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Coagulase-negative staphylococcal bloodstream and prosthetic-device-associated infections: the role of biofilm formation and distribution of adhesin and toxin genes

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Coagulase-negative staphylococci (CNS), especially *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, have emerged as opportunistic pathogens in immunocompromised patients and those with indwelling medical devices. In this study, CNS recovered from patients with bloodstream infections (BSIs) or prosthetic-device-associated infections (PDAIs) were compared in terms of biofilm formation, antimicrobial resistance, clonal distribution, and carriage of adhesin and toxin genes. A total of 226 CNS isolates (168 *S. epidermidis* and 58 *S. haemolyticus*) recovered from hospital inpatients with BSIs (100 isolates) or PDAIs (126 isolates) were tested for biofilm formation, antimicrobial susceptibility, and *mecA*, *ica* operon, adhesin (*aap*, *bap*, *fnbA*, *atlE*, *fbe*) and toxin (*tst*, *sea*, *sec*) genes. The selected CNS were classified into pulsotypes by PFGE and assigned to sequence types by multilocus sequence typing. In total, 106/226 isolates (46.9 %) produced biofilm, whereas 150 (66.4 %) carried the *ica* operon. Most isolates carried *mecA* and were multidrug resistant (90.7 %). CNS recovered from BSIs were significantly more likely to produce biofilm ($P=0.003$), be resistant to antimicrobials and carry *mecA* ($P<0.001$), as compared with isolates derived from PDAIs. CNS from PDAIs were more likely to carry the *aap* and *bap* genes ($P=0.006$ and $P=0.045$, respectively). No significant differences in the carriage of toxin genes were identified ($P>0.05$). Although PFGE revealed genetic diversity, especially among *S. epidermidis*, analysis of representative strains from the main PFGE types by multilocus sequence typing revealed three major clones (ST2, ST5 and ST16). A clonal relationship was found with respect to antimicrobial susceptibility and *ica* and *aap* gene carriage, reinforcing the premise of clonal expansion in hospital settings. The results of this

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Abbreviations: AAP, accumulation-associated protein; BSI, bloodstream infection; CNS, coagulase-negative staphylococci; MLST, multilocus sequence typing; MR-CNS, meticillin-resistant coagulase-negative staphylococci; PDAI, prosthetic-device-associated infection; PIA, polysaccharide intercellular adhesin; ST, sequence type; TSST, toxic shock syndrome toxin.

study suggest that the pathogenesis of BSIs is associated with biofilm formation and high-level antimicrobial resistance, whereas PDAIs are related to the adhesion capabilities of *S. epidermidis* and *S. haemolyticus* strains.

INTRODUCTION

Coagulase-negative staphylococci (CNS), especially *Staphylococcus epidermidis*, have emerged as a significant health problem in hospital settings during the past decades (McCann *et al.*, 2008). CNS are part of the normal skin flora but can cause severe infections, especially in immunocompromised patients or those with prosthetic devices such as intravascular catheters or biomaterials. Of major importance in the initiation of these infections is the ability of staphylococci to adhere to various surfaces, such as host tissues and prosthetic devices, and subsequently to form biofilm. The initial attachment is promoted by adhesins, which are grouped into a single family named 'microbial surface components recognizing adhesive matrix molecules' (Tristan *et al.*, 2003). These adhesins are encoded by a number of genes, such as *aap* (encoding accumulation-associated protein, AAP) (Rohde *et al.*, 2005), *atlE* (major cell-wall autolysin of *S. epidermidis*) (Qin *et al.*, 2007), *bap* (biofilm-associated protein) (Potter *et al.*, 2009), *fnbA* (fibrinogen-binding protein A) (Edwards *et al.*, 2010) and *fbe* (fibronectin-binding protein of *S. epidermidis*) (Arciola *et al.*, 2004). The next step in biofilm formation is bacterial accumulation, which is mediated by the production of polysaccharide intercellular adhesin (PIA), encoded by the *ica* operon (Mack *et al.*, 1996). Biofilm is a microbial-derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced and exhibiting an altered phenotype with respect to growth rate and gene transcription (Donlan & Costerton, 2002).

Another important factor in the pathogenesis of staphylococcal infections is the production of toxins, including enterotoxins and toxic shock syndrome toxin (TSST)-1 (da Cunha Mde *et al.*, 2007), which act as superantigens. They directly bind to major histocompatibility complex class II molecules without undergoing the typical processing of normal antigens, resulting in increased T-cell stimulation and, consequently, excessive production of cytokines such as IL-1, IL-2, IFN-γ and TNF-α (Marrack & Kappler, 1990).

Staphylococci express resistance to many antimicrobials used to treat infections. This is an increasing problem worldwide, especially with respect to nosocomial pathogens (Santos Sanches *et al.*, 2000). The rise of drug-resistant virulent CNS strains, particularly meticillin-resistant CNS (MR-CNS), is a serious problem in the control of staphylococcal infections. Meticillin resistance in staphylococci is known to be associated with the presence of the *mecA* gene, which encodes a penicillin-binding protein with low affinity to β-lactam antibiotics (PBP2a) (Chambers, 1997). MR-CNS cause hard-to-treat infections because they are usually multidrug resistant.

The aim of the present study was to investigate possible differences in biofilm-forming ability, antimicrobial-resistance patterns and genetic backgrounds of CNS recovered from patients with bloodstream infections (BSIs) or prosthetic-device-associated infections (PDAIs) in two hospitals in Greece. Clonal distribution and the frequency of adhesin- and toxin-encoding genes, as well as their contribution to infection and biofilm formation, were determined.

METHODS

Patients and hospitals. A total of 226 CNS isolates retrieved from patients hospitalized in the tertiary-care University General University Hospital of Patras (204 isolates) and Pentelis Paediatric Hospital in Athens (22 isolates), Greece, during a 3-year period (2006–2008) were selected for further analysis. The Ethics Committee of the University General Hospital of Patras approved this study and waived the need for informed consent (Approval no. : 316). A total of 100 isolates were recovered from patients with BSIs (71 from children), as defined by established criteria (clinical symptoms and two or more positive blood cultures drawn on separate occasions no more than 2 days apart) (Horan *et al.*, 2008), and 126 isolates were recovered from patients with PDAIs (60 from children) (intravascular catheters or orthopaedic implants, $\geqslant$15 c.f.u. in semiquantitative catheter culture and local signs of infection without bacteraemia). The Pentelis Paediatric Hospital and the paediatric wards of the University General Hospital of Patras admit patients younger than 14 years.

Phenotype identification and antibiotic susceptibility testing. CNS were identified to the species level using the VITEK 2 Advanced Expert System (bioMérieux) and by RFLP analysis of the amplified *tuf* gene (Kontos *et al.*, 2003). Susceptibility to cefoxitin, erythromycin, clindamycin, kanamycin, tobramycin, gentamicin, ciprofloxacin, fusidic acid and sulfamethoxazole/trimethoprim was tested using the disc-diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2011). The MICs of oxacillin, vancomycin, teicoplanin, linezolid and daptomycin were determined by Etest (bioMérieux). Isolates exhibiting a resistance phenotype to at least three different classes of antimicrobials were considered multidrug resistant. β-Lactamase production was tested using a nitrocefin assay (Becton Dickinson). Biofilm formation was tested with a quantitative microtitre plate assay using the reference *S. epidermidis* ATCC 35984 (RP62A), a well-characterized slime-producing/*ica*-positive strain, as a positive control, and ATCC 12228, a slime-negative/*ica*-negative strain, as a negative control (Stepanović *et al.*, 2007).

Molecular analysis. Amplification of *mecA*, two genes of the *ica* operon (*icaA*, *icaD*), genes encoding TSST (*tst*) and staphylococcal enterotoxins (*sea*, *sec*), and the adhesin genes *atlE* (among *S. epidermidis*), *aap*, *bap* and *fnbA* was performed by PCR with specific primers, as described previously (Arciola *et al.*, 2002; da Cunha Mde *et al.*, 2007; Gomes *et al.*, 2005; Petrelli *et al.*, 2006; Potter *et al.*, 2009).

A pair of gene-specific primers was synthesized for PCR-based detection of the *fbe* gene (among *S. epidermidis*). The primers were as follows: 5′-GAAACCAAATCGGCATCTAC-3′ (forward) and 5′-GTTGATGGCGATTTTGTAGG-3′ (reverse), including a 413 bp region. Thermal cycling conditions included an initial denaturation step (5 min at 94 °C), followed by 30 cycles of amplification (denaturation for 30 s at 94 °C, annealing for 1 min at 56 °C and extension for 1 min at 72 °C). The reaction was terminated with a 7 min final extension step at 72 °C. PCR products were analysed by electrophoresis in 1 % agarose gels.

Clonal identification. CNS were classified into pulsotypes by PFGE of chromosomal DNA *Sma*I digests (Tenover *et al.*, 1995). A dendrogram comparing molecular masses of DNA fragments was created using FPQuest version 4.5 (Bio-Rad). According to criteria established by Miragaia *et al.* (2008), patterns differing by less than 79 % (corresponding to a difference of fewer than seven bands) were considered to belong to the same PFGE type. Selected strains of the main PFGE types among *S. epidermidis* were characterized by multilocus sequence typing (MLST; http://mlst.net) (Thomas *et al.*, 2007). Results were analysed by applying the eBURST algorithm. Clonal complexes were defined using the default setting, in which all sequence types (STs) within a clonal complex differ by no more than one allele from at least one other ST in the clonal complex.

Statistical analysis. Pearson's χ^2 and Fisher's exact tests were used to evaluate differences in the frequencies of variables among the tested strains, conducted using SPSS version 20. The results were considered statistically significant at $P<0.05$. Isolates were assorted according to infection source, biofilm production, and carriage of the *ica* operon and *mecA* gene (MR-CNS and meticillin-susceptible CNS).

RESULTS

The 226 isolates studied comprised two different CNS species: *S. epidermidis* ($n=168$) and *Staphylococcus haemolyticus* ($n=58$). *S. epidermidis* predominated among both BSIs (71/100 isolates, 71 %) and PDAIs (97/126 isolates, 77 %). Overall, 203/226 isolates (89.8 %) were meticillin resistant. All cefoxitin-resistant isolates were also oxacillin resistant and carried the *mecA* gene (MR-CNS). All isolates were susceptible to linezolid (MICs <4 μg ml^{-1}), daptomycin (MICs <1 μg ml^{-1}) and vancomycin (MICs $\leqslant 4$ μg ml^{-1}). No teicoplanin-resistant isolates were found, although one strain displayed reduced susceptibility (MIC 16 μg ml^{-1}). CNS from BSIs expressed higher resistance rates to antimicrobials than those from PDAIs (Fig. 1). Most CNS (205/226, 90.7 %) were multidrug resistant. More specifically, 99/100 isolates (99 %) from BSIs and 106/126 (84.1 %) from PDAIs were multidrug resistant ($P<0.001$). A total of 208/226 isolates (92 %) produced β-lactamase, with similar proportions among the studied groups (91 % of isolates from BSIs and 92.9 % from PDAIs, $P=0.629$; Table 1).

In total, 106/226 isolates (46.9 %) produced biofilm (28 *S. haemolyticus*), while 150/226 (66.4 %) carried *ica* operon (20 *S. haemolyticus*). There was a statistically significant difference between biofilm producers and non-producers with respect to *ica* operon carriage, in favour of the biofilm-positive isolates (75.5 vs 58.3 %, $P=0.007$). No significant difference between biofilm-positive and -negative isolates was found with respect to carriage of adhesin genes ($P>0.05$). Eight out of 25 *ica*-negative, biofilm-producing CNS carried the *fnbA* gene (32 %), all of which were *S. haemolyticus*.

Isolates from BSIs prevailed over those from PDAIs in terms of biofilm formation. In particular, 58/100 (58 %) CNS from BSIs and 48/126 (38.1 %) from PDAIs produced biofilm ($P=0.003$), whereas the presence of *ica* operon did not differ statistically significantly between the two groups ($P>0.05$; Table 1). Moreover, CNS recovered from PDAIs carried in a significant higher rate of the adhesin genes *aap* and *bap*, with *aap* predominating; 30/100 CNS from BSIs (30 %) and 61/126 CNS from PDAIs (48.4 %) carried the *aap* gene ($P=0.006$), while 14/100 (14%) and 32/126 (25.4%) of isolates, respectively, carried the *bap* gene ($P=0.045$; Table 1). Carriage of the aforementioned genes was not associated with meticillin resistance ($P>0.05$). No statistically significant difference was found between BSIs and PDAIs regarding *fnbA*, *atlE* and *fbe* gene carriage ($P>0.05$, Table 1).

Among the toxin genes tested, *tst* predominated. Overall, out of 226 CNS, *tst* was present in 19 isolates (8.4 %), *sea* in 12 isolates (5.3 %) and *sec* in seven isolates (3.1 %). Two *S. epidermidis* and one *S. haemolyticus* isolate carried both *tst* and *sea*, but no other combination of toxin-gene carriage was identified. There was no significant difference in toxin-gene carriage between BSIs and PDAIs ($P>0.05$; Table 1).

PFGE typing revealed a diverse population: 168 *S. epidermidis* and 58 *S. haemolyticus* were grouped into 67 and 12 PFGE types, respectively. Among the *S. epidermidis* isolates two main pulsotypes were characterized (type a, 50 strains; type b, 36 strains), which comprised 51.2 % of the studied *S. epidermidis* population (86/168 strains). *S. haemolyticus* isolates were less diverse, with one major PFGE type (type h) making up 44 of the 58 strains (75.9 %). A dendrogram showing the main *S. epidermidis* pulsotypes is presented in Fig. 2. Compared with pulsotype b strains, type a strains displayed higher resistance rates to clindamycin (94 %), kanamycin (96 %), tobramycin (96 %), gentamicin (90 %), ciprofloxacin (100 %), fusidic acid (98 %) and sulfamethoxazole/trimethoprim (94 %) (all $P<0.05$; Table 2). *S. epidermidis* belonging to pulsotype a also showed a higher rate of *ica* operon carriage compared with type b strains (92 vs 72.2 %, $P=0.019$). In contrast, pulsotype b strains carried the *aap* gene more often than type a strains (66.7 vs 44 %, $P=0.049$; Table 2). All 36 PFGE type b strains were positive for *atlE*; however, there was no significant difference between the two major *S. epidermidis* pulsotypes in *atlE*, *fbe*, *bap*, *fnbA* or toxin-gene carriage ($P>0.05$; Table 2). Pulsotype b *S. epidermidis* strains were identified in both participating hospitals, which are located in different areas of Greece. *S. haemolyticus* strains belonging to PFGE type h displayed a lower rate of *ica* operon carriage (38.6 %) than the two main *S. epidermidis* pulsotypes. A high level of antimicrobial resistance was also present, but, besides SXT (P=0.040), no

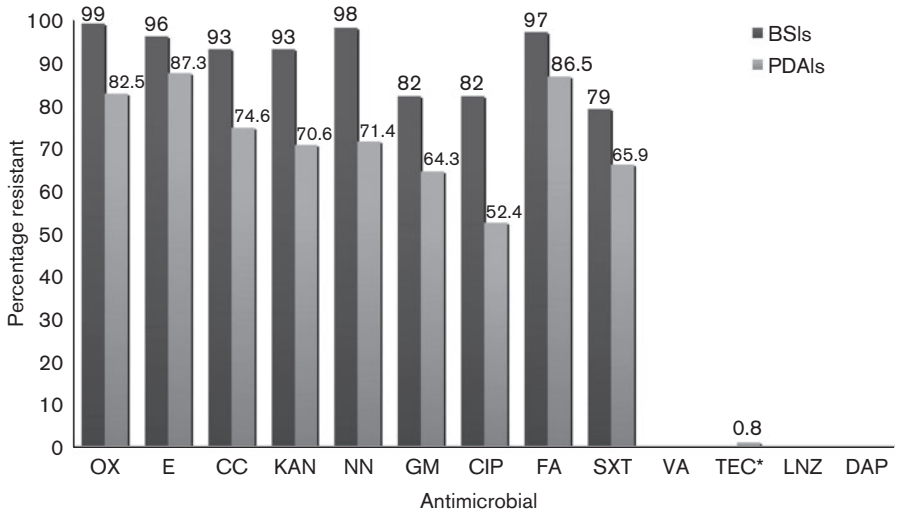

Fig. 1. Comparison of resistance patterns to antimicrobials among CNS isolated from patients with BSIs or PDAIs. *Isolate expressed reduced susceptibility to the antimicrobial agent. CC, Clindamycin; CIP, ciprofloxacin; DAP, daptomycin; E, erythromycin; FA, fusidic acid; GM, gentamicin; KAN, kanamycin; LNZ, linezolid; NN, tobramycin; OX, oxacillin; SXT, sulfamethoxazole/trimethoprim; TEC, teicoplanin; VA, vancomycin.

differences in antibiotic-resistance patterns among the *S. haemolyticus* pulsotypes were identified ($P>0.05$; Table 2).

Analysis of MLST data of tested *S. epidermidis* strains revealed three major STs: ST2, ST5 and ST16. PFGE pulsotype a strains ($n=50$ strains, 29.8 % of the *S. epidermidis* population) were characterized as ST2, whereas type b strains ($n=36$) belonged to ST5 and ST16 (59.6 and 40.4 %, respectively). Analysis using the eBURST algorithm showed that all three STs belonged to the same clonal complex (clonal complex 2), with ST2 being the primary group founder.

DISCUSSION

Although previously considered harmless organisms, CNS, especially *S. epidermidis*, are now recognized as opportunistic pathogens in immunocompromised patients and those with indwelling foreign bodies, particularly prosthetic cardiac valves, cerebrospinal fluid shunts, intravascular catheters and orthopaedic implants (McCann *et al.*, 2008). *S. haemolyticus* is the second most frequently isolated CNS from patients with hospital-associated infections and has the potential to form biofilm without PIA synthesis (Fredheim *et al.*, 2009). Although the role of *S. haemolyticus* in BSIs is well established, data regarding its association with PDAIs are lacking (Barros *et al.*, 2012; Fredheim *et al.*, 2009; Montanaro *et al.*, 2011). In the review article of Montanaro *et al.* (2011), *S. haemolyticus* was reported to account for only 3.3 % of total orthopaedic infections and 1.8 % of infections associated with medical devices. The present study verified the predominance of *S. epidermidis* among BSIs (71/100 isolates, 71 %) and PDAIs (97/126 isolates, 77 %), but also found *S. haemolyticus* to be the second most frequently isolated CNS species in both infection types (29 and 23 %, respectively).

Although polyclonality was observed among isolates recovered from both BSIs and PDAIs, *S. epidermidis* ST2, ST5 and ST16 predominated in this study, with all belonging to clonal complex 2 and disseminated in both participating hospitals. ST2 has been the most prevalent clone observed in previous epidemiological studies of *S. epidermidis* worldwide, including studies from Greece (Liakopoulos *et al.*, 2010; Miragaia *et al.*, 2007). In a study published by Miragaia *et al.* (2007), a representative collection of *S. epidermidis* isolates from diverse geographical and clinical origins was analysed using MLST. ST2 was the most widely disseminated type among both colonizing and infecting isolates, and was identified in strains from 13 countries across South America, Europe, Africa and Asia. In the current study, *S. haemolyticus* isolates were less diverse, with one major PFGE type, type h, making up 75.9 % of the studied population. A low level of genetic diversity has also been reported from Norway, where 72 *S. haemolyticus* isolates were grouped in only seven clusters (Fredheim *et al.*, 2009).

Various studies have demonstrated increasing resistance rates of CNS to antimicrobials (Santos Sanches *et al.*, 2000). This is a global problem, and is particularly apparent among nosocomial pathogens. The majority of CNS in this collection were *mecA* positive (89.8 %) and multidrug resistant (90.7 %), in accordance with previously published data (Santos Sanches *et al.*, 2000). The *mecA* gene was more frequent in CNS isolated from BSIs (99 %) as compared with those from PDAIs (82.5 %). CNS from patients with bacteraemia displayed higher resistance rates to the antimicrobials tested than isolates from PDAIs

Table 1. Characteristics of CNS (*S. epidermidis* and *S. haemolyticus*) isolated from patients with BSIs or PDAIs

Characteristic, *n* (%)	BSIs (*n*=100*)	PDAIs (*n*=126*)	*P* value
S. epidermidis	71 (71)	97 (77)	
S. haemolyticus	29 (29)	29 (23)	
Meticillin resistance	99 (99)	104 (82.5)	<0.001
β-Lactamase production	91 (91)	117 (92.9)	0.629
Clindamycin resistance	93 (93)	94 (74.6)	<0.001
Erythromycin resistance	96 (96)	110 (87.3)	0.032
Kanamycin resistance	93 (93)	89 (70.6)	<0.001
Tobramycin resistance	98 (98)	90 (71.4)	<0.001
Gentamicin resistance	82 (82)	81 (64.3)	0.004
Ciprofloxacin resistance	82 (82)	66 (52.4)	<0.001
Fusidic acid resistance	97 (97)	109 (86.5)	0.008
Sulfamethoxazole/trimethoprim resistance	79 (79)	83 (65.9)	0.037
Biofilm production	58 (58)	48 (38.1)	0.003
ica positivity overall	72 (72)	78 (61.9)	0.121
aap positivity overall	30 (30)	61 (48.4)	0.006
aap positivity among biofilm-producing isolates	20/58 (34.5)	26/48 (54.2)	0.050
aap positivity among non-biofilm-producing isolates	10/42 (23.8)	35/78 (44.9)	0.030
bap positivity overall	14 (14)	32 (25.4)	0.045
bap positivity among biofilm-producing isolates	7/58 (12.1)	10/48 (20.8)	0.290
bap positivity among non-biofilm-producing isolates	7/42 (16.7)	22/78 (28.2)	0.185
fnbA positivity overall	43 (43)	38 (30.2)	0.051
fnbA positivity among biofilm-producing isolates	24/58 (41.4)	14/48 (29.2)	0.225
fnbA positivity among non-biofilm-producing isolates	19/42 (45.2)	24/78 (30.8)	0.162.
tst positivity overall	9 (9)	10 (7.9)	0.813
sea positivity overall	8 (8)	4 (3.2)	0.138
sec positivity overall	3 (3)	4 (3.2)	1.000
atlE positivity among *S. epidermidis*	61/71 (85.9)	87/97 (89.7)	0.478
atlE positivity among biofilm-producing *S. epidermidis*	36/42 (85.7)	35/36 (97.2)	0.116
atlE positivity among non-biofilm-producing *S. epidermidis*	25/29 (86.2)	52/61 (85.2)	1.000
fbe positivity among *S. epidermidis*	61/71 (85.9)	75/97 (77.3)	0.171
fbe positivity among biofilm-producing *S. epidermidis*	37/42 (88.1)	29/36 (80.6)	0.531
fbe positivity among non-biofilm-producing *S. epidermidis*	24/29 (82.8)	46/61 (75.4)	0.589

*Unless otherwise stated.

($P<0.05$); thus, BSIs are considered to be more difficult to treat. This indicates that resistance, by accumulation of resistance genes and probably due to the high antibiotic pressure associated with hospital care, is a key factor in the selection of invasive isolates. The highest resistance percentages in isolates from BSIs were identified for meticillin (99 %), tobramycin (98 %), fusidic acid (97 %) and erythromycin (96 %) (Fig. 1). *S. epidermidis* PFGE type a (ST2) was associated with a higher resistance rate against clindamycin, aminoglycosides, ciprofloxacin, fusidic acid and sulfamethoxazole/trimethoprim, as compared with pulsotype b (ST5 and ST16) (all $P<0.05$; Table 2). Despite the high percentage of resistance to anti-staphylococcal agents, only one isolate displayed reduced susceptibility to teicoplanin. Although all of the isolates were susceptible to vancomycin, MICs of 2–4 μg ml^{-1} render this antibiotic inefficient in the treatment of staphylococcal infections. *S. haemolyticus* isolates were also multidrug resistant, but no statistically significant differences were found between the main PFGE type h and other pulsotypes, with the exception of SXT (Table 2).

A major factor in the pathogenesis of staphylococcal infections is biofilm formation (Donlan & Costerton, 2002). As compared with the planktonic phase, biofilm confers increased mechanical, metabolic, immune and antibiotic resistance on staphylococci (Donlan & Costerton, 2002). The so-far prevalent mechanism of *Staphylococcus* biofilm accumulation is functionally linked to the synthesis of PIA, encoded by the *ica* operon (Mack *et al.*, 1996). Another factor that may play a role in pathogenicity is a 20 kDa polysaccharide consisting mainly of glucose and *N*-acetylglucosamine, which promotes the attachment of *S. epidermidis* to endothelial cells and exhibits antiphagocytic properties (Spiliopoulou *et al.*, 2012). In our study, biofilm formation was directly associated with *ica* operon carriage, since significantly more biofilm-positive than biofilm-negative isolates carried the *ica* operon (75.5 versus 58.3 %, $P=0.007$).

Significantly more isolates from BSIs than from PDAIs formed biofilm (58 vs 38.1 %, $P=0.003$), rendering biofilm an important virulence factor in the pathogenesis of more severe staphylococcal infections. According to Donlan &

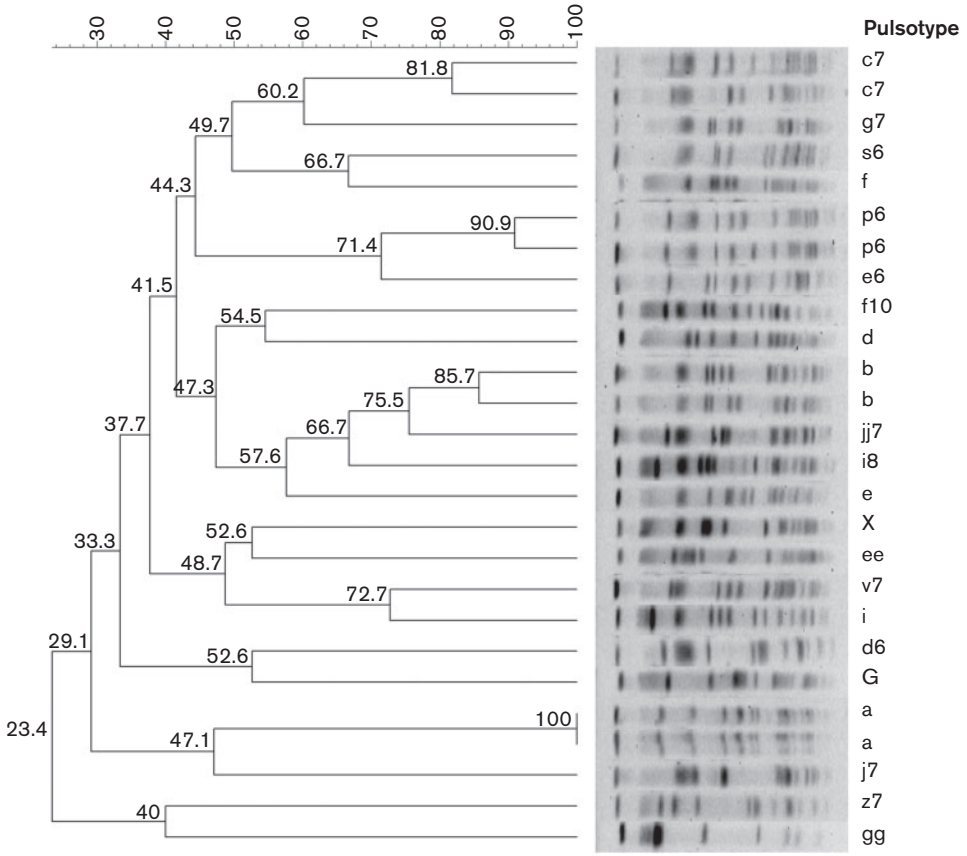

Fig. 2. Dendrogram of *S. epidermidis* isolates after digestion of DNA with *Sma*I and PFGE. The scale bar shows percentages of similarity.

Table 2. Characteristics of the two major *S. epidermidis* and the main *S. haemolyticus* PFGE types

Characteristic, *n* (%)	*S. epidermidis*			*S. haemolyticus*		
	Type a (*n*=50)	Type b (*n*=36)	*P* value	Type h (*n*=44)	Others (*n*=14)	*P* value
Meticillin resistance	48 (96)	34 (94.4)	1.000	44 (100)	14 (100)	–
β-Lactamase production	47 (94)	32 (88.9)	0.446	44 (100)	14 (100)	–
Clindamycin resistance	47 (94)	26 (72.2)	0.012	42 (95.5)	12 (85.7)	0.243
Erythromycin resistance	47 (94)	33 (91.7)	0.691	43 (97.7)	14 (100)	1.000
Kanamycin resistance	48 (96)	23 (63.9)	<0.001	43 (97.7)	14 (100)	1.000
Tobramycin resistance	48 (96)	28 (77.8)	0.015	43 (97.7)	13 (92.9)	0.428
Gentamicin resistance	45 (90)	16 (44.4)	<0.001	42 (95.5)	13 (92.9)	1.000
Ciprofloxacin resistance	50 (100)	21 (58.3)	<0.001	43 (97.7)	12 (85.7)	0.142
Fusidic acid resistance	49 (98)	29 (80.6)	0.008	42 (95.5)	12 (85.7)	0.243
Sulfamethoxazole/trimethoprim resistance	47 (94)	17 (47.2)	<0.001	43 (97.7)	11 (78.6)	0.040
Biofilm formation	22 (44)	18 (50)	0.663	20 (45.5)	8 (57.1)	0.545
ica positivity	46 (92)	26 (72.2)	0.019	17 (38.6)	3 (21.4)	0.338
aap positivity	22 (44)	24 (66.7)	0.049	1 (2.3)	1 (7.1)	0.428
atlE positivity	46 (92)	36 (100)	0.136	–	–	–
fbe positivity	44 (88)	33 (91.7)	0.729	–	–	–
bap positivity	11 (22)	10 (27.8)	0.614	1 (2.3)	–	1.000
fnbA positivity	19 (38)	13 (36.1)	1.000	19 (43.2)	5 (35.7)	0.759
tst positivity	6 (12)	1 (2.8)	0.231	4 (9.1)	1 (7.1)	1.000
sea positivity	4 (8)	2 (5.6)	1.000	2 (4.5)	1 (7.1)	1.000
sec positivity	1 (2)	2 (5.6)	0.569	1 (2.3)	–	1.000

Costerton (2002), bacterial cells may detach individually from biofilms as a result of cell growth and division within the biofilms, or cell aggregates or clusters may detach or be sloughed off from the biofilm and conceivably cause BSI, initiating even from very small numbers of bacteria. A study published by Valour *et al.* (2013) concluded that there was no difference in biofilm formation between nasal colonizing and infecting *S. epidermidis* recovered from patients with orthopaedic device infections. In our study, CNS from PDAIs were not strongly associated with biofilm formation, since only 38.1 % of staphylococci in this group produced biofilm, whereas CNS from BSIs were biofilm-positive at a significantly higher rate (58 %, $P<0.05$).

Although PIA is considered the major functional component for intercellular adhesion in CNS biofilms, clinically significant PIA-negative strains are regularly encountered, suggesting the role of additional factors such as intercellular adhesins. A total of 25 *ica*-negative isolates (six *S. epidermidis* and 19 *S. haemolyticus*) in the current study were biofilm-positive, indicating that other factors besides PIA, such as AAP and biofilm-associated protein (encoded by *aap* and *bap*, respectively), can mediate PIA-independent intercellular accumulation during biofilm development by *ica*-deficient CNS, as reported elsewhere (O'Gara, 2007). Our study detected a low prevalence of *ica* operon-bearing *S. haemolyticus*, in accordance with previously published data (Fredheim *et al.*, 2009). More specifically, even though 28/58 *S. haemolyticus* isolates (48.3 %) produced biofilm, 19 of these biofilm-positive isolates (67.9 %) were *ica*-negative. Silva *et al.* (2013), analysing 27 *S. haemolyticus* isolates from BSIs, determined that 18 isolates produced biofilm, even though *ica* operon was not detected in any of them. Among the PIA-negative, biofilm-producing CNS in our collection, *fnbA* (encoding fibrinogen-binding protein A) was the predominant adhesin gene (8/25, 32 %), detected specifically in *S. haemolyticus*. Fibrinogen-binding protein A facilitates tissue invasion by binding to fibronectin, promotes attachment to the endothelium and triggers the uptake of staphylococci by endothelial cells, which is believed to facilitate bacterial persistence and the establishment of secondary infections (Edwards *et al.*, 2010).

An important factor in the establishment of staphylococcal infections is their ability to attach to various surfaces, including host tissues and medical devices. In this study, isolates derived from PDAIs, compared with those from BSIs, displayed a higher correlation to *bap* (encoding biofilm-associated protein) and *aap* (encoding AAP). Biofilm-associated protein is a surface protein implicated in biofilm formation, whereas AAP acts as a polysaccharide-independent mechanism of CNS biofilm accumulation and intercellular adhesion (Rohde *et al.*, 2005). The *aap* gene predominated among the aforementioned adhesins in both BSIs and PDAIs (30 vs 48.4 %, respectively, $P=0.006$). According to Rohde *et al.* (2007), biofilm formation is a common phenotypic feature in staphylococci isolated from prosthetic joint infections after arthroplasty, and is mediated by PIA and protein factors. Importantly, these different factors appear to have varying importance in the pathogenesis of *S. epidermidis* prosthetic joint infections, with PIA being of particular importance in infections related to total knee arthroplasty. In total hip arthroplasty, protein factors such as AAP seem to be sufficient to establish persistent infections (Rohde *et al.*, 2007).

The *atlE* (encoding the major cell-wall autolysin of *S. epidermidis*) and *fbe* genes were detected in high rates among *S. epidermidis* isolates. The major cell-wall autolysin of *S. epidermidis* has been reported to promote the release of extracellular DNA, a major component required for the initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development by *S. epidermidis* (Qin *et al.*, 2007). Although no clonal relationship was found concerning biofilm formation, a statistically significant difference for *ica* and *aap* gene carriage in favour of specific clones reinforces the characteristic of clonal expansion in the hospital setting. As reported by Cherifi *et al.* (2013), *S. epidermidis* isolates belonging to ST2 that were recovered from patients with catheter-related BSIs were multidrug resistant and *ica* positive. In our study, ST2 was also associated with *ica* operon carriage and high rates of antimicrobial resistance, whereas ST5 and ST16 were significantly related to *aap*.

A number of studies have identified *S. haemolyticus*, mainly from BSIs, as a potential pathogen (Fredheim *et al.*, 2009; Potter *et al.*, 2009; Silva *et al.*, 2013). In the present study, we identified a number of *S. haemolyticus* isolates ($n=29$) contributing to the pathogenesis of PDAIs, in addition to BSIs. Moreover, the presence of potential virulence factors such as biofilm formation, adhesin and toxin genes was evaluated.

Production of toxins, including TSST-1 and staphylococcal enterotoxins A and C, also contributes to the establishment of infection, especially in patients with immunodeficiency. TSST-1 was the predominant toxin found in CNS in the current study, but no correlation between toxin-gene carriage and infection was identified, since isolates from both groups (BSIs and PDAIs) carried the toxin genes with the same frequency. In additional, no patient was identified as having toxic shock syndrome; therefore, no association with disease was proven.

In conclusion, certain *S. epidermidis* and *S. haemolyticus* clones predominated among patients with BSIs and PDAIs in two hospitals located in different areas of Greece. This study found biofilm formation and multidrug resistance to be important determinants of CNS virulence in BSIs, whereas *aap* and *bap* were strongly correlated with PDAIs. Specific phenotypic and genotypic characteristics of CNS, combined with successful clonal expansion, render these bacteria the main cause of BSIs and PDAIs.

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