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NOTE

Antibiotic resistance and biofilm formation ability among coagulase-negative staphylococci in healthy individuals from Portugal

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INTRODUCTION

In the past few years the interest in coagulase-negative staphylococci (CoNS) species has significantly increased due to their impact on human health and disease. CoNS are common bacterial colonizers of the normal human microflora and usually have a benign relationship with the host. However, they are now recognized as opportunistic pathogens, causing infection especially in immunocompromised individuals.[1] CoNS have recently emerged as a leading cause of healthcare-associated infections, particularly when indwelling medical devices are used.[2] The major recognized determinants in the pathogenesis of CoNS infections are their high resistance to several classes of antibiotics along with an exceptional ability to form thick and multilayered biofilms.[1] Biofilms are defined as structured communities of microorganisms embedded in a self-produced matrix of extracellular polymeric components (e.g., polysaccharides, proteins, lipids and nucleic acids). It is well established that bacteria exhibiting a biofilm phenotype are recalcitrant to antimicrobial therapy[3] and therefore, the biofilm formation ability and the resistance to antimicrobial therapy can be intimately related. Nevertheless, some researchers argue that these determinants may in fact reflect the adaptation of CoNS to their particular commensal habitat, the skin.[4]

A large proportion of studies performed on CoNS species have been conducted almost exclusively on clinical strains, and the role of community-associated strains has been neglected. In fact, very little is known regarding CoNS strains isolated from healthy individuals in the community settings. Consequently, a better characterization of CoNS strains in their commensal lifestyle could give us new insights on how harmless commensal bacteria can turn into dangerous pathogens.

Hence, in this work we aimed to evaluate the pathogenic potential of CoNS from the skin of healthy individuals, by assessing their antibiotic susceptibility profiles and biofilm production ability, and analyze the relationship between antibiotic resistance and biofilm production.

EXPERIMENTAL PROCEDURE

A prospective study including 59 healthy volunteers from the Northern region of Portugal was performed. Participants were asked to provide skin samples and to complete a questionnaire recording demographic data (gender, age, occupation and area of residence), hospitalization history and antibiotic use (in the preceding 12 months), and contact with medical staff. All volunteers gave written informed consent. This study was approved by the Ethics Subcommission for Health and Life Sciences (process SECVS 002/2013) of the University of Minho, Portugal.

Skin samples from each participant were obtained by rubbing a sterile cotton-tip swab on the palm and between fingers of both hands, with Amies transport medium and coal (VWR, Lisbon, Portugal). The swabs were inoculated on Tryptic Soy Agar plates (Liofilchem, Teramo, Italy) supplemented with 7% sodium chloride (Sigma-Aldrich, St Louis, MO, USA) for selective growth of *Staphylococcus* spp. After incubation for 72 h at 37 °C, colony morphology and pigment characteristics of all colonies were recorded. Colonies resembling CoNS were selected for further investigation. CoNS were identified at the species level by partial *rpoB* gene sequencing, as previously described.[5] Only one isolate per participant was included in the study, except for two subjects from whom two different CoNS species were isolated.

Antibiotic susceptibility was tested using the broth microdilution method accordingly to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[6] The following antibiotics were tested: ciprofloxacin, erythromycin, gentamicin, penicillin, rifampicin and vancomycin (Sigma-Aldrich). Each isolate was tested in two independent occasions in triplicate. *Staphylococcus aureus* ATCC 29213 was used as a quality control. Multidrug-resistance was defined as resistance to three or more classes of antimicrobial agents.

Quantification of biofilm formation was performed using a modified microtiter-plate assay.[7] *S. epidermidis* 9142 and its isogenic strain *S. epidermidis* 9142-M10 were used as positive and negative controls, respectively.[8] All isolates were tested in eight wells in two parallel runs, and this experiment was repeated on two independent occasions. In addition, Tryptic Soy Broth alone was used as a control to check sterility and nonspecific binding of media. The mean absorbance of non-inoculated wells was calculated and subtracted from all test values. The OD of each isolate (OD_I) was compared with the mean absorbance of the negative control (OD_{NC}).

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Biofilm formation ability of each of the isolates tested was classified as follows: non-biofilm producer ($OD_I \leqslant OD_{NC}$), weak biofilm producer ($OD_{NC} < OD_I \leqslant 2 \times OD_{NC}$), moderate biofilm producer ($2 \times OD_{NC} < OD_I \leqslant 4 \times OD_{NC}$) and strong biofilm producer ($OD_I > 4 \times OD_{NC}$).

All statistical analyses were performed using GraphPad Prism version 6.00 (La Jolla, CA, USA). Chi-square or Fisher's exact test were used to examine the relationship between categorical variables. A $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

A total of 61 unique isolates were included in the study. According to the questionnaire data, 5% of the volunteers were medical care employees, 37% reported having contact with medical staff, 25% were under treatment with antibiotics in the preceding 12 months and 12% were hospitalized during the same period.

Among the 61 CoNS isolates included in this study, *S. epidermidis* accounted for 51% of the strains. A study on community-associated CoNS isolates conducted by Widerström *et al.*[9] reported a similar result. Besides *S. epidermidis*, other CoNS species were isolated: *S. capitis* (15%), *S. hominis* (13%), *S. warneri* (10%), *S. haemolyticus* (6%), *S. equorum* (3%) and *S. pettenkoferi* (2%). Although *S. epidermidis* was the most frequent species isolated, these results revealed a great diversity of the skin staphylococcal flora, which may explain the increasing importance of other non-*S. epidermidis* CoNS in human infections.[10]

The results of antimicrobial susceptibility are shown in Table 1. The highest rates of resistance were detected for erythromycin (44%), penicillin (38%) and gentamicin (25%) whereas the lowest rate was exhibited for ciprofloxacin (7%), rifampicin (0%) and vancomycin (0%). Overall, 69% of the isolates were resistant to at least one class of antibiotics tested, and 39% exhibited resistance toward more than one antimicrobial class. Multidrug-resistance was observed in 15% of CoNS isolates. The frequency of resistance to gentamicin and penicillin was significantly higher in *S. epidermidis* than in other CoNS isolates.

Antibiotic resistance is a multifactorial phenomenon, and high rates of antibiotic consumption along with their misuse are pivotal factors that have created this serious public health issue.[11] Moreover, it is currently known that antibiotic molecules are widely disseminated in a broad range of environmental sources.[12] Hence their presence in different ecological niches may also account for the local selection of resistant bacteria. Recent data from a European surveillance program[13] reported that Portugal was one of the countries with higher rates of antibiotic consumption outside hospitals. Penicillins and macrolides (e.g., erythromycin) were the most prescribed drugs in the past years, which may partially explain the high levels of resistance for these classes of antibiotics found in our study. On the other hand, and in contrast with other studies on CoNS isolated from healthy individuals,[9,14] we observed a higher resistance rate to gentamicin. Furthermore, a considerable proportion of isolates (69%) exhibited a decreased susceptibility to vancomycin (MIC $\geqslant 2\,\mu g\,ml^{-1}$), especially those belonging to *S. epidermidis* species. Nevertheless, we found no significant difference in the distribution of resistant isolates among the different groups of subjects (Supplementary Information). These are worrisome results as it appears that resistance and/or decreased susceptibility to currently available drugs for most staphylococcal infections are becoming spread in our community. Moreover, the wide spread of multiresistant CoNS in the healthy population, colonizing even subjects without contact with hospital environment and/or antibiotic treatment in recent past was also observed in our study.

The study of biofilm formation revealed that 57% of isolates were able to produce biofilm. Of the 61 isolates, 34% were classified as weak, 10% as moderate and 13% as strong biofilm producers. Similar results have been reported by other researchers, who observed a significant number of commensal CoNS isolates capable of producing biofilm.[15,16] Interestingly, no significant difference in biofilm production was observed between *S. epidermidis* and other CoNS isolates ($P=0.300$). This finding supports the idea that biofilm production is also essential in an environment like the skin, where CoNS are exposed to extensive mechanical stress. We also found a significant higher frequency of antibiotic resistance in biofilm producers than in non-biofilm producers ($P=0.018$). Moreover, the frequency of multidrug-resistance among biofilm producers was significantly higher than in non-biofilm producers ($P=0.035$). The distribution of antibiotic resistance between biofilm- and non-biofilm-producing isolates is summarized in Table 2. Overall, our findings demonstrate an association between biofilm production and

Table 1 Antimicrobial resistance profiles to six antimicrobial agents ($\mu g\,ml^{-1}$) in 61 CoNS isolates recovered from the skin of healthy individuals

	S. epidermidis (n = *31*)		*Others CoNS*[a] (n = *30*)			
Antimicrobial agent	*MIC range*	*% Resistance*	*MIC range*	*% Resistance*	*Overall % resistance (n = 61)*	P-*value*[b]
Ciprofloxacin	0.25 to 8	6	⩽0.125 to 2	7	7	1.000
Erythromycin	⩽0.125 to >8	48	⩽0.125 to >8	40	44	0.610
Gentamicin	0.25 to 2	42	⩽0.125 to 2	7	25	0.002
Penicillin	⩽0.125 to >8	52	⩽0.125 to >8	23	38	0.030
Rifampicin	⩽0.015 to 0.03	0	⩽0.015 to 0.06	0	0	n/a
Vancomycin	1 to 4	0	⩽0.125 to 4	0	0	n/a

Abbreviation: n/a, not applicable.
[a]*S. capitis*, $n=9$; *S. hominis*, $n=8$; *S. warneri*, $n=6$; *S. haemolyticus*, $n=4$; *S. equorum*, $n=2$; *S. pettenkoferi*, $n=1$.
[b]Fisher's exact test ($P<0.05$ was considered statistically significant).

Table 2 Distribution of antibiotic resistance between biofilm- and non-biofilm-producing CoNS isolates recovered from the skin of healthy individuals

Antimicrobial resistance	*Biofilm-producing isolates*[a], n *(%)*	*Non-biofilm-producing isolates*[b], n *(%)*	P-*value*[c]
Ciprofloxacin	2 (6)	2 (8)	1.000
Erythromycin	18 (51)	9 (35)	0.148
Gentamicin	12 (34)	3 (12)	0.039
Penicillin	16 (46)	7 (27)	0.101
R = 0	7 (20)	12 (46)	0.049
R = 1	10 (29)	8 (31)	0.516
R = 2	10 (29)	5 (19)	0.298
R ⩾ 3[d]	8 (23)	1 (4)	0.035

[a]$n=35$.
[b]$n=26$.
[c]Fisher's exact test ($P<0.05$ was considered statistically significant).
[d]Multidrug-resistant isolates (R represents the number of antibiotics to which the isolates were found to be resistant).

antibiotic resistance in CoNS isolated from healthy individuals in community settings. A similar study that also included a few commensal CoNS isolated from hospitalized patients also reported the same association.[17]

CONCLUSIONS

To our knowledge, this is the first report on CoNS isolates (and not only *S. epidermidis*) recovered from healthy persons in the community, that correlates their antimicrobial resistance profiles to their biofilm formation ability. The results showed that a great proportion of isolates exhibited resistance to more than one class of antimicrobial agents, as well as high biofilm formation ability. Therefore, this study provides evidences that CoNS strains resistant to antibiotics and with biofilm formation ability are not confined to the hospital setting, but are indeed disseminated in the community. Furthermore, these results may partially explain why CoNS are becoming important nosocomial pathogens, despite their lack of recognized virulence factors other than biofilm formation. We conclude that the rate of antibiotic resistance in CoNS isolated from the skin of healthy individuals in Portugal is relatively high, which may contribute as reservoirs of resistance genes. The issue of antibiotic resistance among CoNS needs to be addressed through a more rational use of existing antibiotics as well as the development of novel antimicrobial agents. In the future, it would be interesting to investigate which of these isolates carry antibiotic resistance and/or biofilm-related genes and whether they have the ability to horizontally transfer these genes to more pathogenic species, particularly *S. aureus*.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)