

Species-Specific Sensitivity of Coagulase-Negative Staphylococci to Single Antibiotics and Their Combinations

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Abstract

The activity of β -lactam antibiotics (oxacillin, cloxacillin, cephalotin), vancomycin, gentamicin and rifampicin applied *in vitro* individually and in combination against 37 nosocomial methicillin-resistant strains of coagulase-negative staphylococci (CNS) was assessed to demonstrate the heterogeneity of this group of bacteria and estimate the chance of the efficacy of such therapy. The strains belonged to four species: *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus cohnii*, *Staphylococcus hominis*. They originated from a hospital environment and from the skin of medical staff of the intensive care unit of a paediatric ward at a university hospital. All strains were methicillin-resistant, according to CLSI standards, but individual strains differed in MIC_{ox} values. Susceptibility to other tested antibiotics was also characteristic for the species. The increased susceptibility to antibiotics in combinations, tested by calculating the fractional inhibitory concentration (FIC) index, concerned 26 out of 37 investigated strains and it was a feature of a particular species. Combinations of vancomycin and cephalotin against *S. epidermidis* and oxacillin with vancomycin were significant, as well as cephalotin and rifampicin in growth inhibition of multiresistant *S. haemolyticus* strains.

Key words: *Staphylococcus* sp., antibiotic synergism, methicillin-resistant CNS, staphylococci infections treatment

Introduction

Coagulase-negative staphylococci (CNS) have been regarded as a threat since the 80s of the last century due to advances in medicine and implementation of new medical methods. This type of infection is difficult to diagnose as it is often not easy to distinguish an etiological disease factor from a natural flora contamination. Recently, much more attention has been paid to the need of CNS species identification and searching for a connection between species affiliation and the clinical importance of particular staphylococci (Tan *et al.*, 2006; Hamels *et al.*, 2001).

CNS are the leading causes of nosocomial bloodstream infections (Chandran and Rennie, 2005; Frigatto *et al.*, 2005). According to Finkelstein *et al.*, (2002) morbidity among patients with CNS bacteremia amounts to 16%, hence investigations concerning differences in resistance profiles of particular species of this group, especially multi-resistant strains are of particular significance. The increase of bacterial resistance forces rational antibiotic therapy in order to maintain the efficacy of these drugs for as long as possible (Wang and Lipstich, 2006). Methods of alternative therapy against these infections are being sought. One of them can be

combination therapy (Dawis *et al.*, 2003; Guerrero and Gorgolas, 2006; Miranda-Nowales *et al.*, 2006).

In the presented investigations, the activity of β -lactam antibiotics (oxacillin, cloxacillin, cephalotin), vancomycin, gentamicin and rifampicin applied *in vitro* as single or in combination against nosocomial methicillin-resistant CNS belonging to *S. epidermidis*, *S. haemolyticus*, *S. cohnii* and *S. hominis* was investigated.

Experimental

Materials and Methods

37 methicillin-resistant CNS from our own collection were tested. They belonged to *S. epidermidis* (n = 12), *S. haemolyticus* (n = 9), *S. cohnii* (n = 10), *S. hominis* (n = 6). The strains originated from hospital environment and from the skin of medical staff of the intensive care unit at a paediatric ward of a university hospital. Identification to the species level was performed with API-Staph System (BioMérieux). Only a single isolate per patient was tested. Methicillin resistance was detected phenotypically with a ceftoxitin test (FOX₃₀ – Becton-Dickinson) and confirmed by *mecA*

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gene detection in PCR method (PK14 kit – DNA-GdańskII) (Van Griethuysen *et al.*, 1999). β -lactamase was detected in a cefinase test (Becton-Dickinson). Strains were stored frozen in glycerol-broth at the temperature of -70°C .

Strain susceptibility tests were performed according to the standards of the CLSI (2008) by the MIC microdilution tests using oxacillin (OX), cloxacillin (CX), cephalotin (CF), gentamicin (GM), vancomycin (VA) and rifampicin (RA) (Fluka and Sigma) in Mueller-Hinton broth (BioMérieux) without 2% NaCl. MIC is the lowest concentration of antibiotic that yielded no visible growth after incubation at 35°C for 24 h.

Activity of antibiotics in combination was checked, at first using disc sensitivity tests, where discs (all from Becton-Dickinson) were placed on Mueller-Hinton agar plates at a distance of 19–20 mm in pairs: $\text{OX}_1 + \text{VA}_{30}$, $\text{CF}_{30} + \text{VA}_{30}$, $\text{CF}_{30} + \text{RA}_5$, $\text{CF}_{30} + \text{GM}_{10}$ and $\text{CF}_{30} + \text{CX}_1$. Subsequently, strains forming characteristic elongated shape of inhibit growth zones around discs with antibiotics were tested in a microdilution synergy test. Combinations of antibiotics at appropriate concentrations in Mueller-Hinton broth were tested in 96-well microtiter plates (Kartell). Plates were incubated at 35°C for 24 h and Fractional Inhibitory Concentration (FIC) index was calculated. FIC is MIC of drug in combination divided by the MIC of drug acting alone (MIC of drug A in combination with drug B/MIC of drug A alone). The sum of the FICs of both antibiotics gave the FIC index. Synergism was identified when the FIC index was ≤ 0.5 (Climo *et al.*, 1999; Dawis *et al.*, 2003). All tests were performed at least twice. Reference strains were *S. aureus* ATCC 29213 and ATCC 25923. Quantitative variables were calculated using the Kruskal-Wallis test.

Results

All investigated strains were methicillin-resistant according to CLSI standards but individual strains presented different levels of oxacillin susceptibility, expressed in MIC_{ox} values (Table I). The greatest number of strains with low MIC (≤ 4 mg/l) was found among *S. hominis*,

with an average MIC (8–128 mg/l) among *S. epidermidis* and with high MIC (≥ 256 mg/l) among *S. cohnii*. There were significant differences in MIC values (from 0.5 to 2048 mg/l) within species, particularly in *S. haemolyticus*. Nevertheless, the differences in antibiotic-resistance between species were clearly visible and statistically important in the Kruskal-Wallis test.

Ranges of MIC values for the tested antibiotics: cloxacillin, cephalotin, gentamicin, vancomycin and rifampicin were also different for individual species (Fig. 1). Resistant strains with high MIC values were the most numerous among *S. haemolyticus*. In spite of great standard deviations, it can be stated that statistically significant differences among species in their sensitivity to particular antibiotics not only concerned differences in absolute MIC values, but also sensitivity or resistance in the clinical sense, thus placing above or below the breakpoint values for individual antibiotics.

Using UPGMA (unweighted pair group mathematical averages), a dendrogram was created. The dendrogram grouped all investigated strains in relation to their antibiotic-susceptibility and the level of their resistance was expressed in MIC values. Strains formed several clusters and particular species were located in separated areas on the axis. *S. haemolyticus* strains were located mostly on the left side, *S. epidermidis* on the right, whereas *S. cohnii* and *S. hominis* in the central part of the dendrogram (Fig. 2).

In further experiments, the activity of antibiotics in combination was tested. Disc-diffusion method allowed to observe the occurrence of synergism between cephalotin and vancomycin (CF+VA), cephalotin and rifampicin (CF+RA), oxacillin and vancomycin (OX+VA), cephalotin and cloxacillin (CF+CX). In none of the investigated combinations either antagonism or addition occurred. For the strains in which, on the basis of growth inhibition zone shape around discs, the beneficial activity of antibiotics used in combination was observed, FIC index was calculated confirming or excluding synergism. Synergistic action was confirmed in most cases. It concerned 26 out of 37 investigated strains (Fig. 3). Most susceptible strains were among *S. epidermidis* – 10 out of 12 of the tested ones. The

Table I
MIC values of investigated species to oxacillin

Species (number of strains examined)	MIC_{ox} [mg/l] range in species	Number of strains of low, medium and high MIC_{ox} values		
		≤ 4 mg/l	8–128 mg/l	≥ 256 mg/l
<i>S. epidermidis</i> (12)	1–256	5 (42%)	6 (50%)	1 (8%)
<i>S. haemolyticus</i> (9)	0.5–2048	4 (45%)	2 (22%)	3 (33%)
<i>S. cohnii</i> (10)	4–512	1 (10%)	3 (30%)	6 (60%)
<i>S. hominis</i> (6)	0.5–64	5 (83%)	1 (17%)	0

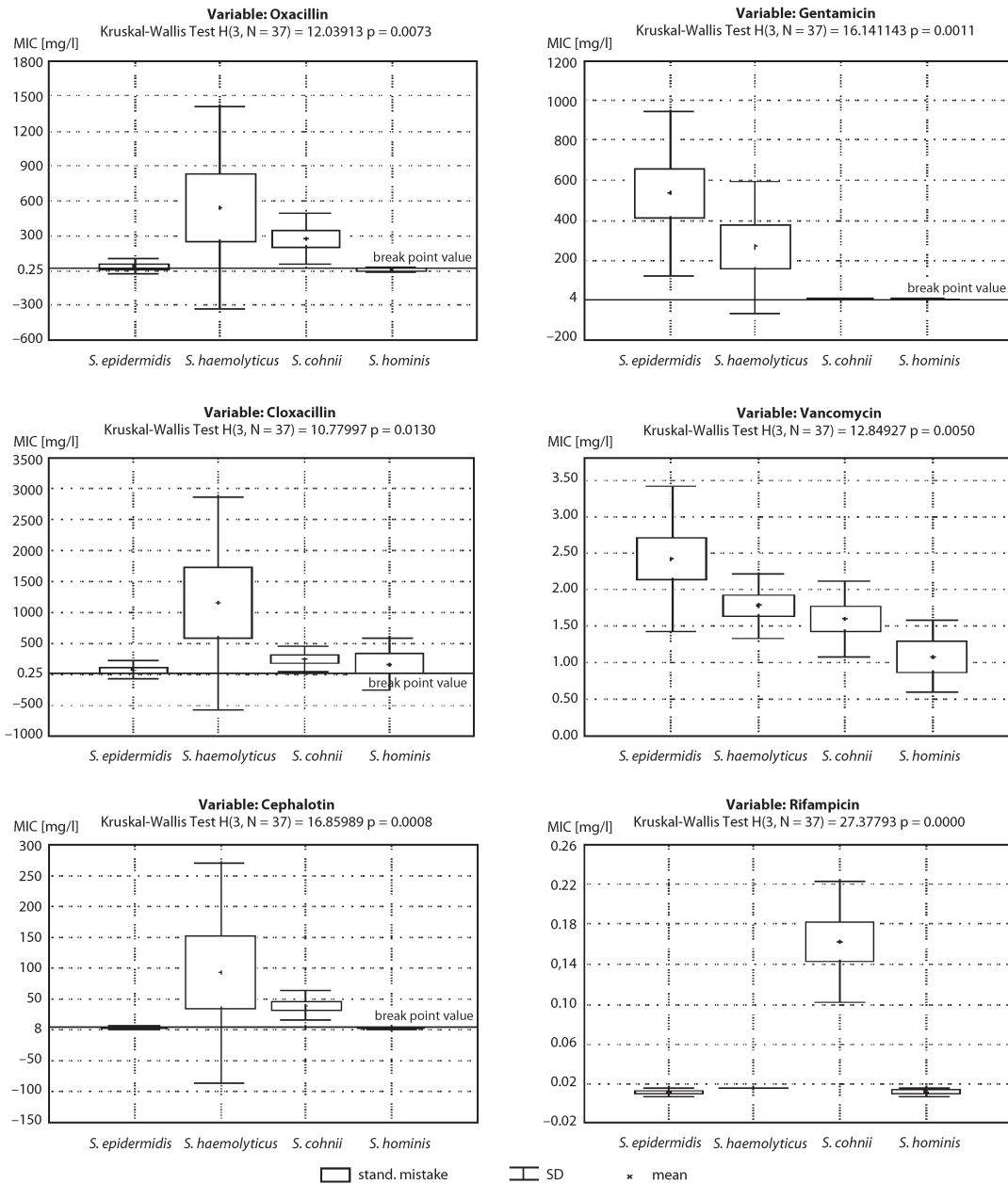


Fig. 1. Differences in MIC values of the tested antibiotics for strains of the investigated species. In the case of vancomycin and rifampicin, breakpoints were higher than the maximum points on a chart scale.

majority of them reacted this way to two or three pairs of antibiotics. Mostly, it was the combination of CF+VA. The same effect was achieved for most of the strains by CF+RA binding. Only for *S. epidermidis* CF+CX combination synergism occurred. However, for none of the *S. epidermidis* strains, synergism of β -lactams and gentamicin was determined. Synergistic activity of antibiotics against *S. haemolyticus* was observed for six out of nine tested strains. Mostly, combination of OX+VA (5 strains) presented synergism, less frequently

combination of CF+RA (3 strains). Synergistic activity in eight out of ten of the tested *S. cohnii* strains presented CF+RA and in half of the tested strains a combination of CF+GM occurred. In *S. hominis* synergism was noticed only against two strains and only for one pair of antibiotics (CF+ GM). Sensitivity to particular pairs of antibiotics was a feature connected with species. The combination presenting a synergistic effect in relation to the greatest number of tested strains was CF+RA. This combination was effective when MIC of

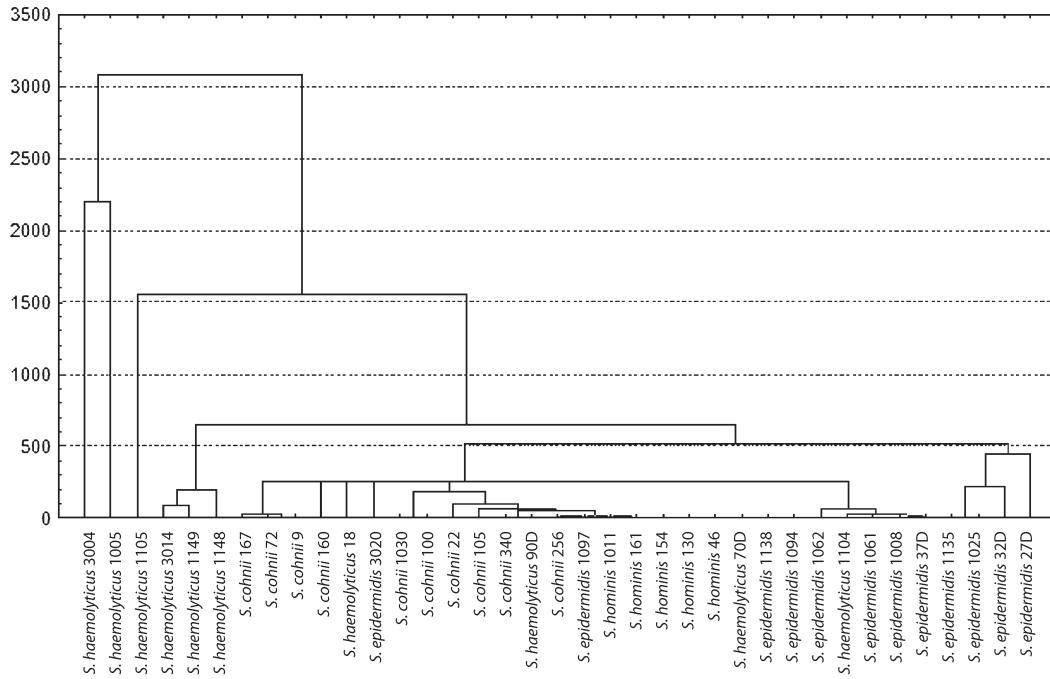


Fig. 2. Dendrogram presenting similarity in susceptibility to 6 antibiotics of 37 strains belonging to four tested species.

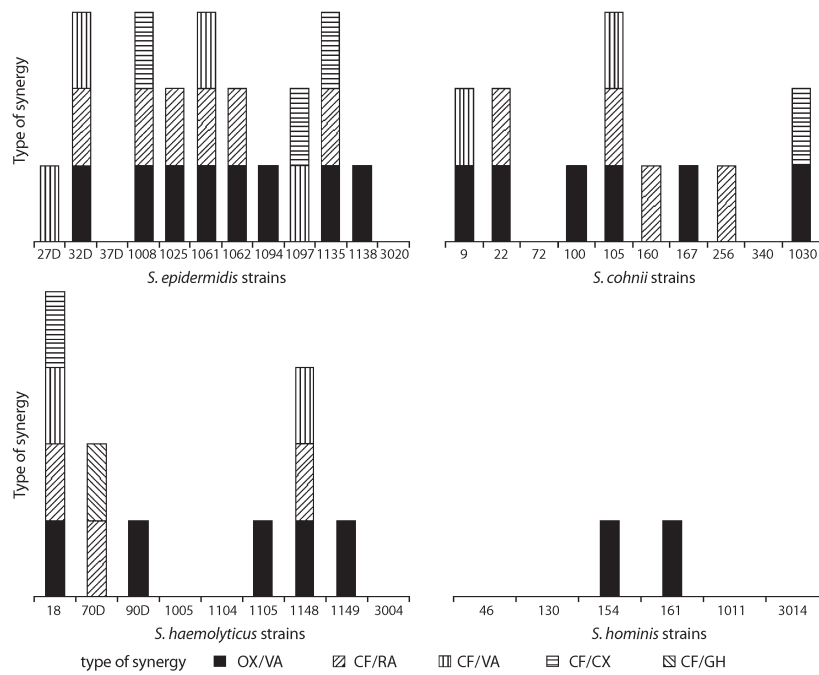


Fig. 3. Synergistic effect of antibiotics in combination against strains of the investigated CNS.

cephalotin as a single antibiotic was $\leq 8 \mu\text{g/ml}$ (sensitive strain) but also when it was $\geq 32, \mu\text{g/ml}$ (resistant strain). Production of β -lactamases was a feature characteristic for almost all strains of the three tested species. The exception was *S. cohnii* strains, the majority

of which did not produce β -lactamases. There was no connection between synergism occurrence and production of those enzymes.

Strains presenting a synergistic reaction to the highest number of antibiotics combinations are shown in

Table II
Sensitivity of strains susceptible to a number of antibiotic combinations

Strain	MIC [mg/l]						β -lactamase production	Antibiotics in synergistic combinations MIC [mg/l]
	OX	CF	CX	VA	RA	GM		
CLSI breakpoints	0.25	8	0.25	4	1	4		
<i>S. epidermidis</i> 32D	256 R	8 S	128 R	2 S	0.008 S	1024 R	+	OX+VA (16+0.25) CF+VA (2+0.125) CF+RA (1+0.0019)
<i>S. epidermidis</i> 1008	8 R	8 S	32 R	4 S	0.008 S	512 R	-	CF+VA (2+0.25) CF+CX (1+1) CF+RA (2+0.0005)
<i>S. epidermidis</i> 1061	16 R	8 S	32 R	2 S	0.008 S	512 R	+	OX+VA (4+0.25) CF+VA (2+0.0625) CF+RA (2+0.0019)
<i>S. epidermidis</i> 1135	64 R	2 S	32 R	2 S	0.016 S	1024 R	+	CF+VA (0.25+0.25) CF+CX (0.25+8) CF+RA (0.5+0.00312)
<i>S. haemolyticus</i> 18	512 R	1 S	8 R	2 S	0.016 S	0.25 S	+	OX+VA (128+0.5) CF+VA (0.125+0.0625) CF+CX (0.125+0.125) CF+RA (0.25+0.0019)
<i>S. haemolyticus</i> 1148	128 R	32 R	1024 R	2 S	0.016 S	256 R	+	OX+VA (4+0.5) CF+VA (2+0.5) CF+RA (8+0.00375)
<i>S. cohnii</i> 105	64 R	32 R	64 R	2 S	0.125 S	1 S	-	OX+VA (16+0.5) CF+GM (4+0.125) CF+RA (4+0.0312)

R – resistant, S – sensitive

Table II, in which MIC values for single antibiotics, strain sensitivity in clinical sense and antibiotic combinations giving synergistic effect and MIC values achieved for antibiotics in pairs are presented.

The use of combinations of antibiotics gave an effective growth inhibition of the tested bacteria with simultaneously lowered antibiotic concentration. For *S. epidermidis* strains vancomycin concentration could be lowered 8–64 fold and for *S. haemolyticus* and *S. cohnii* 4-fold. Cephalotin could be applied mostly in the dose of 4–16 times lower than when it was applied individually.

Discussion

CNS include several dozens of species and are a heterogeneous group of microorganisms, but only some of them are frequently isolated from humans. There is no doubt nowadays that these bacteria, especially methicillin-resistant strains, are important nosocomial pathogens. In this research strains belonging to four of these species were investigated. Three of those are species considered the most important in hospital infections

(*S. epidermidis*, *S. haemolyticus*, *S. hominis*), relatively often isolated from clinical samples (Kloos and Bannerman, 1994; Weinstein *et al.*, 1998) and numerous represented in the hospital environment. *S. cohnii* strains are less frequent in clinical materials, but often occur in the hospital environment and on the skin of medical personnel. *S. hominis* may be considered a resistance reservoir in the environment (Kloos, 1997; Szweczyk *et al.*, 2004). All strains were multiresistant and it could be assumed that this resistance was a result of selective pressure. Potentially, each of them could become the cause of a hospital infection.

The need to identify CNS species, wrongly treated as the same, is often perceived. It is stressed by many authors that such approach is a significant obstacle in research and diagnostics (Tacconelli *et al.*, 2003). Nevertheless, there are still papers, even quite recent ones, in which all isolates from this group are treated equally (Kuti *et al.*, 2008). There were also some radical opinions questioning antibiotic resistance evaluation of microorganisms from this group (Chandran and Rennie, 2005). This work was to defy such approach by showing the results of our research.

Methicillin resistance estimation of CNS still rises doubts and discussion. This stems from the differences among particular species. The differences between novobiocin-sensitive and novobiocin-resistant species are clearly visible, however, the similarity of *S. haemolyticus* to the latter group is noticeable (Nowak *et al.*, 2006; Frigatto *et al.*, 2005). This is presumably connected with the content of different cassettes including *mecA* gene in staphylococci (Martins and Maria de Lourdes Cunha, 2007) and perhaps also with other features of these bacteria. There is an urgent need for research in this matter.

The multiresistant strains tested herein clearly show the differences in antibiotic susceptibility profiles of the tested species. It may be suspected that they also differ in genetic equipment. The results showed the noticeable resistance of strains of *S. haemolyticus* and *S. cohnii*. John *et al.*, 2002, who conducted a research on the spectrum of 658 clinical staphylococcal isolates belonging to 13 species, pointed out that the highest levels of participation of isolates resistant to oxacyllin, erythromycin, klindamycin and telithromycin were presented by *S. haemolyticus* and *S. epidermidis* while for *S. cohnii* to quinupristin/dalfopristin. The presence of multiple plasmids in the cells was shown in the latter species (Szewczyk *et al.*, 2004).

Sensitivity to cephalosporins maintained in methicillin-resistant staphylococci strains (*S. epidermidis*, *S. hominis*) was observed previously. In the research of Krediet *et al.*, (1999) this feature was characteristic for 25 (almost 70%) of the tested strains including all from *S. epidermidis* and *S. hominis*, five strains of *S. haemolyticus* and two of *S. cohnii*. The use of this antibiotic in CNS infection therapy should be analysed thoroughly, particularly because of earlier suggestions to apply it. All environmental strains of staphylococci tested by Szewczyk *et al.*, (2000) were susceptible to cefuroxime and cefotaxime. 87% and 71% isolates from infants were respectively susceptible to two of these cephalosporins. All investigated strains of *S. epidermidis* from both groups were susceptible to cephalotin.

All of the strains from the collection tested in this research were sensitive to vancomycin and rifampicin, though clear differences in MIC values among species were visible (Fig. 1). Vancomycin is still frequently used in treatment of serious infections caused by gram-positive microorganisms, but stepping away from vancomycin therapy and replacing it with, *e.g.* cloxacillin both in cases of infections caused by *S. aureus* and CNS is recommended (Lawrence *et al.*, 2005). As can be seen in the results presented in this work, therapy with cloxacillin would not meet the expectations in case of all species investigated CNS except *S. haemolyticus*, especially in combination with cephalotin. Lowered sensitivity to glycopeptides of *S. aureus* and *S. epidermidis* strains was

described by authors who analysed CNS clinical isolates' sensitivity to vancomycin and claimed appearance of tolerance to that antibiotic and low sensitivity level (Walsh *et al.*, 2001; Bourgeois *et al.*, 2007).

Several studies have previously reported the synergistic effect of antibiotics on methicillin-resistant strains of staphylococci. Such studies have been undertaken mostly towards infections caused by multiresistant clinical strains *i.e.* MRSA (Dawis *et al.*, 2003). Also research on treatment of infections with those bacteria in experimental models has been carried out. Fox *et al.* (2006), showed that using nafcillin and vancomycin in combination cleared bloodstream infections in experimental endocarditis in rabbit models caused by vancomycin-resistant *S. aureus*. Efficacy of treatment with vancomycin in combination with aminoglycosides against MRSA has also been tested (Lee *et al.*, 2003). In the study of Miranda-Nowales (2006), synergy was evident for dicloxacillin or cephalotin in combination with amikacin against methicillin-resistant *Staphylococcus* spp.

In this research it has been shown that there is a chance to obtain an efficient antibiotic therapy in combination also in infections caused by CNS. Combinations of vancomycin and cephalotin against *S. epidermidis*, staphylococcus most frequently isolated from gram-positive hospital infections, seem to be particularly promising. A synergistic effect on growth inhibition of multiresistant *S. haemolyticus* strains was obtained using oxacillin and vancomycin as well as cephalotin and rifampicin. However, clinical research including species identification of CNS causing infections is necessary, because as can be seen, individual species of this group differ in antibiotic resistance.

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