Coagulase-negative staphylococci: pathogenesis, occurrence of antibiotic resistance genes and \textit{in vitro} effects of antimicrobial agents on biofilm-growing bacteria

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Coagulase-negative staphylococci (CoNS) are opportunistic pathogens that particularly cause infections in patients with implanted medical devices. The present research was performed to study the virulence potential of 53 clinical isolates of \textit{Staphylococcus capitis}, \textit{Staphylococcus auricularis}, \textit{Staphylococcus lugdunensis}, \textit{Staphylococcus simulans}, \textit{Staphylococcus cohnii} and \textit{Staphylococcus caprae}. All clinical strains were clonally unrelated. Isolates carried genes encoding resistance to \(\beta\)-lactam (\textit{mecA} (15\%)), aminoglycoside \([\text{aac}(6')/\text{aph}(2')]\) (11\%), \textit{aph} (3')-Ila (15\%), \textit{ant}(4')-la (19\%) and macrolide, lincosamide and streptogramin B (MLS\(_B\)) \([\text{erm} (A) \,(4\%), \text{erm}(B) \,(13\%), \text{erm}(C) \,(41\%), \text{msr}(A) \,(11\%)]\) antibiotics. CoNS isolates (64\%) were able to form biofilms. Confocal laser scanning microscopy revealed that these biofilms formed a three-dimensional structure composed mainly of living cells. All biofilm-positive strains carried the \textit{ica} operon. \textit{In vitro} studies demonstrated that a combination treatment with tigecycline and rifampicin was more effective against biofilms than one with ciprofloxacin and rifampicin. The minimum biofilm eradication concentration values were 0.062–0.5 \(\mu\text{g ml}^{-1}\) for tigecycline/rifampicin and 0.250–2 \(\mu\text{g ml}^{-1}\) for ciprofloxacin/rifampicin. All CoNS strains adhered to the human epithelial cell line HeLa, and more than half of the isolates were able to invade the HeLa cells, although most invaded relatively poorly. The virulence of CoNS is also attributed to their cytotoxic effects on HeLa cells. Incubation of HeLa cells with culture supernatant of the CoNS isolates resulted in cell death. The results indicate that the pathogenicity of \textit{S. capitis}, \textit{S. auricularis}, \textit{S. lugdunensis}, \textit{S. cohnii} and \textit{S. caprae} is multi-factorial, involving the ability of these bacteria to adhere to human epithelial cells, form biofilms and invade and destroy human cells.

INTRODUCTION

Coagulase-negative staphylococci (CoNS) are normal inhabitants of the human skin and mucous membranes, but can also cause a variety of infections, particularly in immunocompromised patients and those with implanted medical devices (Piette & Verschraegen, 2009). \textit{Staphylococcus capitis}, \textit{Staphylococcus cohnii}, \textit{Staphylococcus auricularis}, \textit{Staphylococcus simulans}, \textit{Staphylococcus caprae} and \textit{Staphylococcus lugdunensis} have been implicated in catheter-related bloodstream infections, osteomyelitis, bone and joint infections, prosthetic valve endocarditis, early-onset sepsis and wound infections (Piette & Verschraegen, 2009). \textit{S. lugdunensis} is more virulent than other CoNS species and can cause aggressive skin and soft tissue infections (Frank et al., 2008). The ability to form biofilms appears to play an essential role in staphylococcal virulence. Biofilms are surface-associated bacterial communities that are enclosed in a self-synthesized extracellular polymeric matrix composed of polysaccharides, such as poly-N-acetylglicosamine (PIA) encoded by icaADBC genes, cell wall-associated proteins, extracellular DNA and teichoic acid. Formation of biofilms protects micro-organisms from the host’s immune defence mechanisms and antibacterial therapies (Otto, 2004, 2012; Mack et al., 2006; Rodhe et al., 2006; Cerca et al., 2014; Le et al., 2014). Biofilm-associated infections are of particular concern because they are very difficult to treat using standard antibiotic-based therapies. In vitro data indicate that rifampin diffuses very well within
staphylococcal biofilms, but development of rifampicin resistance is common, caused by a single amino acid change in the β-subunit of RNA polymerase (Coiffier et al., 2013). Therefore, rifampicin is always used in combination with other anti-staphylococcal antibiotics. Tigecycline, a novel glyccycline antibiotic, is a promising antimicrobial agent with excellent in vivo and in vitro effects against meticillin-resistant Staphylococcus aureus (MRSA) strains (Aybar et al., 2012). Ciprofloxacin similarly displays potency against staphylococci, including meticillin-resistant strains (Fey, 2010).

Although staphylococci have long been recognized as extracellular pathogens, recent evidence points to S. aureus invasion of various types of host cells, including endothelial cells, bovine epithelial cells, keratinocytes and osteoblasts (Almeida & Oliver, 2001; Anaya-López et al., 2006; Hirshhausen et al., 2010; Shi & Zhang, 2012). Furthermore, certain staphylococci express a number of virulence factors such as tissue-damaging toxins and extracellular enzymes (i.e. protease, lipase and esterase), which play a role in local tissue destruction and invasion (Edward et al., 2010). Clinical isolates of S. lugdunensis were reported to produce synergistic haemolysis (Pereira et al., 2012). Synergistic haemolysis has similarly been observed in S. capitis, S. cohnii and S. simulans isolates (Donvito et al., 1997; Różalska & Szewczyk, 2008; Cui et al., 2013). Additionally, β-haemolysis was observed from S. caprae that was plated on blood agar (Ross et al., 2005).

In addition to their potential as pathogens, CoNS commonly develop resistance to multiple antibiotics and are a reservoir for resistance genes, which can be transmitted to other pathogens (Otto, 2012). The major mechanism of resistance to β-lactam antibiotics in CoNS is the expression of the mecA gene, which encodes an alternative penicillin-binding protein (PBPa) with a low affinity to these antibiotics (Geha et al., 1994). Aminoglycoside resistance is generally conferred by aminoglycoside-modifying enzyme (AME) activity inactivating the drug. Three AMEs are of particular importance, as these modify the therapeutically important aminoglycosides: the bifunctional enzyme AAC(6ʹ)/APH (2”) encoded by the aac(6ʹ)/aph(2”) gene, APH(3ʹ)-III encoded by the aaph(3’) gene and ANT(4’)-I encoded by the ant(4’)-Ia gene (Ardic et al., 2006). In staphylococci, resistance to the macrolide, lincosamide and streptogramin B (MLSb) antibiotics is generally due to three mechanisms: active efflux mediated by the mcr(A) gene, ribosomal target modification mediated by erm genes and enzymic drug inactivation mediated by the Inv(A) gene (Le Bouter et al., 2011).

In the present study, we evaluated the pathogenic potentials of S. capitis, S. auricularis, S. lugdunensis, S. simulans, S. cohnii and S. caprae, by assessing their biofilm formation abilities and their capacities for adhesion, invasion and cytotoxic activity on human epithelial cells. Moreover, we sought to determine the genomic relatedness of CoNS strains isolated from wounds, abscesses and blood of hospitalized patients and the presence of antibiotic resistance genes and biofilm-associated genes. We also investigated the efficacy of tigecycline/rifampicin and ciprofloxacin/rifampicin combinations on biofilm-growing bacteria.

METHODS

Bacterial strains. In total, 53 strains were isolated from wounds, abscesses and blood of hospitalized patients. These human specimens were collected to perform diagnostic tests on hospitalized patients. They were collected in a hospital laboratory, which is subject to the national registry of the National Chamber of Laboratory Diagnosticians and its code of ethics. All strains were identified using the VITEK 2 system (bioMérieux). Resistance to β-lactams was determined by the cefoxitin (30 µg) screen test as well as by amplification of the meca gene by a PCR technique. Analysis of susceptibility to antibiotic agents was also performed using the Vitek 2 system (bioMérieux). All strains were susceptible to vancomycin. We also used Escherichia coli K12C600 (Polish Collection of Microorganisms), Yersinia enterocolitica O:3/4 (pYV”) (Polish Collection of Microorganisms), Staphylococcus epidermidis ATCC 35984 and S. epidermidis strain ATCC 12228 (American Type Culture Collection). The isolates were stored in 50% (v/v) glycerol broth at −70°C until commencement of the study.

Box-PCR analysis. PCR was performed using a primer (5’-CTACGGCAAGGGCAGCTGCG-3’) complementary to BOX elements of bacterial genomic DNA (Versalovic et al., 1991). The results of BOX-PCR analysis were evaluated using GelCompar II (version 3.0; Applied Maths) software. The percentages of DNA fingerprint similarities were analysed using the Dice coefficient. The unweighted pair group method with arithmetic mean (UPGMA) clustering method was used to create a dendrogram.

Quantitative determination of biofilm formation. Biofilm formation was evaluated by the polystyrene microtitre plate method using crystal violet as described previously (Kim et al., 2008; Fredheim et al., 2009). All isolates were tested in six wells in two parallel runs. The highest and the lowest OD values were removed to exclude outliers, and the remaining values were averaged. Cells were grown overnight at 37°C in tryptic soy broth (TSB; Difco, Beckton Dickinson) with 0.25% glucose, diluted 1:100 in medium, and 100 µl of the bacterial suspension was seeded into 96-well plates. After overnight incubation at 37°C, the medium was gently removed from each well and the wells were washed three times with PBS to remove free-floating bacteria. The biofilms formed by adherent bacteria were stained with 0.4% crystal violet solution for 10 min and washed with water. The crystal violet that remained in the wells was dissolved using an ethanol/acetone mixture (70:30, v/v), and absorbance at 490 nm was determined. The strains were scored positive for biofilm formation if they had an OD490 >0.250. S. epidermidis strains ATCC 35984 and ATCC 12228 were used as positive and negative controls, respectively for biofilm formation.

Confocal laser scanning microscopy (CLSM). Overnight cultures of bacteria were added to Lab-TekII cell-culture chamber wells (Nunc) and incubated for 24 h (Qin et al., 2007). After removing the medium and washing gently three times with PBS to remove planktonic cells, adherent cells were stained using SYTO and PI (Live/Dead BacLight Bacterial Viability kits; Invitrogen) for 15 min and observed by fluorescence microscopy (LSM 510 Axiosvert 200M; Carl Zeiss). The Carl Zeiss confocal software and computer program COMSTAT was used for the analysis of three-dimensional biofilm images and data analysis (Heydorn et al., 2000; www.comstat.dk).
Biofilm susceptibility assay. Bacterial biofilms were formed on a modified polystyrene microtitre lid (TSP system, Nunc). The effects of treatment with tigecycline/rifampicin and ciprofloxacin/rifampicin were studied according to the procedure described by Moskowitz et al. (2004). Briefly, bacterial strains were grown for 22 h, adjusted to a turbidity of 0.5 McFarland, and 100 µl culture was transferred to the wells of a 96-well microtitre plate, covered with 96-peg lids and incubated for 20 h to allow biofilm development. The peg lids were rinsed three times and placed in a new microtitre plate containing twofold dilutions of antibiotics and incubated for 20 h at 37 °C. Cells were dislodged by sonication at room temperature for 5 min using a Polonic (Son-10) and transferred from the pegs into recovery media, and the OD was measured at 650 nm with a plate reader. The plate was incubated for 6 h, and subsequent growth was measured by OD_{650}. Growth was measured as the mean OD_{650} difference (OD_{650} at 6 h − OD_{650} at 0 h); values ≥0.05 were scored as positive growth. The minimum biofilm eradication concentration (MBEC) values were defined as the lowest concentration of antibiotics with no bacterial growth. We calculated a general linear model to determine the changes in growth due to the antibiotics. All statistical tests were performed using STATISTICA software (version 10.00; StatSoft). A P value of <0.05 was considered significant.

Adhesion and invasion assay. Adhesion to and invasion of HeLa cells by staphylococci were determined using a gentamicin/lysostaphin protection assay (Krzymińska et al., 2015). HeLa cells were infected with bacteria at an m.o.i. of 5 (6 × 10^4 cells were infected with approx. 3 × 10^6 bacteria) and cultivated for 90 min at 37 °C. To assess the total number of bacteria that adhered to epithelial cells, the infected monolayer was washed three times with PBS and lysed with 1 % Triton X-100, and c.f.u. were determined by plating serial dilutions on brain heart infusion (BHI) agar (Becton Dickinson GmbH). To assess the number of bacteria that had invaded the HeLa cells, infected HeLa cells were incubated for 2 h with 100 µg gentamicin ml^-1 and 20 µg lysostaphin ml^-1. Gentamicin does not penetrate eukaryotic cells, and lysostaphin is both unable to enter eukaryotic cells and is effective in eradicating extracellular bacteria, even those with aminoglycoside resistance. After 2 h incubation, cells were washed three times with PBS and lysed with 1 % Triton X-100. The number of invasive bacteria was determined by plating serial dilutions of the lysates on BHI agar. The number of attached staphylococci was determined by subtracting the number of intracellular bacteria from total bacterial counts.

Cytotoxic assay. Cytotoxic activity was assessed in a neutral red retention assay (Krzymińska et al., 2015). Bacteria were grown overnight in TSB media on a rotary shaker at 37 °C. After incubation, bacterial cultures were centrifuged at 2000 g for 30 min. Bacteria were removed from supernatants by filtration through membranes with a pore size of 0.22 µm (Roth). Monolayers of HeLa cells in 6-well dishes were incubated with 100 µl of supernatants for 24 h. Supernatants were removed, and 200 µl of neutral red (50 µg ml^-1) was added to each well and incubated for 3 h at 37 °C. Excess dye was removed, and cells were fixed with formalin in calcium chloride solution. Incorporated dye was eluted from the cells by adding a mixture of ethanol/acetate (70:30, v/v). The absorbance was measured at 540 nm using a plate reader. The results are the means of two separate experiments performed in triplicate. As a negative control, cells treated with non-pathogenic E. coli K12C600 filtrate were used.

Detection of the ica operon and the antibiotic resistance genes. Bacterial DNA was extracted using a Genomic DNA Prep Plus kit (A and A Biotechnology), and target genes were detected by PCR. The icaA/DBC genes were amplified using four primer pairs complementary to four regions in the ica operon (Chokr et al., 2006). To detect MLSβ resistance genes [erm(A), erm(B), erm(C)], msr(A) and lun(A)], the β-lactam resistance gene ( mecA) and aminoglycoside resistance genes [aac(6’)/aph(2’), aph (3’)-IIIa and ant(4’)-Ia], PCR assays were performed as previously described (Geha et al., 1994; Ardiz et al., 2006; Le Bouter et al., 2011). Amplification products were separated by electrophoresis in a 1.5 % agarose gel. DNA in the gel was stained with ethidium bromide, visualized on a UV light transilluminator and documented with a V.99 Bio-Print system (Vilber Lourmat).

RESULTS

Population structure

To characterize 53 CoNS strains isolated from patients treated at the Regional Hospital in Poznań, we generated a BOX PCR fingerprint for each strain; the resulting ampli- cons ranged in size from 250 to 2000 bp. Each strain produced a unique genomic profile. The DNA pattern similarity levels ranged from 25 to 87 % (Fig. 1). The results revealed considerable genomic diversity among our CoNS isolates.

CoNS biofilm formation and sensitivity to rifampicin in combination with tigecycline or ciprofloxacin in vitro

A total of 34 (64 %) isolates adhered to polystyrene surfaces and formed biofilms (Table 1), including 87.5 % of S. capitis, 80 % of S. lugdunensis, 64 % of S. auricularis and 40 % of S. cohnii strains. The icaA/DBC genes were present in all biofilm-positive strains. Although a small number of S. capræ isolates were recovered from clinical specimens in this study (four isolates), three of these isolates were positive for biofilm formation. In contrast, none of the S. simulans isolates produced biofilms. To obtain more detailed information about biofilms formed by CoNS, we analysed their structures by CLSM (Fig. 2). The images revealed that at 24 h, biofilms have complex, three-dimensional structures composed mainly of living cells. The thickness of biofilms ranged from 16 to 19 µm. All strains efficiently colonized the entire substratum (92–100 % coverage). The surface area to biovolume ratio of biofilms ranged from 3.17357 to 6.75788 µm^2 µm^-3 and biomass (i.e. volume of biomass divided by the area of view) ranged from 12.53526 to 20.86062 µm^3 µm^-2.

We selected 19 strains that created dense biofilm structures and represented different species, to estimate the most effective antibiotic combination for treating biofilm-associated infections. Planktonic forms of these strains were sensitive to rifampicin, tigecycline and ciprofloxacin. The MIC value for tigecycline ranged from ≤0.03 to 0.125 µg ml^-1, for rifampicin from 0.062 to 0.250 µg ml^-1 and for ciprofloxacin from 0.125 to 0.5 µg ml^-1. We found that rifampicin with tigecycline was more effective than ciprofloxacin/ rifampicin after 20 h of treatment (P=0.012; Table 2). The MBEC values for tigecycline/rifampicin ranged from 0.062 to 0.5 µg ml^-1, whereas the MBEC values for ciprofloxacin/ rifampicin were 0.250 to 2 µg ml^-1. None of the biofilm-forming isolates exhibited resistance to tigecycline (based on EUCAST planktonic susceptibility breakpoints). Sixteen isolates had MBEC values ≤1 µg ml^-1, which we
Fig. 1. Clinical isolates of CoNS strains have unique BOX-PCR profiles. Dendrogram generated from BOX-PCR profiles of CoNS strains using the UPGMA clustering method. The Dice band-based similarity coefficient was calculated with a band position tolerance of 1%.
### Table 1. Biofilm formation, adhesion, invasion, cytotoxic effects and presence of antibiotic resistance genes in CoNS strains

<table>
<thead>
<tr>
<th>Species (number of isolates)</th>
<th>Source (strain no.)</th>
<th>No. biofilm-positive strains*</th>
<th>No. strains showing cytotoxic activity; range (%)†</th>
<th>No. strains with ability to adhere to human epithelial cells (HeLa); range‡</th>
<th>No. strains with ability to invade human epithelial cells (HeLa); range, (median, %)§</th>
<th>Number of isolates with ica genes and antibiotic resistance genes¶</th>
<th>Number of isolates with ica genes and antibiotic resistance genes¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. capitis (16)</td>
<td>Blood (MPU S. capitis 1,3,4,5,8,10,13,15,16); abscess (MPU S. capitis 2,6,9,12); wound (MPU S. capitis 7,11,14)</td>
<td>14</td>
<td>16</td>
<td>16</td>
<td>9</td>
<td>14 4 3 4 3 0 3 7 1</td>
<td></td>
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<tr>
<td>S. auricularis (11)</td>
<td>Blood (MPU S. auricularis 1,2,5,6,7,8,10); abscess (MPU S. auricularis 3,4,9,11)</td>
<td>7</td>
<td>11</td>
<td>11</td>
<td>8</td>
<td>7 0 1 0 1 1 0 1 6 2</td>
<td></td>
</tr>
<tr>
<td>S. lugdunensis (10)</td>
<td>Blood (MPU S. lugdunensis 1,5,6,8); abscess (MPU S. lugdunensis 2,3,7,9,10); wound (MPU S. lugdunensis 4)</td>
<td>8</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>8 2 1 1 2 1 2 1 2 4 2</td>
<td></td>
</tr>
<tr>
<td>S. simulans (4)</td>
<td>Blood (MPU S. simulans 1,2,3,4,5,6,7)</td>
<td>0</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0 1 1 1 2 3 0 1 2 1 1</td>
<td></td>
</tr>
<tr>
<td>S. cohnii (5)</td>
<td>Blood (MPU S. cohnii 1,2,3,4,5)</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>2 1 0 1 0 0 1 1 0</td>
<td></td>
</tr>
<tr>
<td>S. caprae (4)</td>
<td>Blood (MPU S. caprae 1,3,4,5; MPU S. caprae 2)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3 0 0 0 1 0 0 2 0</td>
<td></td>
</tr>
</tbody>
</table>

*Biofilm formation was examined by crystal violet assay. All isolates were tested in six wells in two parallel runs. For each run, the highest and lowest OD values were removed to exclude outliers, and the remaining values were averaged. The strains were considered biofilm-positive when OD490 > 0.250.
†The percentage of cells destroyed due to extracellular toxins was assessed with a neutral red retention assay. All results are the means of two separate experiments performed in triplicate.
‡The mean c.f.u. number of adhered bacteria per 1×10⁶ HeLa cells.
§Percentage of invasive bacteria relative to the number of adherent cells. Invasion was determined using a gentamicin/lysostaphin protection assay.
¶Presence of genes encoding antibiotic resistance genes and ica genes in CoNS strains was detected using PCR assays. The primer sequences were as follows: for ica1 5′-AAGGTATTATGCGTTA TCAA-3′ (forward) and 5′-TCGTGTCTGGGTTTGAC-3′ (reverse), for ica2 5′-GAAAATAAGGTTATTGCGTTAATACTTA-3′ (forward) and 5′-TTAATACAGCAGATTTACCTATTCG-3′ (reverse), for ica3 5′-CAGTGTTCTATTTATATGAGCTGTGCTAG-3′ (forward) and 5′-AGCCACTTTCTTCCTTTTTAAATTCTTAG-3′ (reverse), and for ica4 5′-ATCAATATCATCCAA TTTACAGTTGCTTA-3′ (forward) and 5′-GAAATAAAGGTTATTGCGTTAATACTTA-3′ (reverse). Multiple-PCR amplifications were performed as follows: initial denaturation step of 5 min at 94°C; 25 cycles consisting of denaturation for 60 s at 94°C, annealing for 45 s at 51.5°C for ica1, 50.7°C for ica2, 52.4°C for ica3, 52.4°C for ica4 and extension of 2 min at 72°C. The primer sequences were as follows: for aac(6)/aph(2') 5′-GAAGTACGCAGAAGAGA-3′ (forward) and 5′-GAAGTACGCAGAAGAGA-3′ (reverse), for aph(3')-IIIa 5′-CATACTCTTTCCGAGCAA-3′ (forward) and 5′-CATACCTTTCCGAGCAA-3′ (reverse), for ant(4')-Ia 5′-AATCGTGGATAAGGCCCCA-3′ (forward) and 5′-GGACCTTGCGTATTGCA-3′ (reverse). PCR amplifications were performed as follows: initial denaturation step of 5 min at 94°C; 30 cycles consisting of denaturation for 2 min at 95°C, annealing for 1 min at 54°C and extension for 1 min at 72°C; and a final extension step of 7 min at 72°C. The primer sequences were as follows: for erm(A) 5′-CTCTAAAAGAATGTTAAAAAGAAA-3′ (forward) and 5′-CGATACCTTTGGTGTACCTTC-3′ (reverse), for erm(B) 5′-CGATACCTTTGGTGTACCTTC-3′ (forward) and 5′-CGATACCTTTGGTGTACCTTC-3′ (reverse), for erm(C) 5′-GCTATAGTTATATGCGTTAATTCC-3′ (forward) and 5′-GCTATAGTTATATGCGTTAATTCC-3′ (reverse), for msr(A) 5′-TGCTGAGAAAAGGACATTTAC-3′ (forward) and 5′-TGCTGAGAAAAGGACATTTAC-3′ (reverse), for msr(A) 5′-TGCTGAGAAAAGGACATTTAC-3′ (forward) and 5′-TGCTGAGAAAAGGACATTTAC-3′ (reverse), for lnu(A) 5′-GGTGGGTGGGTGGTGGTAGTTAATACCTGG-3′ (forward) and 5′-GGTGGGTGGGTGGTGGTAGTTAATACCTGG-3′ (reverse). PCR amplifications were performed as follows: initial denaturation step of 5 min at 95°C; 30 cycles consisting of denaturation for 30 s at 95°C, annealing for 30 s at 52°C for erm(A) and erm(C), 55°C for erm(B), 54°C for msr(A), 57°C for lnu(A) and extension for 30 s at 72°C; and a final extension step of 5 min at 72°C.
categorized as ciprofloxacin-sensitive (based on the standard for planktonic growth of MIC \( \leq 1 \) \( \mu \text{g ml}^{-1} \)). The MBEC values for tigecycline/rifampicin were twofold lower for nine Staphylococcus strains growing as biofilms than the corresponding MBEC values for ciprofloxacin/rifampicin. For seven isolates, tigecycline/rifampicin was only slightly more effective than ciprofloxacin/rifampicin. In the case of three strains, ciprofloxacin/rifampicin had the same effect on staphylococcal growth in biofilms as tigecycline/rifampicin.

**Adherence of CoNS to human epithelial cells**

All CoNS strains tested adhered successfully to HeLa cells, ranging from \( 2 \times 10^2 \) to \( 2.1 \times 10^4 \) c.f.u. per \( 10^5 \) HeLa cells (Table 1). The highest adhesion index was observed for S. lugdunensis (2 isolates), S. auricularis (1 isolate) and S. cohnii (1 isolate), ranging from \( 1 \times 10^3 \) to \( 2.1 \times 10^4 \) c.f.u. per \( 10^5 \) HeLa cells (Table 1). Non-pathogenic E. coli K12C600 was used as a negative control and had an adhesion index of 70 c.f.u. per \( 10^5 \) HeLa cells, whereas our positive control, Yersinia enterocolitica O:3/4, had an adhesion index of \( 2 \times 10^5 \) c.f.u. per \( 10^5 \) HeLa cells. Analysing the results of both assays, we found that the ability of CoNS to adhere to HeLa cell lines was correlated with the ability to form biofilm on polystyrene surfaces (P=0.012).

**Invasion of HeLa cells by CoNS**

We found that 27 (51 %) staphylococcus strains successfully invaded HeLa cells (Table 1). The percentage of internalized bacteria relative to the number of adherent cells ranged from 1 to 32 %. The control strains E. coli K12C600 and Y. enterocolitica O:3/4 demonstrated 0 and 57.3 % internalized cells relative to total adherent cells, respectively. Three isolates of S. auricularis demonstrated the greatest invasion activity (23–32 %) and S. capitis, S. lugdunensis, S. simulans, S. cohnii and S. caprae species exhibited a significantly lower invasive ability (1–10 %). Twenty-six (49 %) strains of S. capitis, S. auricularis, S. lugdunensis, S. simulans and S. caprae did not display any invasive ability. No invasion was observed for any of the S. simulans isolates tested.

**Cytotoxic activity**

We observed a cytotoxic effect on HeLa cells from the cell-free supernatants of CoNS strains, with 23 to 87 % cells destroyed after 24 h of incubation (Table 1). The highest activity (>76 % of cells destroyed) was observed for 23 (43 %) strains, including S. capitis (6 isolates), S. simulans (5 isolates), S. auricularis (3 isolates), S. lugdunensis (4 isolates), S. cohnii (1 isolate) and S. caprae (4 isolates) species. The lowest cytotoxicity (less than 20 % of cells destroyed) was observed for 4 isolates (7.5 %). The cell-free supernatant from the E. coli K-12 C600 strain, used as a negative control, demonstrated no cytotoxicity. Preheating the cell-free supernatants of 41 (77 %) strains at 56 °C for 20 min resulted in reduction of the cytotoxic effect from 3 to 87 % after 24 h.

**Antibiotic resistance genes**

The mecA gene, which confers resistance to almost all \( \beta \)-lactam antibiotics, was detected in only eight strains (15 %; Table 1). All isolates carrying the mecA gene also expressed a -MR-resistant phenotype. The most important gene conferring aminoglycoside resistance [\( \text{aac}(6^\prime)/\text{aph}(2^\prime) \)] was detected in six isolates (11 %). Eight isolates (15 %) harboured the \( \text{aph}(3^\prime) \)-IIIa gene, encoding phosphotransferase, and 10 isolates (19 %) carried the \( \text{ant}(4^\prime) \)-Ia gene encoding nucleotidyltransferase. Sixteen isolates were resistant to gentamicin and tobramycin. Resistance to erythromycin was detected in 19 isolates of CoNS, while none of isolates were resistant to clindamycin. The MLS\(_B\) resistance genes erm (A), erm(B), erm(C) and msr(A) were detected in 2 (4 %), 7 (13 %), 22 (41 %) and 6 (11 %) isolates, respectively. No isolates harboured the Inu(A) gene.

**DISCUSSION**

S. capitis, S. auricularis, S. lugdunensis, S. simulans, S. cohnii and S. caprae are rarely isolated from clinical specimens, and receive less attention than more frequently encountered staphylococcal species (i.e. S. epidermidis and Staphylococcus haemolyticus). The molecular bases for S. epidermidis and S. haemolyticus virulence generally, and in the context of biofilms, are well established, whereas the pathogenic potential of other CoNS is almost unknown (Krzymińska et al., 2012; Otto, 2012; Szcuka et al., 2015). The bacterial strains examined in this study were genetically diverse and carried genes coding for resistance to \( \beta \)-lactam, aminoglycoside and MLS\(_B\) antibiotics. However, the prevalence of these genes was low. A recent investigation in France concerning 53 S. capitis bloodstream isolates found that all meticillin-resistant S. capitis isolates from infants were clonally related. Isolates collected from other pediatric patients and adults exhibited high genetic diversity (Ragi-gade et al., 2007). In other studies, S. capitis strains obtained from blood cultures of neonates over a 5-year period were separated in five major clusters. The great majority of these isolates were meticillin-resistant and positive for the mecA gene (Cui et al., 2013). Other studies carried out in the neonatal intensive care unit showed that all S. caprae strains isolated from infants, during an 8-month period, belonged to a single clone. All of these were mecA-gene-positive (Ross et al., 2005). Significantly, we observed that all of the CoNS strains in the study were able to adhere to epithelial cells (HeLa). Adherence is considered an essential step in colonization and/or dissemination of bacteria into surrounding tissues and in the establishment of infections. Furthermore, the attachment of bacteria to a eukaryotic cell or polymeric surfaces is necessary to initiate the process of biofilm formation. In this study, we observed a high prevalence of biofilm formation in CoNS isolates.
formation among clinical isolates representing five species and originating from different sources. The biofilms formed by CoNS isolates appeared as a confluent mass of bacteria that was several layers thick and composed mainly of living cells. We detected the ica operon in all S. capitis, S. auricularis, S. lugdunensis, S. cohnii and S. caprae isolates, which all had the ability to form biofilms in vitro. These observations add to a growing body of research demonstrating widespread biofilm formation in CoNS isolates. It should be emphasized that PIA is a major functional component mediating intercellular adhesion in staphylococcal biofilm and protects bacteria from effectors of the immune response of the host (Mack et al., 2006). Biofilm formation and the presence of the ica operon have been demonstrated in S. lugdunensis (Frank et al., 2008). Recently, Greco-Stewart et al. (2013) showed that three ica-positive S. capitis isolates recovered from platelet concentrates had the ability to form biofilms under standard assay conditions. The presence of ica genes was also reported in S. caprae strains originating from human specimens or goat milk (Allignet et al., 2001). In contrast, none of the S. simulans isolates tested formed biofilms or carried ica genes. However, some isolates produced slime and formed biofilm (Koksal et al., 2009; Simojoki et al., 2012; Rumi et al., 2013; Osman et al., 2015).

As biofilms provide an extracellular barrier to antibiotics and the host’s immune system, it has been suggested that strains capable of forming biofilms may persist within the host, contributing to chronic and difficult-to-treat infections. In this study, we compared the effectiveness of rifampin in combination with tigecycline or ciprofloxacin against bacteria growing in a biofilm. Our study demonstrated that the MBEC range of tigecycline/rifampin was ≤0.5 µg ml⁻¹. If we apply conventional planktonic breakpoints, the biofilms studied here would be considered susceptible to these antibiotics. It should be added that the MIC values for all strains tested were very low. Previous studies have demonstrated that the tigecycline/rifampin combination is effective against PIA-dependent and PIA-independent biofilms formed by S. epidermidis and S. haemolyticus, respectively (Szczyka & Kaznowski, 2014; Szczyka et al., 2015). Experiments with an experimental rabbit model similarly revealed the positive effects of rifampin/tigecycline therapy on the treatment of osteomyelitis connected with MRSA strains (Yin et al., 2005). Our findings therefore add to the evidence that rifampin/tigecycline is a promising treatment option for biofilm-associated infections with various CoNS species. We found that the combination of tigecycline/rifampin was more effective than that of ciprofloxacin/rifampin for the isolates studied growing in biofilms. However, in the case of three strains, ciprofloxacin/rifampin displayed the same effect on staphylococcal biofilms as tigecycline/rifampin. Similarly, Qu et al. (2010) found that the effectiveness of ciprofloxacin on biofilm-growing strains varies depending on the strain. Clinical studies have shown the efficacy of ciprofloxacin and rifampin combination therapy in treating right-sided endocarditis (Heldman et al., 1996), and a study by Berdal et al. (2005) demonstrated that ciprofloxacin/rifampin combination therapy was successful for 24 out of 29 patients with prosthetic joint infections. Taken together, it appears that CoNS infections in patients may be treated by either tigecycline/rifampin or ciprofloxacin/rifampin combination, depending on the characteristics of the strain.

All CoNS strains examined in this study adhered to HeLa cells, whereas only 27 (51 %) strains were able to invade. These findings are important as internalization plays an important role during both the establishment and persistence of infections (Garzoni & Kelley, 2009). According to our knowledge, this is the first report demonstrating the ability of S. capitis, S. auricularis, S. cohnii and S. caprae to invade human epithelial cells. Szabados et al. (2011) described the invasion of endothelial cell line EA.hy 926 and the bladder carcinoma cell line 5637 by S. lugdunensis. Another study demonstrated that five S. lugdunensis isolates (of 23 tested) invaded human epithelial lung A549 cells (Pereira et al., 2012). In our study, five of ten S. lugdunensis strains tested invaded HeLa cells. Importantly, most CoNS strains demonstrated low internalization activity. Recently, Valour et al. (2013) demonstrated that S. epidermidis invades osteoblasts at an extremely low rate, suggesting that bone cell invasion is not a major pathophysiological mechanism in orthopaedic infections. In contrast, several studies have demonstrated a high S. epidermidis internalization rate within

**Fig. 2.** CoNS biofilms contain mostly living cells at 24 h. Confocal laser scanning micrographs of 24 h biofilms of S. capitis (a), S. auricularis (b) and S. lugdunensis (c) stained with SYTO9 (green) and PI (red).
endothelial cells and bovine mammary epithelial cells (Almeida & Oliver, 2001; Anaya-López et al., 2006; Hirschhausen et al., 2010). Thus, the rates of cell invasion may be specific to the CoNS strain and host cell type.

The virulence of S. capitis, S. auricularis, S. lugdunensis, S. simulans, S. cohnii and S. caprae is attributed to their cytotoxic effects towards epithelial cells. We observed a cytotoxic activity that was unstable after heat treatment, indicating that some bacterial toxins and extracellular enzymes are heat-labile proteins.

This study contributes to our understanding of the virulence mechanisms of CoNS species, such as S. capitis, S. auricularis, S. lugdunensis, S. simulans, S. cohnii and S. caprae. The pathogenesis of these bacteria is multi-factorial, involving adherence to the human epithelial cells and abiotic surfaces, biofilm formation and the ability to invade and destroy human cells. This study also demonstrated that the combination of tigecycline/rifampicin was more effective against bacterial residents than that of ciprofloxacin/rifampicin.

### REFERENCES


### Table 2. In vitro antimicrobial susceptibilities of staphylococci biofilm

<table>
<thead>
<tr>
<th>MBEC (µg ml⁻¹) of the following antimicrobial agents</th>
<th>Number of strains</th>
<th>Strain no.</th>
</tr>
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<tbody>
<tr>
<td>Tigecycline/rifampicin</td>
<td>0.062 0.250</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>MPU S. capitis 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPU S. auricularis 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPU S. auricularis 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPU S. caprae 3</td>
<td></td>
</tr>
<tr>
<td>Tigecycline/rifampicin</td>
<td>0.125 0.250</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>MPU S. capitis 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MPU S. auricularis 3</td>
<td></td>
</tr>
<tr>
<td>Tigecycline/rifampicin</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>MPU S. lugdunensis 8</td>
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</tr>
<tr>
<td></td>
<td>MPU S. lugdunensis 4</td>
<td></td>
</tr>
<tr>
<td>Tigecycline/rifampicin</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>MPU S. auricularis 15</td>
<td></td>
</tr>
<tr>
<td>Tigecycline/rifampicin</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>MPU S. capitis 7</td>
<td></td>
</tr>
<tr>
<td>Tigecycline/rifampicin</td>
<td>0.5 1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>MPU S. auricularis 8</td>
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<tr>
<td>Tigecycline/rifampicin</td>
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<td>1</td>
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<tr>
<td></td>
<td>MPU S. capitis 5</td>
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</tr>
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<td>Tigecycline/rifampicin</td>
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<td>3</td>
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<tr>
<td></td>
<td>MPU S. capitis 13</td>
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<td>Tigecycline/rifampicin</td>
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<td></td>
<td>MPU S. lugdunensis 6</td>
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</tr>
<tr>
<td>Tigecycline/rifampicin</td>
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<td>MPU S. lugdunensis 7</td>
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<tr>
<td></td>
<td>MPU S. lugdunensis 3</td>
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</tr>
<tr>
<td></td>
<td>MPU S. capitis 1</td>
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</tr>
</tbody>
</table>
Pathogenesis and antibiotic resistance of CoNS strains


