Characterization of Staphylococcus epidermidis and Staphylococcus warneri small-colony variants associated with prosthetic-joint infections

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We determined the frequency of isolation of staphylococcal small-colony variants (SCVs) from 31 culture-positive patients undergoing revision of total hip prosthesis for aseptic loosening or presumed prosthetic-joint infection (PJI). We analysed auxotrophy of cultured SCVs, their antimicrobial susceptibility profiles and their biofilm-forming capacity. Eight SCV strains were cultivated from six (19%) patients. All SCVs were coagulase-negative staphylococci (CNS) with Staphylococcus epidermidis as the predominant species; there was also one Staphylococcus warneri SCV. The SCVs were auxotrophic for haemin, with one strain additionally auxotrophic for menadione. We noted the presence of two phenotypically (differences concerning antimicrobial susceptibility) and genetically distinct SCV strains in one patient, as well as the growth of two genetically related SCVs that differed in terms of their morphology and the type of auxotrophy in another. Seven out of eight SCVs were resistant to meticillin and gentamicin. In addition, antibiotic sensitivity testing revealed three multidrug-resistant SCV–normal-morphology isolate pairs. One S. epidermidis SCV harboured icaADBC genes and was found to be a proficient biofilm producer. This paper highlights the involvement of CNS SCVs in the aetiology of PJIs, including what is believed to be the first report of a S. warneri SCV. These subpopulations must be actively sought in the routine diagnosis of implant-associated infections. Moreover, in view of the phenotypic and genetic diversity of some SCV pairs, particular attention should be paid to the investigation of all types of observed colony morphologies, and isolates should be subjected to antimicrobial susceptibility testing.

INTRODUCTION

The introduction of total joint arthroplasty into the clinical practice represented a milestone in the development of orthopaedic surgery. Although prosthetic-joint implants significantly improve quality of life among patients suffering from degenerative joint diseases and bone fractures, these procedures are associated with a risk of complications, including aseptic loosening (AL) and prosthetic-joint infection (PJI). The diagnostic yield of PJIs is hampered by the phenotypic change of bacteria into a sessile and resistant form strictly adherent to the surface of the device called a biofilm, the development of small-colony variants (SCVs) or by the possibility of intracellular persistence of causative micro-organisms (Nelson et al., 2005; Sendi et al., 2010; Trampuz & Zimmerli, 2005).

SCVs are naturally occurring subpopulations of bacteria demonstrating distinctive phenotypic characteristics and pathogenic traits. Phenotypically, SCVs have a slow growth...
rate, atypical colony morphology associated with the formation of pinpoint or ‘fried egg’ colonies and unusual biochemical features, which pose a challenge in terms of their laboratory isolation, identification and susceptibility testing. SCVs have been described for a wide spectrum of bacterial genera and species; however, they have been most extensively studied for staphylococcii, especially for *Staphylococcus aureus* (Maduka-Ezech et al., 2012; Proctor et al., 2006; von Eiff, 2008).

The tiny size of SCVs on solid agar is often due to auxotrophy for haemin and/or menadione, two compounds involved in the biosynthesis of electron transport chain components. Auxotrophy to the above-mentioned compounds is associated with defects in electron transport and, consequently, altered membrane potential. The abnormal membrane potential, in turn, may confer on these variants innate resistance to aminoglycosides, since the ability of these antibiotics to gain access to intracellular target sites depends on the proton motive force. Resistance to folic acid antagonists such as trimethoprim-sulfamethoxazole has been observed in thymidine-dependent SCVs, which may bypass the effect of these antimicrobial agents by uptake of exogenous thymidine. Moreover, it has been suggested that antibiotics administered systemically or locally may actually select for SCVs (Besier et al., 2008; Kahl et al., 1998; Maduka-Ezech et al., 2012; Proctor et al., 2006; von Eiff, 2008; von Eiff et al., 1997).

Several studies have also demonstrated the enhanced biofilm-forming ability of laboratory-derived SCVs of *S. aureus* and *Staphylococcus epidermidis* compared with normal-morphology counterparts (Al Laham et al., 2007; Singh et al., 2009, 2010). Another aspect of the pathogenic potential of SCVs associated with their ability to cause persistent or relapsing infections is the ability of these subpopulations to invade and survive inside non-professional phagocytes, such as epithelial and endothelial cells, where they are protected against the host immune system and antimicrobial agents (Maduka-Ezech et al., 2012; Proctor et al., 2006; Sendi et al., 2010; Sendi & Proctor, 2009; Tuchscherr et al., 2010; von Eiff et al., 2001).

According to the available literature data, the pathogenesis of PJIs has been associated with SCVs populations produced by *S. aureus*, *S. epidermidis* and *Escherichia coli* (Maduka-Ezech et al., 2012; Proctor et al., 1995; Roggenkamp et al., 1998; Sendi et al., 2006, 2010). However, the number of reports in this field is unsatisfactory and the prevalence of staphylococcal SCVs, especially those produced by non-*S. aureus* species in orthopaedic infections, is still largely unknown.

The purpose of the study was to determine the relative frequency of isolation of SCVs from patients undergoing surgical revision of prosthetic hip joints due to implant loosening. We also studied the characteristics of the isolates including their auxotrophy, antimicrobial susceptibility and biofilm-forming capacity.

**METHODS**

From April 2010 to January 2012, bacterial isolates were obtained from culture-positive patients undergoing removal of a total hip prosthesis for AL or presumed PJI at the University Hospital in Lublin, Poland, and at the Public Clinical Hospital in Otwock, Poland, respectively. The two groups enrolled in the study included 12 patients (AL) and 19 patients (presumed PJI).

The preliminary criteria taken into account for the clinical classification of implant failure as associated with PJI (19 patients) was the development of a sinus tract communicating with the prosthesis. This is a definitive symptom of a periprosthetic infectious process according to recommendations published by Parvizi et al. (2011). The microbiological diagnostic proceedings were supplemented by the analysis of systemic markers of inflammation [erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) concentration] in the direct preoperative period in order to interpret the clinical significance of positive culture results.

Another group of 12 patients initially qualified as suffering from AL experienced a prosthesis failure in the absence of a sinus tract and/or purulence in the affected joint. The microbiological diagnostic proceedings were supplemented by the analysis of local (synovial fluid leukocyte count and differential) and systemic markers of inflammation in order to verify the clinical significance of positive culture results. Synovial fluid samples were collected intraoperatively, whereas serum ESR and CRP concentration were determined in the direct preoperative period.

In general, the clinical significance of positive culture results was inferred on the basis of recommendations published by Parvizi et al. (2011), according to which a definite diagnosis of PJI can be made when the following conditions are met: (i) There is a sinus tract communicating with the prosthesis; or (ii) a pathogen is isolated by culture from two separate tissue or fluid samples obtained from the affected prosthesis joint; or (iii) four of the following six criteria exist: (a) elevated ESR or serum CRP concentration*; (b) elevated synovial leukocyte count**; (c) elevated synovial neutrophil percentage*; (d) presence of pusulence in the affected joint; (e) isolation of a microorganism in one culture of periprosthetic tissue or fluid; (f) greater than five neutrophils per high-power field in five high-power fields observed from histological analysis of periprosthetic tissue at \( \times 400 \) magnification*** [2], parameters of serum ESR and CRP exceeding 30 mm h\(^{-1}\) and 10 mg l\(^{-1}\), respectively, were interpreted as elevated and indicative of an ongoing inflammation (Parvizi et al., 2011; Trampuz et al., 2007); **, cut-off values concerning the synovial leukocyte count (>1700 cells \( \mu \)l\(^{-1}\)) and synovial neutrophil percentage (>65%) were adopted from Trampuz et al. (2007); ***, pathohistological examination was not performed in the study; criteria (a)–(e) of the recommendations by Parvizi et al. (2011) were taken into account for the final interpretation of culture results.

**Sample collection.** Intraoperatively, tissue samples from close proximity to the implant and demonstrating the most obvious inflammatory changes were collected for microbiological studies. At least three tissue samples were collected from each patient. The synovial fluid was collected intraoperatively from patients with the clinical diagnosis of AL for leukocyte count and differential, as well as for microbiological culture.

The explanted prosthetic components were placed in 1 l, straight-sided, wide-mouthed polypropylene jars that had been autoclaved at 132 °C for 15 min. The specimens were processed by the microbiology laboratory within 2 h.

**Conventional microbiological culture.** Synovial fluid was inoculated in 100 μl aliquots onto a set of routine aerobic and anaerobic bacteriological media plates. Tissue specimens were inoculated into
thioglycollate broth and incubated at 35–37 °C. Cloudy thioglycollate broth was subcultured onto conventional bacteriological media plates. The microbiological cultures of synovial fluid and subcultured tissue specimens lasted for up to 14 days.

**Sonication of removed prostheses.** Briefly, the explanted prostheses were placed in sterile polystyrene containers. Five hundred millilitres of sterile saline were added to each container. The container was vortexed for 30 s and subsequently subjected to sonication (Bransonic ultrasonic cleaner; G. Heinenmann) for 7 min at a temperature of 20 °C. Sonication was followed by additional vortexing for 30 s. The resulting sonicate fluid was removed under aseptic conditions and placed into 50 ml sterile Falcon tubes. Samples were then centrifuged at 4200 r.p.m. for 20 min. Aliquots (100 μl) of the sedimented sonicate fluid were inoculated onto a set of routine aerobic and anaerobic bacteriological media plates. Incubation (at 35–37 °C) lasted for up to 14 days. The sonication procedure was based on the publications of Monsen et al. (2009) and Trampuz et al. (2007).

All media plates were inspected daily for microbial growth. The culture result was considered positive when there were at least 5 c.f.u. of the same organism on either plate. Normal-morphology staphylococci were identified to the species level using the commercially available biochemical test API Staph (bioMérieux).

**SCV identification.** The identification of SCVs growing in cultures was initiated with the use of conventional laboratory methods including Gram staining, catalase reaction, and growth on sheep blood agar and mannitol salt agar (MSA), as well as the coagulase test. SCVs were defined as pinpoint colonies, 1/10th or less than the size of normal staphylococcal colonies when grown on sheep blood agar. The growth of the SCV colonies was observed after at least 48 h of incubation in air. Stimulation of SCV growth was observed on sheep blood agar and on Schaedler agar incubated under microaerophilic and anaerobic conditions, respectively. The size of colonies growing on the Schaedler agar was similar to the size of colonies characteristic of the normal phenotype (NP).

The identification of the SCVs to the species level was performed with the GenoType Staphylococcus assay (Hain Lifescience) based on PCR followed by hybridization. The assay was performed according to the manufacturer’s instructions.

**Repetitive sequence-based PCR (rep-PCR).** For SCVs that were co-isolated with normal colony-morphology micro-organisms belonging to the same species and cultured from the same patient, relatedness between the SCV and the normal-morphology isolates was assessed using a commercially available DNA fingerprinting assay based on rep-PCR technology.

DNA from staphylococcal colonies was extracted using a MoBio Ultra Clean microbial DNA isolation kit (Mo Bio Laboratories) according to the manufacturer’s instructions. The extracted DNA was amplified using a DiversiLab Staphylococcus DNA fingerprinting kit (bioMérieux), which included rep-PCR master mix 1, Staphylococcus primers and kitspecific positive and negative controls, in accordance with the manufacturer’s product insert. Briefly, 2 μl genomic DNA (concentration of 25–50 ng μl⁻¹), 0.5 μl AmpliTaq DNA polymerase (concentration of 5 U μl⁻¹) and 2.5 μl 10 x PCR buffer (Applied Biosystems) were added to the rep-PCR master mix to achieve a total volume of 25 μl.

Thermal cycling parameters for a Sensique thermocycler were as follows: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, extension at 70 °C for 90 s and a final extension at 70 °C for 3 min.

Analysis of rep-PCR products was implemented using a DiversiLab system in which the amplified fragments of various sizes and intensities are separated and detected using a microfluidics Labchip with an Agilent 2100 Bioanalyser (Agilent Technologies). The relatedness of isolates was determined by cluster analysis and guidelines provided by the manufacturer. In general, isolates were categorized as indistinguishable (when there were no band differences), similar (1–2 band differences) or different (≥ 3 band differences).

**Auxotrophy testing.** Auxotrophy for haemin, menadione and thymidine was evaluated using the disc method. Overnight cultures were diluted to a 0.5 McFarland standard and inoculated onto tryptic soy agar (TSA; Becton Dickinson) and Mueller–Hinton II agar (MHA; Becton Dickinson). Discs impregnated with 15 μl menadione and thymidine (Sigma-Aldrich) at a concentration of 100 μg ml⁻¹ were placed on the TSA and MHA media, respectively. Haeminauxotrophy was investigated on the TSA medium using commercially available haemin (X factor) discs (Sigma-Aldrich). Isolates were considered auxotrophic when they demonstrated increased growth around the impregnated disc compared with the periphery. Auxotrophy testing methodology was based on the following publications: Maduka-Ezeh et al. (2012), Proctor et al. (1995) and von Eiff et al. (1999).

**Antimicrobial susceptibility.** Sensitivity of SCVs and their normal-morphology counterparts to antimicrobial drugs was evaluated by disc diffusion tests and Etests (bioMérieux). The Etests were applied to determine susceptibilities to co-trimoxazole, gentamicin, vancomycin, teicoplanin and linezolid. The disc diffusion method was used for cefoxitin, erythromycin, clindamycin, rifampicin, ciprofloxacin and fusidic acid susceptibility testing. The tests were performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations, except that in addition to reading susceptibilities at 16–20 h they were also read at 48 h to accommodate the slow growth rate of SCVs. Molecular detection of the mecA gene was performed to verify meticillin resistance among SCV isolates (GenoType Staphylococcus; Hain Lifescience) according to the manufacturer’s instructions.

**Biofilm production.** The ability of the cultured SCVs and their NP counterparts to produce biofilm was assessed by the molecular detection of icaABDC genes, which are involved in the biosynthesis of the polysaccharide intercellular adselin (PIA), and by the quantitative microtitre plate assay to verify the ability of isolates to produce biofilm in vitro. The microtitre plate assay was followed by the addition of trypsin in order to investigate the possibility of protein-mediated mechanisms of biofilm production. For all methods, a reference strain of Staphylococcus epidermidis ATCC 35984 was used as a positive control.

Bacterial DNA for the PCR detection of genes belonging to the ica operon was isolated using a procedure recommended for the GenoType Staphylococcus assay (Hain Lifescience). Briefly, staphylococcal colonies grown on blood agar medium were collected with an inoculation loop and suspended in 150 μl water. The solution was incubated at 95 °C for 10 min followed by 10 min incubation in an ultrasonic bath. The solution was then centrifuged for 5 min at maximum speed and the supernatant was transferred to a new tube.

Primer sequences and amplification conditions used in the PCR assay are shown in Table 1. Amplification products were analysed by 2% agarose gel electrophoresis.

**Microtitre plate assay.** Quantitative determination of biofilm production was performed with the use of a microtitre plate assay with crystal violet. Briefly, for each isolate representing the NP, the overnight bacterial culture was adjusted with tryptic soy broth (TSB; bioMérieux) to match the turbidity of the 0.5 McFarland standard. The suspension was subsequently incubated overnight at 37 °C. The solution was diluted 1:100 in TSB and 200 μl aliquots were
inoculated into three wells each of a 96-well sterile microtitre plate. The plates were incubated overnight at 37 °C in air, washed and stained with 0.1% crystal violet. The optical density was measured at 570 nm (Paluch-Oles et al., 2011).

In the case of isolates representing the SCV phenotype, cultures were adjusted to TSB to match the turbidity of the 0.5 McFarland standard. The subsequent steps of the applied procedure were analogous to those described above for the NP counterparts, with the exception of a modification concerning the conditions of incubation of the diluted bacterial suspensions in the microtitre plate. Namely, the incubation was conducted for 48 h and 72 h at 37 °C due to the slow growth rate of SCVs. Both aerobic and microaerophilic conditions were applied in the experiment due to the fact that the growth of the isolated SCVs was stimulated in the presence of higher CO₂ concentration.

In order to identify the ica-independent, protein-mediated mechanisms of biofilm formation, trypsin at a concentration of 100 μg ml⁻¹ (Rohde et al., 2007) was added to the wells after overnight incubation (NP counterparts), and after 48 and 72 h of incubation (SCVs), for 16 h at 37 °C. The plates were subsequently washed and stained with 0.1% crystal violet. The optical density was measured at 570 nm.

Classification of the degree of bacterial adherence to the wells of the microtitre plate was based on the comparison of the mean optical density value obtained for each isolate tested to the cut-off optical density calculated with the use of the negative control (Paluch-Oles et al., 2011). ODc—the mean optical density of the negative control + 3 × SD.

The bacterial isolate was identified as not capable of biofilm production when its mean optical density was ≤ ODc; a moderate biofilm producer was identified when the mean optical density was > ODc but < 2 × ODc; whereas the ability of the isolate to produce a strong biofilm was identified when the mean optical density was ≥ 2 × ODc. Wells containing sterile TSB served as a negative control.

RESULTS

Eight SCV strains were cultivated from six (19%) patients including those with the clinical diagnosis of aseptic and septic implant failure (Table 2). All SCVs were represented by coagulase-negative staphylococci (CNS). S. epidermidis was the predominant species – it was isolated from five patients, whereas one patient was infected with Staphylococcus warneri SCV.

The SCVs were isolated from various types of clinical samples including the sonicate fluid and periprosthetic tissue cultures. The sites of their isolation are shown in Table 2. Only one patient (no. 49) was infected with S. epidermidis SCV growing in monoculture. The remaining patients were infected with a mixture of SCVs and their NP counterparts. Fig. 1 shows differences concerning the size of colonies produced on sheep blood agar by the SCV and NP isolates cultured from one of the patients.

Auxotrophy testing revealed that all SCVs were auxotrophic for haemin. One S. epidermidis SCV isolate (57 SCV2) was additionally auxotrophic for menadione.

Additionally, we noted the growth of pairs of SCVs with different appearances in two patients (Table 2). (i) Isolate no. 57 SCV1 produced larger colonies, grew faster (its observation on blood agar was possible after 48 h incubation at the primary isolation from the sonicate fluid culture) and was auxotrophic for haemin; isolate no. 57 SCV2 produced smaller colonies, exhibited a very slow growth rate (its observation on blood agar was possible after 96 h incubation at the primary isolation from the sonicate fluid culture) and was auxotrophic both for haemin and menadione. (ii) O21 SCV1 was resistant to co-trimoxazole and demonstrated a high gentamicin MIC (> 256 μg ml⁻¹), whereas O21 SCV2 was sensitive to co-trimoxazole and although resistant, had a lower MIC for gentamicin (48 μg ml⁻¹).

The rep-PCR molecular fingerprinting of co-isolated SCVs and normal-colony isolates revealed the following (Fig. 2): (i) five indistinguishable pairs (no band difference): 4 NP–4SCV, 57 NP–57 SCV1, O16 NP–O16 SCV, O22 NP–O22 SCV and O21 NP–O21 SCV1; (ii) one related pair (one band difference): 57 NP–57 SCV2; (iii) one unrelated pair (more than three band differences): O21 NP–O21 SCV2.

**Table 1. Primer sequences and amplification conditions used for the PCR detection of the icaADBC genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (S. epidermidis template)</th>
<th>PCR conditions</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaA</td>
<td>Forward: 5'-GACCTCGAAGTC AATAGAGGT-3'</td>
<td>60 s, 94 °C</td>
<td>814</td>
<td>Ziebuhr et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CCCATATACAGTTAAGTAC-3’</td>
<td>60 s, 60 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaB</td>
<td>Forward: 5’-ATGCTTTAGACAGCAGGTC-3’</td>
<td>2.5 min, 72 °C</td>
<td>526</td>
<td>Ziebuhr et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TATCGGCTATGTTGAGTAC-3’</td>
<td>60 s, 94 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaC</td>
<td>Forward: 5’-ATACCTGATTAGTATTATT-3’</td>
<td>60 s, 94 °C</td>
<td>989</td>
<td>Ziebuhr et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-ATACCTGATTAGTATTATT-3’</td>
<td>60 s, 45 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaD</td>
<td>Forward: 5’-GGGCTTAAAGCACGACGC-3’</td>
<td>2.5 min, 72 °C</td>
<td>282</td>
<td>de Silva et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GGGCTTAAAGCACGACGC-3’</td>
<td>60 s, 94 °C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Antimicrobial-susceptibility testing results**

Among eight staphylococcal SCVs, seven were meticillin-resistant, which was confirmed by the molecular detection
Table 2. Characterization of SCVs and their normal-morphology counterparts cultured from patients included in the study

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Species</th>
<th>Site of isolation (no.)</th>
<th>Genetic relatedness of SCV/NP pairs</th>
<th>Gentamicin MIC (µg ml(^{-1})) [S/R]</th>
<th>TMP–SXT MIC (µg ml(^{-1})) [S/R]</th>
<th>Cefoxitin</th>
<th>meca</th>
<th>Resistance to other antibiotics</th>
<th>Auxotrophy</th>
<th>Type of implant loosening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small- and normal-colony isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 NP</td>
<td><em>S. epidermidis</em></td>
<td>SF, T (1)</td>
<td>Indistinguishable</td>
<td>24 [R]</td>
<td>0.064 [S]</td>
<td>R</td>
<td>ND</td>
<td>MLS(_B), RA</td>
<td>–</td>
<td>Aseptic</td>
</tr>
<tr>
<td>4 SCV</td>
<td>T (1)</td>
<td></td>
<td></td>
<td>&gt;256 [R]</td>
<td>0.094 [S]</td>
<td>R</td>
<td>Positive</td>
<td>MLS(_B), RA</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>57 NP</td>
<td><em>S. epidermidis</em></td>
<td>SF, T (2)</td>
<td>Indistinguishable</td>
<td>&gt;256 [R]</td>
<td>&gt;4 [R]</td>
<td>R</td>
<td>ND</td>
<td>Positive</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>57 SCV1</td>
<td>SF</td>
<td></td>
<td></td>
<td>&gt;256 [R]</td>
<td>0.38 [S]</td>
<td>R</td>
<td>Positive</td>
<td>–</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>57 SCV2</td>
<td>SF</td>
<td>Related</td>
<td></td>
<td>&gt;256 [R]</td>
<td>0.38 [S]</td>
<td>S</td>
<td>Positive</td>
<td>–</td>
<td>H + M</td>
<td></td>
</tr>
<tr>
<td>O16 NP</td>
<td><em>S. warneri</em></td>
<td>T (1)</td>
<td>Indistinguishable</td>
<td>12 [R]</td>
<td>0.012 [S]</td>
<td>R</td>
<td>ND</td>
<td>MLS(_B)</td>
<td>–</td>
<td>Septic</td>
</tr>
<tr>
<td>O16 SCV</td>
<td>SF, T (1)</td>
<td></td>
<td></td>
<td>48 [R]</td>
<td>0.064 [S]</td>
<td>R</td>
<td>Positive</td>
<td>MLS(_B)</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>O21 NP</td>
<td><em>S. epidermidis</em></td>
<td>T (2)</td>
<td>Indistinguishable</td>
<td>&gt;256 [R]</td>
<td>12 [R]</td>
<td>R</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>Septic</td>
</tr>
<tr>
<td>O21 SCV1</td>
<td>T (1)</td>
<td></td>
<td></td>
<td>&gt;256 [R]</td>
<td>12 [R]</td>
<td>R</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>Septic</td>
</tr>
<tr>
<td>O21 SCV2</td>
<td>SF, T (1)</td>
<td>Unrelated</td>
<td></td>
<td>48 [R]</td>
<td>0.094 [S]</td>
<td>R</td>
<td>Positive</td>
<td>–</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>O22 NP</td>
<td><em>S. epidermidis</em></td>
<td>T (1)</td>
<td>Indistinguishable</td>
<td>0.016 [S]</td>
<td>0.125 [S]</td>
<td>R</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>Septic</td>
</tr>
<tr>
<td>O22 SCV</td>
<td>SF, T (1)</td>
<td></td>
<td></td>
<td>0.38 [S]</td>
<td>0.125 [S]</td>
<td>R</td>
<td>Positive</td>
<td>–</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Small-colony isolate in monoculture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49 SCV</td>
<td><em>S. epidermidis</em></td>
<td>SF</td>
<td>–</td>
<td>&gt;256 [R]</td>
<td>0.125 [S]</td>
<td>S</td>
<td>Negative</td>
<td>–</td>
<td>H</td>
<td>Aseptic</td>
</tr>
</tbody>
</table>

H, haemin; M, menadione; MLS\(_B\), resistance to macrolides, lincosamides and group B streptogramins; ND, not done; R, resistant; RA, resistance to rifampicin; S, sensitive; SF, sonicate fluid; T (no.), periprosthetic tissue (no. of tissues from which growth of the given staphylococcal isolate was obtained); TMP–SXT, trimethoprim–sulfamethoxazole.
of the mecA gene. One isolate was sensitive to meticillin as detected by the use of the disc diffusion method (cefoxitin disc), but the mecA gene was detected by PCR assay. As many as seven out of eight SCVs were resistant to gentamicin, which was accompanied by haemin auxotrophy in all of the isolates. There were three multidrug resistant (i.e. resistant to at least three distinct groups of antimicrobial drugs) SCV–NP pairs. The results of the antibiotic-sensitivity testing of SCV isolates are shown in Table 2.

In general, antibiotic-resistance profiles of SCVs and their co-isolated NP counterparts were concordant with the exception of sensitivity to co-trimoxazole demonstrated by the 57 SCV1 and 57 SCV2 isolates, whereas the normal-morphology isolate was resistant to this drug.

In addition, for three out of six and two out of six SCV–NP pairs, the small-colony subpopulations had higher gentamicin and co-trimoxazole MIC values, respectively, than their normal-morphology counterparts even when they remained within the resistance/susceptibility range (Table 2).

### Biofilm production

Genes belonging to the ica operon were detected in only one SCV S. epidermidis isolate (no. 49 SCV, ica ADBC+). This isolate was a single proficient biofilm producer in the applied microtitre biofilm assay. The ability of the isolate to produce biofilm was not affected by the application of the proteolytic enzyme.

The remaining SCVs demonstrated a moderate degree of adhesion. Biofilm production was observed in all of the SCVs after incubation under microaerophilic conditions irrespective of the time of incubation (48 or 72 h). It was further assumed that the production of biofilm by these SCV isolates could have been protein mediated since the adhesion properties were lost after the application of trypsin. It has also been concluded that the majority of SCVs did not demonstrate enhanced biofilm-forming capacity compared with their NP counterparts. The exception was S. warneri SCV with a moderate ability to produce biofilm in the microtitre assay, whereas its parental strain was non-adherent in vitro. The reaction of the SCV–normal-morphology isolate pairs to trypsin was
also similar – isolates with the NP lost (with the exception of one isolate, no. 4), as did the corresponding SCVs, the adhesion ability after the application of the enzyme (Table 3).

**DISCUSSION**

The study was focused on the determination of the frequency of isolation of SCVs from patients undergoing revision of hip prostheses due to loosening. Among 31 culture-positive patients enrolled in the study, microbiological investigation revealed 6 (19%) cases of infection associated with staphylococcal SCVs. The subpopulations were cultured both from patients with the clinical diagnosis of AL and those who met the clinical criteria of PJI. At the same time, these findings highlight the need for thorough microbiological diagnostic proceedings in every case of loosened total hip prosthesis, since ‘aseptic’ implant failure may in fact be associated with a subclinical infection. Of note, only CNS SCVs were cultured. They were predominantely represented by *S. epidermidis*; one patient was infected with *S. warneri* SCV.

Maduka-Ezeh et al. (2012) reported the isolation of SCVs of *S. epidermidis* in 12 out of 31 patients who had explanted prosthetic joints due to infection. These authors also revealed three cases of PJI associated with *S. aureus* SCVs. Sendi et al. (2006) in turn reported the ratio of SCV infections to all infections in patients with total hip arthroplasty as 5:66 (7.5%) during a 4-year period. These authors identified SCVs produced only by *S. aureus*. Generally, there is a scarcity of literature data on the involvement of SCVs produced by staphylococci other than *S. aureus* in the aetiology and pathogenesis of human infections. In addition to the above-mentioned publication (Maduka-Ezeh et al., 2012), the only available data in this field include a few reports dealing with the isolation of SCVs produced by *S. epidermidis*, *Staphylococcus capitis* and *Staphylococcus lugdunensis* from cases of pacemaker-related bloodstream infections (Seifert et al., 2005; von Eiff et al., 1999). Adler et al. (2003) described a teicoplanin-resistant SCV of *S. epidermidis* that emerged during vancomycin therapy for a catheter-associated bloodstream infection in a neutropenic patient. To the best of the knowledge of the authors of this publication, this is the first report on the isolation of SCVs produced by *S. warneri* from a patient suffering from PJI.

All SCVs reported in this study were auxotrophic for haemin, whereas one isolate was additionally auxotrophic for menadione. This double auxotrophy (associated with electron transport deficiencies) was the probable cause of an extremely slow growth rate and the production of very small colonies by the isolate. The auxotrophy testing results obtained in this study are in agreement with the characteristics of CNS SCVs reported by von Eiff et al. (1999), who also revealed haemin dependencies for all isolated CNS SCVs, but they stand in contrast to results of auxotrophy testing conducted by Maduka-Ezeh et al. (2012). These authors revealed that none of their *S. epidermidis* SCVs was auxotrophic for haemin, menadione or thymidine. Moreover, among the 11 SCV isolates tested, 5 demonstrated reduced susceptibility to at least one aminoglycoside tested. This observation led the authors to the conclusion that aminoglycoside resistance may not be a uniform property of staphylococcal SCVs and may be limited to SCVs of certain species and/or to SCVs that are menadione or haemin auxotrophic.

In our study, all SCV isolates demonstrated haemin auxotrophy and the overwhelming majority (7 out of 8)

**Table 3. Characterization of the ability of SCVs and their normal-morphology counterparts to produce biofilm**

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Species</th>
<th>icaADBC genes</th>
<th>Biofilm production</th>
<th>Sensitivity of biofilm to protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 NP</td>
<td><em>S. epidermidis</em></td>
<td>–</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td>4 SCV</td>
<td><em>S. epidermidis</em></td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>57 NP</td>
<td><em>S. epidermidis</em></td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>57 SCV1</td>
<td>–</td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>57 SCV2</td>
<td>–</td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>O16 NP</td>
<td><em>S. warneri</em></td>
<td>ND</td>
<td>None</td>
<td>–</td>
</tr>
<tr>
<td>O16 SCV</td>
<td><em>S. epidermidis</em></td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>O21 NP</td>
<td><em>S. epidermidis</em></td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>O21 SCV1</td>
<td>–</td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>O21 SCV2</td>
<td>–</td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>O22 NP</td>
<td><em>S. epidermidis</em></td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>O22 SCV</td>
<td>–</td>
<td>–</td>
<td>Moderate</td>
<td>Yes</td>
</tr>
<tr>
<td>Small-colony isolate in monoculture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49 S SCV</td>
<td><em>S. epidermidis</em></td>
<td>+</td>
<td>Strong</td>
<td>No</td>
</tr>
</tbody>
</table>

ND, not detected (primers used in the assay were designed for *S. epidermidis*).
were resistant to gentamicin in vitro. In general, the antibiotic susceptibility testing revealed consistent results in terms of resistance profiles of SCVs and the co-isolated normal-morphology isolates. Nevertheless, in three out of six and in two out of six SCV–NP pairs the small-colony subpopulations had higher gentamicin and co-trimoxazole MIC values, respectively. In spite of these differences, however, the pairs remained in the same resistance and sensitivity range, respectively. Kahl et al. (1998) reported MIC differences concerning S. aureus SCV–normal strains pairs isolated from patients with cystic fibrosis. Namely, 11 out of 12 SCVs had higher gentamicin MICs than did their corresponding normal strains. Furthermore, all S. aureus SCVs were resistant to antifolate antibiotics, while the corresponding parent strains were susceptible. Analysis of the underlying auxotrophism of SCVs revealed haemin, thymidine and/or menadione dependencies. von Eff et al. (1997), in turn, recovered S. aureus SCVs from four patients with osteomyelitis that were haemin or menadione auxotrophic and whose MICs of gentamicin were up to 32-fold higher than for the parent strains.

Another important aspect of our investigation is that we identified three SCV–NP pairs that demonstrated multidrug resistance, including one pair resistant to rifampicin, considered among the best anti-staphylococcal drugs used in the therapy of PJIs due to its effectiveness against slow-growing and adherent bacteria (Senneville et al., 2011; Trampuz & Zimmerli, 2005). This raises significant concerns in terms of treatment of PJIs as SCVs can cause refractory, difficult-to-treat infections not only due to their ‘inherent’ decreased susceptibility or resistance to antimicrobial agents associated with their metabolic defects. Like NP isolates, SCVs can also acquire and express all classical mechanisms of resistance to antimicrobials (Garcia et al., 2013).

Enhanced biofilm-forming capacity has been described for laboratory-derived menadione auxotrophs of S. aureus (Singh et al., 2009, 2010) and haemin auxotrophs of S. epidermidis (Al Laham et al., 2007). Maduka-Ezech et al. (2012) analysed the ability of clinical isolates of S. epidermidis SCVs to produce biofilm and found no difference between the SCVs and the corresponding normal-morphology isolates in terms of their biofilm-forming capability. Among 11 SCVs only 2 were found to be proficient biofilm producers, 7 were poor biofilm formers, whereas the remaining 2 isolates demonstrated intermediate ability to produce biofilm. Similar conclusions were drawn in this study. Namely, S. epidermidis SCV–NP pairs did not differ in terms of their biofilm-forming capacity. S. warneri SCV, in turn, was found to be a moderate biofilm producer in the applied in vitro assay, whereas its parent strain was non-adherent. Moreover, only one S. epidermidis SCV, whose growth was observed in the sonicate fluid, was identified as a proficient biofilm producer as it harboured the icaADBC genes.

The production of the PIA synthesized by the icaADBC operon has been regarded as the principal mechanism mediating biofilm accumulation in clinically significant S. epidermidis isolates (Fey & Olson, 2010; Rohde et al., 2010). Recently, proteinaceous factors have gained attention as alternative mechanisms of staphylococcal biofilm formation. Rohde et al. (2007) reported that 27% of biofilm-positive S. epidermidis produced PIA-independent biofilms, in part mediated by the accumulation of associated protein (Aap). Moreover, protein-dependent biofilms were exclusively found in S. epidermidis strains from total hip arthroplasty. The study, however, was focused only on the NP strains.

In our study, S. epidermidis SCVs that did not harbour genes encoding the PIA adhesin did not exceed the moderate degree of biofilm production in the microtiter assay. We assume that in the case of ica-negative SCVs (and the majority of their normal-morphology counterparts) the biofilm production could have been protein-mediated as their biofilms were sensitive to treatment with a protease. The biofilm production by the above-mentioned icaADBC-positive SCV strain, in turn, was not affected by the application of trypsin, which was evidence of the major role of the PIA in this process. Nevertheless, identification of specific proteins involved in biofilm formation by the isolates is beyond the scope of the study. Moreover, we have not investigated how other compounds such as extracellular DNA or teichoic acids contribute to the process of biofilm formation in the SCVs. Hence, we consider the results obtained as preliminary. Further studies focusing on the determination of the nature of biofilms produced by SCVs are necessary.

Bacteria within biofilms develop into organized, complex communities with structural and functional heterogeneity. Sendi et al. (2010) discussed the phenotypic, biochemical and genotypic properties of E. coli variants including SCVs that were involved in a PJI. These variants were isolated from periprosthetic tissue and by sonication of the explanted prosthesis, whereas isolates from synovial fluid revealed a NP. Interestingly, CNS SCVs reported in our study have also been cultured from sonicate fluid and/or periprosthetic tissues.

Molecular typing conducted by Sendi et al. (2010) enabled the assignment of all strains to the same clinical origin although non-identical banding patterns were yielded by PFGE. The authors found the results indicative of the possibility of the initiation of the infectious process by one clone (seeded from the urinary tract in the case of the reported E. coli), followed by genetic diversification associated with a horizontal gene transfer facilitated by close contact between bacterial cells within the biofilm.

In our study, the sonicate fluid culture obtained from one patient revealed the growth of two SCVs of different appearance, which additionally demonstrated different types of auxotrophy. They did not demonstrate an identical fingerprinting pattern but were genetically related. This observation corroborates the hypothesis mentioned above – when patients are infected with a single clone, different
phenotypes may arise as a result of mutation, rearrangement, loss or transposition of DNA (Galdbart et al., 1999). Moreover, we detected a non-related SCV pair in another patient. Only one of these SCVs was clonally related to the normal-morphology strain. The SCV pair differed in terms of sites of their isolation and antibiotic susceptibility profiles. As Galdbart et al. (1999) noted, the presence of multiple Staphylococcus epidermidis strains following implantation of joint prostheses may be due to either the genomic instability of a single infectious clone or an infection caused by a polyclonal mixture of strains. It is conceivable that both phenomena could be associated with infections in which SCV subpopulations are involved.

This paper highlights the involvement of CNS SCVs in the aetiology of PJIs, including what is believed to be the first report of S. warneri SCV isolation. These subpopulations must be actively sought in the routine diagnosis of implant-associated infections, and multiple clinical samples must be subjected to culture in order to increase the chance of SCV cultivation and characterization. Moreover, in view of the phenotypic and genetic diversity of some SCV pairs isolated from individual patients, antimicrobial susceptibility testing must be performed on all colony morphologies, which, unfortunately, further complicates the diagnostic process. In view of the scarcity of data on the prevalence of CNS SCVs in the aetiology of human infections we consider our findings – isolation of SCVs – as a new finding in the diagnostic process. In view of the phenotypic and genetic diversity of some SCV pairs isolated from individual patients, antimicrobial susceptibility testing must be performed on all colony morphologies, which, unfortunately, further complicates the diagnostic process. In view of the scarcity of data on the prevalence of CNS SCVs in the aetiology of human infections we consider our findings – isolation of SCVs – as a new finding in the diagnostic process.

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