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# In vitro antimicrobial synergy testing of coagulase-negative staphylococci isolated from prosthetic joint infections using Etest and with a focus on rifampicin and linezolid

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**Abstract** In recent years, coagulase-negative staphylococci (CoNS) have been increasingly recognised as causative agents of various infections, especially in immunocompromised patients and related to implanted foreign body materials. CoNS, and especially *Staphylococcus epidermidis*, transform into a stationary growth phase and produce biofilm when involved in a foreign body infection, making them difficult to eradicate with antimicrobials. Rifampicin has the ability to penetrate biofilm, but resistance may develop rapidly. To reduce the emergence of resistance, rifampicin should be combined with additional antimicrobials, of which several different ones have been proposed, including the relatively new class of antimicrobials, oxazolidinones, represented by linezolid. Thirty-seven CoNS isolates from patients with prosthetic joint infection were investigated by synergy testing using Etest. Nine antimicrobial combinations, based on either rifampicin or linezolid, were tested. For 16 (43%) of the isolates, a synergistic ( $n=5$ ), additive ( $n=14$ ) and/or antagonistic ( $n=11$ ) effect were identified. In conclusion, Etest is an objective and easily performed in vitro method for antimicrobial synergy testing. However, each isolate requires testing for the specific combination considered for treatment.

## Introduction

Prosthetic joint infections (PJIs) are uncommon complications of prosthetic device surgery procedures, but represent harmful conditions with high morbidity and substantial costs. Coagulase-negative staphylococci (CoNS), and especially *Staphylococcus epidermidis*, is the most important pathogen involved in delayed or late onset PJIs [1]. There are no standards for the antimicrobial treatment of PJIs [2] and, during the recent decades, the treatment consisted predominately of cell wall antimicrobials such as isoxazolyl penicillin or glycopeptides [3]. However, without prosthetic joint exchange surgery, the long-term outcome has been disappointing, with a high rate of failures following finite treatment regimens [4–6]. *S. epidermidis* involved in a foreign body infection transforms into a stationary growth phase and produces biofilm. Therefore, cell wall antimicrobials may not be effective, since they do not efficiently penetrate the biofilm and have only a modest effect on bacteria in a non-growing phase [7]. Accordingly, additional antimicrobial agents and/or combinations of antimicrobials for the treatment of foreign body infections have been assessed. Predominantly antimicrobial combinations including rifampicin, which penetrates the biofilm and is effective also against stationary phase bacteria, have been investigated in experimental studies [8–10] as well as in clinical trials [6, 11, 12]. Resistance to rifampicin may rapidly emerge and, therefore, monotherapy should be avoided. To reduce the emergence of resistance to rifampicin, it is, accordingly, recommended to combine it with additional antimicrobials and several antimicrobials have been proposed, including fluoroquinolones [6], linezolid [13] and clindamycin [12]. In addition, linezolid may be a treatment option for multidrug-resistant (MDR) *S. epidermidis*, as monotherapy [13] or in combination with

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rifampicin [14]. However, concerns have been raised due to supposed in vitro antagonism between rifampicin and other antimicrobial agents [3]. This strengthens the need for synergy testing of antimicrobial combinations including rifampicin that are considered for treatment, which may also be needed for all antimicrobial combinations.

The aim of this study was to investigate antimicrobial combinations against *S. epidermidis* that may be of clinical interest for the long-term treatment of PJIs, focusing on

rifampicin and linezolid. In vitro synergy testing was performed using Etest.

## Materials and methods

Thirty-seven clinical CoNS isolates obtained during revision surgery for PJIs with extraction or exchange of the prosthetic devices were analysed. Multiple tissue biopsy

**Table 1** Minimum inhibitory concentrations (MICs; µg/ml), using Etest, of six antimicrobials in coagulase-negative staphylococcal isolates ( $n=37$ ) from prosthetic joint infections

	Rifampicin	Linezolid	Ciprofloxacin	Fusidic acid	Clindamycin	Trimethoprim–sulfamethoxazole
<i>S. epidermidis</i>	>32	0.19	8	0.047	0.094	>640
<i>S. epidermidis</i>	>32	0.047	>32	6	>256	>640
<i>S. epidermidis</i>	0.002	0.19	16	12	>256	>640
<i>S. epidermidis</i>	>32	0.19	>32	6	0.023	>640
<i>S. epidermidis</i>	>32	0.19	>32	0.047	>256	>640
<i>S. epidermidis</i>	<0.002	0.125	>32	6	0.047	>640
<i>S. epidermidis</i>	>32	0.19	>32	12	>256	>640
<i>S. epidermidis</i>	0.25	0.125	>32	12	>256	>640
<i>S. epidermidis</i>	0.003	0.19	>32	16	>256	>640
<i>S. epidermidis</i>	>32	0.19	>32	0.047	>256	>640
<i>S. epidermidis</i>	0.003	0.19	>32	0.032	>256	2.5
<i>S. epidermidis</i>	0.003	0.25	>32	12	0.064	>640
<i>S. epidermidis</i>	>32	0.25	>32	0.047	>256	>640
<i>S. epidermidis</i>	6	0.125	3	0.032	>256	>640
<i>S. epidermidis</i>	0.003	0.19	>32	0.032	>256	>640
<i>S. epidermidis</i>	0.003	0.25	>32	0.023	>256	320
<i>S. epidermidis</i>	>32	0.125	>32	6	>256	>640
<i>S. epidermidis</i>	>32	0.25	>32	0.047	>256	>640
<i>S. epidermidis</i>	0.003	0.25	>32	0.032	>256	>640
<i>S. epidermidis</i>	0.002	0.25	>32	6	>256	>640
<i>S. epidermidis</i>	>32	0.19	0.19	0.023	>256	>640
<i>S. epidermidis</i>	>32	0.19	6	0.023	>256	>640
<i>S. epidermidis</i>	>32	0.19	0.047	0.047	>256	>640
<i>S. epidermidis</i>	0.002	0.38	16	6	0.094	480
<i>S. epidermidis</i>	0.003	0.25	0.19	0.047	0.125	5
<i>S. epidermidis</i>	0.003	0.38	16	0.064	0.064	>640
<i>S. epidermidis</i>	0.003	0.25	>32	4	>256	>640
<i>S. epidermidis</i>	0.003	0.25	0.19	0.047	0.094	0.64
<i>S. epidermidis</i>	0.003	0.25	>32	0.016	0.064	480
<i>S. epidermidis</i>	0.003	0.19	>32	8	0.094	>640
<i>S. epidermidis</i>	0.003	0.25	0.25	0.125	>256	0.94
<i>S. epidermidis</i>	0.004	0.19	0.19	0.032	>256	3.8
<i>S. epidermidis</i>	0.003	0.38	0.38	0.032	0.125	0.94
<i>S. capitis</i>	0.012	0.38	0.125	0.064	0.19	1.88
<i>S. haemolyticus</i>	0.006	0.38	>32	12	>256	240
<i>S. simulans</i>	0.006	0.5	4	0.047	0.19	15
<i>S. lugdunensis</i>	<0.002	0.094	0.25	0.047	0.064	1.88

samples, usually five or more, were taken perioperatively. The isolates were collected from patients with infected hip ( $n=27$ ), knee ( $n=9$ ) and elbow joint ( $n=1$ ) prostheses. The isolates comprised *S. epidermidis* ( $n=33$ ), *S. capitis* ( $n=1$ ), *S. haemolyticus* ( $n=1$ ), *S. simulans* ( $n=1$ ) and *S. lugdunensis* ( $n=1$ ). The minimum inhibitory concentrations (MICs,  $\mu\text{g/mL}$ ) of all antimicrobials were determined using the Etest method (AB Biodisk, Solna, Sweden). Rifampicin and linezolid were tested in combination with each other and with clindamycin, fusidic acid, ciprofloxacin and trimethoprim–sulfamethoxazole (SXT), respectively. First, the MIC for each antimicrobial was determined according to the guidelines from the Swedish Reference Group for Antibiotics (SRGA; <http://www.srga.org>). For the combination (synergy) testing, two methods, fixed ratio and MIC/MIC ratio, were performed [15–17]. Briefly, in the fixed ratio method, an Etest strip of the first antimicrobial was placed on an agar plate, its exact location marked on the back of the plate, and incubated for 1 h at room temperature. Then, the Etest strip was removed and an Etest strip of the second antimicrobial was placed at the same location on the agar plate as the first strip. In the MIC/MIC ratio method, an Etest strip of the first antimicrobial was placed on an agar plate, with the location and the MIC value from the single testing marked on the back of the plate, and incubated for 1 h at room temperature. Then, the Etest strip was removed and an Etest strip of the second antimicrobial was superimposed so that the respective MIC values from the single testing were aligned. All tests were performed on IsoSensitest agar (Oxoid, Hampshire, England) and at 36°C aerobic atmosphere for 18–24 h.

In order to determine whether each specific combination of antimicrobials resulted in a synergistic, additive, indifferent or antagonistic effect, the fractional inhibitory concentration (FIC) index was calculated as previously described [15]. The following cut-off values and interpretative criteria in regards of the achieved effect were used; FIC index  $\leq 0.5$  indicated a synergistic effect, FIC index  $>0.5$  and  $\leq 1.0$  an additive effect, FIC index  $>1.0$  and  $\leq 4.0$  indifferent effect, and FIC index  $>4.0$  an antagonistic effect [15]. Random isolates were repeatedly analysed in order to assess the reproducibility.

## Results and discussion

The MIC values for all isolates and all antimicrobial agents in single use are shown in Table 1.

Altogether, 888 MIC determinations were performed using the fixed ratio method and the MIC/MIC ratio method. The results of the MIC/MIC ratio method were more reproducible and easier to interpret, especially when evaluating two antimicrobials with substantial differences in their MIC values. Accordingly, the results of the antimicrobial synergy testing, using Etest and the MIC/MIC ratio method, of all isolates and antimicrobial combinations are summarised in Table 2.

Overall, 16 (43%) of the isolates displayed a synergistic ( $n=5$ ), additive ( $n=14$ ) or antagonistic ( $n=11$ ) effect when combining two antimicrobials (Table 2).

The combination of rifampicin and ciprofloxacin displayed no synergistic effect in vitro. Instead, there were two (5%)

**Table 2** Antimicrobial synergy testing, using Etest, of coagulase-negative staphylococcal isolates ( $n=37$ ) from prosthetic joint infections

Antimicrobial combination <sup>a</sup>	Number (%) of isolates			
	Synergism	Addition	Indifference	Antagonism
RI + LZ	0	0	36 (97%)	1 (3%)
RI + CI	0	0	35 (95%) <sup>b</sup>	2 (5%)
RI + FU	2 (5%)	0	35 (95%)	0
RI + CM	0	4 (11%) <sup>c</sup>	33 (89%)	0
RI + SXT	0	1 (3%)	36 (97%) <sup>d</sup>	0
LZ + CI	0	1 (3%)	35 (95%)	1 (3%)
LZ + FU	1 (3%)	6 (16%)	30 (81%) <sup>e</sup>	0
LZ + CM	2 (5%)	2 (5%)	30 (81%)	3 (8%)
LZ + SXT	0	0	33 (89%)	4 (11%)

<sup>a</sup> RI, rifampicin; LZ, linezolid; CI, ciprofloxacin; FU, fusidic acid; CM, clindamycin; SXT, trimethoprim–sulfamethoxazole

<sup>b</sup> Including one strain not possible to distinguish between indifferent and additive effect

<sup>c</sup> Including two strains not possible to distinguish between additive and synergistic effect

<sup>d</sup> Including two strains not possible to distinguish between indifferent and additive effect

<sup>e</sup> Including one strain not possible to distinguish between indifferent and additive effect and one strain not possible to completely distinguish between additive and synergistic effect

isolates showing an antagonistic effect with an increase of the ciprofloxacin MIC from 6 µg/ml and 8 µg/ml, respectively, to >32 µg/ml. For rifampicin and fusidic acid, two isolates (5%) displayed a synergistic effect, with a decrease of the rifampicin MIC from >32 µg/ml to 8 µg/ml and 0.125 µg/ml, respectively, as well as of fusidic acid from 6 µg/ml and 12 µg/ml to 1.5 µg/ml and 0.064 µg/ml, respectively. For rifampicin and clindamycin, four isolates (11%) displayed an additive effect, with a decrease of the clindamycin MIC from >256 µg/ml ( $n=3$ ) to 0.94 µg/ml ( $n=2$ ) and 128 µg/ml, respectively, and one additional isolate displayed a decrease from 0.19 µg/ml to 0.064 µg/ml.

Linezolid comprises no known cross-resistance to other antimicrobials and is highly effective against Gram-positive bacteria. However, the treatment is expensive and long-term use may cause adverse effects [13, 18]. For the combination rifampicin and linezolid, no synergistic or additive effects were found in the present in vitro test, but in a previous study by Jacqueline et al. [19], using the time–kill curve method, an additive effect could be seen. This has also been indicated in clinical studies [13, 14]. Accordingly, this combination treatment may have a synergistic effect in vivo. Regarding linezolid and fusidic acid, almost 20% of the isolates displayed a positive effect (synergistic or additive). For linezolid and clindamycin, all three effects were found, i.e. two isolates (5%) displayed synergistic effect, two (5%) additive effects and three (8%) antagonistic effects. Clindamycin might be of clinical interest as the empirical treatment of PJIs, since it has a good effect on aerobic as well as anaerobic bacteria. Linezolid in combination with trimethoprim–sulfamethoxazole displayed the highest percentage of antagonistic effect (four isolates; 11%), with an increase of linezolid MIC by 2 to 3 whole MIC steps, while rifampicin and trimethoprim–sulfamethoxazole displayed no antagonistic effect and one isolate (3%) displayed an additive effect.

The reproducibility of the antimicrobial synergy testing using Etest was high (data not shown). Ideally, the Etest method for synergy testing of staphylococci should be further validated to a “gold standard,” such as checkerboard titration or any in vitro kinetic model for synergy testing. However, there have been studies comparing Etest, the time–kill curve method and/or checkerboard methods for organisms such as *Acinetobacter baumannii* [20], *Candida* species [21] and *Burkholderia cepacia* [15]. All of these studies concluded that Etest is simple to use, time-efficient, reproducible and has good agreement to other methods for synergy testing. However, additional investigations are needed. A limitation of this study was that only bacteria in the growth phase, and not in the stationary phase protected by biofilm, were examined. Accordingly, the results may not completely reflect the situation in vivo during a PJI,

but examine interesting antimicrobial combinations and their interactions in vitro.

In conclusion, Etest is an objective and easily performed in vitro method for the antimicrobial synergy testing of CoNS. However, the effects of different combinations of antimicrobials were strain-dependent and, accordingly, each isolate needs to be tested for the specific antimicrobial combination considered for treatment.

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