions | PCR fragment size (bp) |
|------|------------------|----------------|------------------------|
| ermA | 5’-TCTAAAAAGCATGTTAAAAAGGA-3’ | (30 s at 94°C, 1 min at 48°C, 2 min at 72°C) | 645 |
|      | 5’-CTTCGATAGTTATATATATATAGT-3’ | | |
| ermB | 5’-GAAAAGGATCTACACCAATAA-3’ | (30 s at 94°C, 30 s at 50°C, 2 min at 72°C) | 639 |
|      | 5’-AGTACAGGTACTTAAATTTGAC-3’ | | |
| ermC | 5’-ATCTCCTGCTATTTAAAGG-3’ | (55 s at 94°C, 1 min at 53°C, 1 min at 72°C) | 642 |
|      | 5’-AGTACAGGGTGTTATATATATATAGT-3’ | | |
| msrA | 5’-GGGACAAATAGCCTATTCATAGCCTTAC-3’ | (1 min at 94°C, 1 min at 50°C, 90 s at 72°C) | 530 |
|      | 5’-AGTACAGGGTGTTATATATATATAGT-3’ | | |
| mphC | 5’-GAGACTACGACCTGACG-3’ | (1 min at 94°C, 1 min at 59°C, 1 min at 72°C) | 323 |
|      | 5’-CATACGGCGATTCTTCTGAT-3’ | | |
| linA/A’ | 5’-GGTTGGCTGGGGGTAGTATATACCTG-3’ | (30 s at 94°C, 30 s at 57°C, 1 min at 72°C) | |
|      | 5’-GGTTGGCTGGGGGTAGTATATACCTG-3’ | | |

* Sutcliffe et al. (1996) and Lina et al. (1999).
phenotype. The MIC\textsubscript{50} and MIC\textsubscript{90} values were also calculated. Strains with cMLS\textsubscript{B} and iMLS\textsubscript{B} phenotypes exhibited MIC\textsubscript{50} and MIC\textsubscript{90} values > 128 mg/L, whereas the MIC\textsubscript{50} and MIC\textsubscript{90} values for the MS\textsubscript{B} strains were determined to 4 mg/L (Table II). Moreover, for the 11 (78.6%) strains exhibiting iMLS\textsubscript{B} phenotypes, the noninhibition zone around the spiramycin disc was found together with a D-shaped zone around the clindamycin disk.

As shown in Table III, among the strains with cMLS\textsubscript{B} resistance, the predominant genes were \textit{ermC} and \textit{mphC} in 23 (85.2%) and 24 (88.9%) strains, respectively. \textit{linA/A}' was found to occur in 14 (51.8%) strains. The presence of other genes (e.g., \textit{ermA} and \textit{ermB}) was detected in a few strains; two strains did not possess any of the \textit{erm} genes. The isolates with iMLS\textsubscript{B} possessed the following genes: \textit{ermC} - 14 (100%) strains, \textit{msrA} - 7 (50%) strains, \textit{mphC} - 13 (92.9%) strains, and \textit{linA/A}' - 10 (71.4%) strains; \textit{ermA} and \textit{ermB} were not detected. The strains exhibiting MS\textsubscript{B} resistance were found to possess the following genes: \textit{ermC} - 20 (58.8%) strains, \textit{msrA} - 32 (94.1%) strains, \textit{mphC} in 23 (92%) strains, and \textit{linA/A}' in 24 (70.6%) strains; these strains did not carry \textit{ermA} or \textit{ermB}. The strains exhibiting L-phenotypes contained \textit{linA/A}' in 24 (96%) strains, \textit{mphC} in 23 (92%) strains, and \textit{ermC} in 24 (96%) strains. \textit{ermA}, \textit{ermB}, and \textit{msrA} were not detected in the isolates with L-phenotypes. One strain did not carry any of the evaluated genes.

Table IV shows the combination of genes responsible for resistance to MLS\textsubscript{B} antibiotics among staphylococci. In isolates exhibiting cMLS\textsubscript{B} resistance, 11 different combinations were detected. The most frequent gene combination was \textit{ermC}, \textit{mphC}, and \textit{linA/A}', which was found in 10 (37%) strains. Among the strains exhibiting iMLS\textsubscript{B} resistance, four gene combinations were evaluated. The most frequent combinations contained the following genes: \textit{ermC}, \textit{mphC}, and \textit{linA/A}' in five (35.7%) isolates and \textit{ermC}, \textit{msrA}, \textit{mphC}, and \textit{linA/A}', also in five (35.7%) isolates. The MS\textsubscript{B} positive strains contained six different
gene combinations in three major groups: \textit{ermC}, \textit{msrA}, \textit{mphC}, and \textit{linA/A’} in 14 (41.2\%) strains; \textit{msrA}, \textit{mphC}, and \textit{linA/A’} in nine (26.5\%) strains, and \textit{ermC}, \textit{msrA}, and \textit{mphC} in six (17.6\%) strains. In the isolates with L-phenotypes, the most significant three-gene combination was \textit{ermC}, \textit{mphC}, and \textit{linA/A’} in 21 (84\%) strains.

**DISCUSSION**

CoNS are potential reservoirs of antibiotic resistance genes, which can be transferred to \textit{S. aureus} not only in vitro but also in vivo (Reyes et al. 2007, Otto 2013). Erythromycin resistance among CoNS was previously reported to result from a methylase encoded by different \textit{erm} family genes that can be horizontally transferred to recipient strains (Zmantar et al. 2011, Vitali et al. 2014). Hence, surveillance of erythromycin resistance and MLS\textsubscript{B} resistance in CoNS at phenotypic and genetic levels can provide important information regarding their current epidemiology.

Among the \textit{S. epidermidis} strains studied, the most frequently identified gene in strains exhibiting both cMLS\textsubscript{B} and iMLS\textsubscript{B} phenotypes was \textit{ermC}, which is consistent with previous reports (Reyes et al. 2007, Gherardi et al. 2009, Coutinho et al. 2010, Bouchami et al. 2011, Brzychczy- Wloch et al. 2013, Heb & Gallert 2014). Only a few \textit{S. epidermidis} exhibiting cMLS\textsubscript{B} phenotypes possessed \textit{ermA} and/or \textit{ermB}. Similar data have been previously reported (Bouchami et al. 2011, Teodoro et al. 2012, Szczyka et al. 2016). Moreover, the presence of other \textit{erm} genes (e.g., \textit{ermF}) has been rarely detected in \textit{Staphylococcus} spp (Roberts 2004). Notably, the distribution of \textit{erm} genes depends on the bacterial species. For example, \textit{ermA} is more characteristic of \textit{S. aureus}, whereas \textit{ermB} is more characteristic of beta-haemolytic streptococci (Roberts 2004, Buter et al. 2010, Meehan et al. 2014, Vitali et al. 2014). Moreover, among CoNS, the type of \textit{erm} gene also depends on the geographical region of their isolation. For example, \textit{ermC} was previously detected in 50\% of the strains exhibiting MLS\textsubscript{B} resistance in Great Britain, whereas it was detected 90\% of those in Denmark (Lim et al. 2002, Gatermann et al. 2007, Cetin et al. 2010, Bouchami et al. 2011) and in Mexico, \textit{ermA} was reported as predominant in \textit{S. epidermidis} (Castro-Alarcón et al. 2011).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Gene combinations} & \textbf{cMLSB\textsubscript{B}} (n = 27) & \textbf{iMLSB\textsubscript{B}} (n = 14) & \textbf{MS\textsubscript{B}} (n = 34) & \textbf{L-phenotype} (n = 25) \\
\hline
\textit{ermC} & 1 (3.7) & 1 (7.1) & 0 (0) & 0 (0) \\
\textit{mphC} & 0 (0) & 0 (0) & 2 (5.9) & 0 (0) \\
\textit{ermC, mphC} & 4 (14.8) & 3 (21.4) & 0 (0) & 0 (0) \\
\textit{ermB, mphC} & 1 (3.7) & 0 (0) & 0 (0) & 0 (0) \\
\textit{ermC, linA/A’} & 1 (3.7) & 0 (0) & 0 (0) & 0 (0) \\
\textit{ermA, mphC} & 1 (3.7) & 0 (0) & 0 (0) & 0 (0) \\
\textit{msrA, mphC} & 0 (0) & 0 (0) & 2 (5.9) & 0 (0) \\
\textit{msrA, linA/A’} & 0 (0) & 0 (0) & 1 (2.9) & 0 (0) \\
\textit{mphC, linA/A’} & 0 (0) & 0 (0) & 0 (0) & 1 (4) \\
\textit{ermC, msrA, mphC} & 3 (11.1) & 0 (0) & 6 (17.6) & 0 (0) \\
\textit{ermC, mphC, linA/A’} & 10 (37) & 5 (35.7) & 0 (0) & 21 (84) \\
\textit{msrA, mphC, linA/A’} & 1 (3.7) & 0 (0) & 9 (26.5) & 0 (0) \\
\textit{ermA, ermC, mphC} & 2 (7.4) & 0 (0) & 0 (0) & 0 (0) \\
\textit{ermC, msrA, mphC, linA/A’} & 1 (3.7) & 5 (35.7) & 14 (41.2) & 0 (0) \\
\textit{ermA, ermC, mphC, linA/A’} & 1 (3.7) & 0 (0) & 0 (0) & 0 (0) \\
Without genes & 1 (3.7) & 0 (0) & 0 (0) & 1 (4) \\
\hline
\end{tabular}
\caption{The prevalence of gene combinations responsible for resistance to macrolide, lincosamide, and streptogramins B (MLS\textsubscript{B}) antibiotics among erythromycin-resistant \textit{Staphylococcus epidermidis}}
\end{table}

\begin{flushleft}
cMLSB\textsubscript{B}: constitutive resistance to MLS\textsubscript{B} antibiotics; iMLSB\textsubscript{B}: inducible resistance to MLS\textsubscript{B} antibiotics; MS\textsubscript{B}: resistance of MS\textsubscript{B} type.
\end{flushleft}
studied exhibited resistance to lincomycin, but susceptibility to clindamycin as a result of increased enzyme affinity for lincomycin (Achard et al. 2005). Resistance both to lincomycin and clindamycin may be a consequence of the presence of other \textit{lin} family genes or \textit{vga(A)} \textit{LC}, which encodes a “new” variant of the SgA protein that is responsible for cross-resistance to streptogramins A and all lincosamides (Novotna & Janata 2006).

Among the iMLS\textsubscript{B} and cMLS\textsubscript{B} \textit{S. epidermidis} strains, the \textit{erm} genes do not exist separately, but in combination with others (predominantly with \textit{mphC}). Notably, other \textit{erm} genes (e.g., \textit{ermF}), which are rarely detected in \textit{Staphylococcus} spp, may encode both the inducible or constitutive MLS\textsubscript{B} phenotypes (Roberts 2004). In MS\textsubscript{B}-positive \textit{S. epidermidis} strains, the \textit{msrA} genes predominantly coexist with \textit{ermC}, \textit{mphC}, and \textit{linA/A’}, and the coexistence of \textit{msrA} and \textit{ermC} has also been previously reported (Roberts 2004, Novotna et al. 2007, Wang et al. 2008, Teodoro et al. 2012). Moreover, the presence of the \textit{linA/A’} gene in \textit{msrA}-positive strains results in resistance to lincomycin. The \textit{S. epidermidis} strains exhibiting \textit{L-phenotypes} correlated with the presence of the \textit{linA/A’} gene in most of the strains that also contained the \textit{ermC} and \textit{mphC} genes, whereas those strains did not contain the \textit{msrA} gene. Notably, the \textit{ermC} genes were also detected in both of the MS\textsubscript{B} and \textit{L-phenotype} \textit{S. epidermidis} strains - but without its expression - suggesting a defect in \textit{ermC} expression.

Previous studies have reported (Leclercq 2002, Coutinho et al. 2010) that 16-membered macrolides (e.g., spiramycin) are not inducers of MLS\textsubscript{B} resistance in staphylococci. According to our data, spiramycin is able to induce resistance to clindamycin among the iMLS\textsubscript{B} \textit{S. epidermidis} isolates examined. Moreover, iMLS\textsubscript{B} \textit{S. epidermidis} strains, which contain \textit{ermC}, exhibited resistance to spiramycin in vitro. These observations contradict previous reports that 16-membered macrolides remain active against staphylococci that exhibit iMLS\textsubscript{B} phenotypes (Leclercq 2002, Szczuka et al. 2016). Notably, resistance to spiramycin appears to be characteristic of iMLS\textsubscript{B} streptococci containing \textit{ermB} (Leclercq 2002, Acikgoz et al. 2003).

The diversity of genes involved in different mechanisms that are responsible for the resistance of \textit{S. epidermidis} to MLS\textsubscript{B} antibiotics suggests that the insensitivity of CoNS strains to these antibiotic drugs is not necessarily a unidirectional process and that the coexistence of various genes may influence the nature of their resistance.

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