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In vitro synergy of oxacillin and gentamicin against coagulase-negative staphylococci from blood cultures of neonates with late-onset sepsis

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Coagulase-negative staphylococci (CoNS) are the leading cause of late-onset sepsis (LOS) in neonates. Increasing resistance of CoNS to beta-lactams and aminoglycosides has led to widespread use of vancomycin, which in turn may lead to resistance to vancomycin. Thus, combination therapy of LOS has been advocated. We aimed to determine the interaction of oxacillin and gentamicin against CoNS. In 2005, 34 isolates of oxacillin- and gentamicin-resistant CoNS were obtained from blood samples of neonates with LOS. Combination effect was tested using the checkerboard method, E-test with the other antibiotic incorporated in the medium (E-test-1) and two E-test strips placed in a cross-formation (E-test-2). Of 34 isolates 61.8%, 53% and 73.5% revealed synergy or an additive effect when tested by the checkerboard method, E-test-1 and E-test-2, respectively. Results of all three tests were concordant for six (17.6%) isolates, four showing synergy, and two indifference. Our *in vitro* results support that combination therapy with penicillinase-resistant penicillin and aminoglycoside can be an alternative to vancomycin.

Key words: *In vitro* synergy; oxacillin; gentamicin; coagulase-negative staphylococci.

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Coagulase-negative staphylococci (CoNS) are the main cause of late-onset sepsis (LOS) in neonates (1, 2). Although only <1% of LOS episodes have a fulminant course (3), the management of sepsis is complicated. Difficulties in distinguishing between contamination and invasive disease have led to widespread and often unnecessary use of antibiotics resulting in high-level resistance of CoNS to semisynthetic penicillins and aminoglycosides (3–6). The latter has prompted the use of vancomycin as a first-line therapy of presumed LOS (7, 8).

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However, in the light of emerging resistance to vancomycin (9, 10) restriction of its use and combining penicillinase-resistant penicillins and aminoglycosides in empiric therapy of LOS has been advocated (11). The rationale of the idea is low mortality from CoNS sepsis permitting initiation of vancomycin later in the course if needed (3). In response to the proposal, a few small retrospective studies conducted in neonatal intensive care unit (NICU)

have demonstrated similar duration and mortality of sepsis when penicillinase-resistant penicillin plus aminoglycoside are used instead of vancomycin in empirical treatment of LOS, even if the majority of CoNS are resistant to the antibiotics administered (3, 6, 12). The effect could be partly explained by a synergistic interaction of the antimicrobial agents supported by *in vitro* experiments demonstrating synergy between oxacillin and fosfomycin or dicloxacillin and amikacin (13, 14). However, we are not aware of studies looking at the potential combination effect of oxacillin and gentamicin.

To determine whether there is any synergy we aimed to investigate the *in vitro* activity of oxacillin in combination with gentamicin against CoNS isolates obtained from blood cultures of neonates with LOS.

MATERIALS AND METHODS

Isolates

Altogether 34 clinical CoNS isolates resistant to both oxacillin and gentamicin [MIC $\geq$ 0.25 μg/mL and MIC $\geq$ 4 μg/mL, respectively, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (15)] collected in 2005 from blood samples of neonates with LOS and stored at −80 °C were analyzed. Identification to species level was performed by the API Staph system (bioMérieux S.A., Marcy-l'Etoile, France) according to the manufacturer's instructions. DNA was extracted with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

MICs of oxacillin and gentamicin were determined by E-test (AB BIODISK, Solna, Sweden) and broth microdilution according to the CLSI guidelines (15).

Synergy testing

Interaction between oxacillin and gentamicin was evaluated by three different methods. All assays were performed in duplicate. The results are reported as the mean of the two assays.

Checkerboard method – In a 96-well microtiter plate 50 μL of both antibiotics in concentrations of 0.25, 0.5, 1.0, or 1.5 of MIC of each microorganism was mixed and inoculated with 100 μL of bacterial suspension (0.5 McFarland) (16). Microbial growth was evaluated by visual inspection for cloudiness after 18 h of incubation at 35 °C in ambient air. For determination of the interaction, the fractional inhibitory concentration (FIC) index was calculated using the following formula: FIC of drug A = MIC of drug A in combination/MIC of drug A alone, FIC of drug B = MIC of drug B in combination/MIC of drug B alone, and FIC index = FIC of drug A + FIC of drug B. Synergy was defined as a FIC index of ≤ 0.5, an additive effect as >0.5 but ≤ 1, indifference as >1 but ≤ 4, and antagonism as >4 (17).

E-test-1 – Gentamicin E-test strips (AB BIODISK) were placed onto inoculated (0.5 McFarland) Mueller-Hinton agar (Oxoid, Hampshire, UK) plates supplemented with oxacillin at concentrations of 0.25, 0.5, 1.0, or 1.5 of MIC of each organism (18). The agar plates were incubated at 35 °C in ambient air for 24 h. Synergy was defined as a decrease in MIC of gentamicin by >3 fold on the agar plates containing oxacillin at concentration of 0.25× MIC, an additive effect as a >2 but ≤ 3 fold decrease, and indifference as a ≤ 2 fold decrease up to <3 fold increase. Antagonism was defined as ≥ 3 fold increase in MIC.

E-test-2 – Oxacillin and gentamicin E-test strips (AB BIODISK) were placed in cross-formation at the intersection formed at MICs of oxacillin and gentamicin onto inoculated (0.5 McFarland) Mueller-Hinton agar plates and incubated at 35 °C for 18 h as described elsewhere (16). The MICs were interpreted at the point of intersection between the E-test strip and the bacterial growth inhibition zone. For evaluation of the interaction, the FIC index was calculated as described above.

Detection of penicillin binding protein

The phenotypic expression of the *mecA* gene, that is, the presence of penicillin binding protein (PBP2a), was detected by latex agglutination test (Oxoid, Cambridge, UK) according to the manufacturer's instructions.

Detection of the mecA gene

The presence of the *mec*A gene was detected by PCR as described elsewhere (19).

Statistical analysis

For statistical analysis R software [version 2.15.1 (22.06.2012; accessed 26.06 2012)] and Statistica version 8.0 (StatSoft Inc., Tulsa, OK, USA) were used. Univariate logistic regression with binomial charac-

teristics (synergy plus additive effect vs indifference plus antagonism) was applied to test concordance between different methods. Spearman rank order correlation was applied to detect relationships between FIC index and MIC change values.

RESULTS

Of 34 isolates 23 were *Staphylococcus epidermidis*, three *S. haemolyticus*, and two *S. hominis*. *S. lentus*, *S. capitis*, *S. xylosus*, and *S. saprophyticus* accounted for one each. Two isolates could not be identified at species level. All CoNS isolates except one were *mecA*-positive, and PBP2a was detectable in 16 strains. Of note, PBP2a was detected also in one *mecA*-negative isolate.

According to the CLSI criteria (15) all CoNS isolates were resistant to oxacillin and gentamicin by E-test (Table 1). Two isolates were deemed susceptible to gentamicin, but none to oxacillin by broth microdilution. No correlation between oxacillin and gentamicin MICs or between oxacillin MICs and expression of PBP2a was observed.

Synergy was observed in 9/34, 16/34, and 22/34 isolates by checkerboard method, E-test-1 and E-test-2, respectively (Table 2). The proportion of isolates demonstrating indifferent effect varied within a smaller range, from 9/34 isolates (E-test-2) to 13/34 (checkerboard) and 15/34 (E-test-1). Only E-test-1 revealed antagonism in one isolate that, however, exhibited synergy in checkerboard method and E-test-2; no antagonistic interaction was obtained in the two other assays. The interaction between oxacillin and gentamicin was dependent on method – results were concordant in all three tests for a total of 6/34 (17.6%) isolates, comprising four isolates showing synergy and two isolates showing indifference.

Binomial logistic regression revealed no association between the results of the three interaction testing methods. However, there was trend between the FIC index values, measured by checkerboard and E-test-2 method (Spearman rank order correlation, $R = 0.328$; $p = 0.057$), and a significant negative correlation ($R = -0.495$; $p = 0.003$) between the FIC index measured by E-test-2 and MIC change measured by E-test-1, that is, the lower the FIC index was in E-test-2, the larger decrease in gentamicin MIC in E-test-1.

No correlation was observed between MIC of oxacillin and interaction in any synergy testing method. However, the MIC of gentamicin was related to the MIC change measured by E-test-1 ($R = 0.465$; $p = 0.006$), that is, the higher the MIC of gentamicin, the larger decrease in E-test-1.

DISCUSSION

Our results demonstrate *in vitro* synergistic or additive interaction between oxacillin and gentamicin against more than half of the CoNS isolates studied. Although agreement between different methods was poor, there was a trend between the FIC index values measured by checkerboard and E-test-2 and correlation between the gentamicin MIC decrease measured by E-test-1 and the FIC index measured by E-test-2.

In vitro studies investigating the interaction of penicillinase-resistant penicillins and aminoglycosides against CoNS are scarce, but similarly to the present study they have demonstrated strain-dependent synergistic or additive effect. In time-kill assay oxacillin in combination with fosfomycin demonstrated synergistic interaction against 6 of 12 methicillin-resistant *S. epidermidis* isolates (13). In checkerboard method dicloxacillin and amikacin exhibited synergy or additivity against all the CoNS tested (14). Neither of these studies including ours reported antagonistic interaction. Although extrapolation of *in vi-*

Table 1. Antibiotic susceptibility of coagulase-negative staphylococci (n = 34)

	E-test				Broth microdilution			
	Susceptibility (%)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	Susceptibility (%)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)
Oxacillin	0	128	>256	2 to >256	0	128	>256	4 to >256
Gentamicin	0	24	>256	6 to >256	5.9	64	>256	4 to >256

Table 2. Interaction between oxacillin and gentamicin assessed by three different synergy testing methods

	Checkerboard method[1,2]	E-test-1[1,3]	E-test-2[2,3]
Synergy – n (%)	9 (26.5)	16 (47.1)	22 (64.7)
Additivity – n (%)	12 (35.3)	2 (5.9)	3 (8.8)
Indifference – n (%)	13 (38.2)	15 (44.1)	9 (26.5)
Antagonism – n (%)	0	1 (2.9)	0

Note. E-test-1, E-test with one antibiotic incorporated in the medium; E-test-2, two E-test strips placed in a cross-formation (see text).
[1]OR (95% CI) for checkerboard vs E-test-1 results 0.57 (0.14–2.32), Spearman rank order correlation coefficient R = −0.080 (p = 0.65).
[2]OR (95% CI) for checkerboard vs E-test-2 results 1.42 (0.30–6.69), Spearman rank order correlation coefficient R = 0.33 (p = 0.057).
[3]OR (95% CI) for E-test-1 vs E-test-2 results 0.87 (0.19–4.01), Spearman rank order correlation coefficient R = −0.495 (p = 0.003).

tro results to *in vivo* is highly questionable, it is interesting to note that no difference in mortality was found in retrospective studies of empiric treatment of LOS, half of which caused by CoNS (1), with oxacillin or cloxacillin plus gentamicin instead of vancomycin (3, 6, 12).

It is well known that *in vitro* synergy tests lack standardization for reproducibility and interpretation, likely explaining the discrepancies in concordance of the methods. Both lack of agreement (20) as well as good correlation (17) between E-test assays and checkerboard have been reported. The latter technique has been criticized for the cumulative error in the FIC index score due to the variability in MIC determination – true MIC may lay within a three-dilution range (mode ± 1 dilution) (21). In our study, the two E-test methods correlated with each other more closely than either of these with checkerboard, possibly exemplifying this criticism. In addition to the lack of standardization, the comparison of the correlation of the methods used in our study with results demonstrated by others is complicated by the multitude of E-test methods (16–18, 22), possible inconsistencies between the definitions of interactions measured by the FIC index score (14, 16, 17, 23), multiple approaches to interpret checkerboard results (24), and the impact of antibiotics studied on the correlation of tests (17, 23, 25). The evaluation of the methods is further compounded by the wide range of microorganisms studied (14, 16, 17, 23, 25) and the uncertainty about the agreement of tests in studies conducted on small number of isolates (17, 26, 27). However, in view of the potential role of combination therapy as a treatment option against resistant pathogens, standardization of the synergy assays is warranted.

Some limitations of our study should be noted. First, time-kill assay, which has been used for validation of synergy testing by E-test and checkerboard technique (17), was not performed in our study. However, it should not be a critical issue, as E-test and checkerboard are widely used to assess synergy with the advantage of ease, speed, and low cost. Second, molecular typing of CoNS isolates was not performed, and therefore related isolates may have been tested which can be a source of bias (14, 17). This may, however, not be of major concern as correlation between antibiograms and molecular types is arguable (28). Finally, although our study included 34 isolates, conclusions about interaction between antimicrobials have been drawn from a considerably smaller number of tested microorganisms (16, 27).

In conclusion, oxacillin in combination with gentamicin exhibited synergy against oxacillin- and gentamicin-resistant CoNS isolates *in vitro*, but this effect was strain dependent. Clinical studies, although retrospective, have obtained concordant results. A combination of penicillinase-resistant penicillin and aminoglycoside could be considered as a potential alternative to vancomycin against CoNS. However, further studies in animal models and clinical settings are warranted to demonstrate that the synergistic activity demonstrated *in vitro* also exist *in vivo*.

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