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ARTICLE

Virulence factors among *Staphylococcus lugdunensis* are associated with infection sites and clonal spread

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Abstract *Staphylococcus lugdunensis* has emerged as a significant human pathogen, with distinct clinical and microbiological characteristics. Our goal was to identify the virulence factors in *S. lugdunensis* recovered from infected patients of two Greek hospitals during a six-year period (2008–2013). A collection of 38 *S. lugdunensis* was tested for biofilm formation, antimicrobial susceptibility, clonal distribution, virulence factors (*ica* operon, *fbl*, *atlL*, *vwbl*, *slush*) and antibiotic resistance genes (*mecA*, *ermC*) carriage. Strains were classified into pulsotypes by pulsed-field gel electrophoresis (PFGE) of *Sma*I DNA digests. The majority (22) was isolated from skin and soft tissue infections (SSTIs), nine from deep-sited infections (DSIs), including three bacteraemias and seven from prosthetic device-associated infections (PDAIs). All isolates were oxacillin-susceptible, *mecA*-negative and *fbl*-positive. The highest resistance rate was detected for ampicillin (50 %), followed by erythromycin and clindamycin (18.4 %). Fourteen isolates (36.8 %) produced biofilm, whereas 26/38 (68.4 %) carried the *ica* operon. Biofilm formation was more frequent in isolates from PDAIs. Thirty-six strains (94.7 %) carried *atlL* and 31 (81.6 %) carried *vwbl*, whereas *slush* was detected in 15 (39.5 %). PFGE revealed a low level of genetic diversity: strains were classified into seven pulsotypes, with two major clones (C: 22 and D: nine strains). Type C strains recovered from all infection sites prevailed in biofilm formation and *ermC* carriage, whereas type D strains associated with SSTIs and DSIs carried more frequently *vwbl*, *slush* or both genes. Despite susceptibility to antimicrobials, the clonal expansion and carriage of virulence factors, combined with biofilm-producing ability, render this species an important pathogen that should not be ignored.

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Introduction

Coagulase-negative staphylococci (CNS), a part of the normal skin flora, have been recognised as opportunistic pathogens in patients with low immune response or indwelling medical devices. Among them, *Staphylococcus lugdunensis* has emerged lately as a significant human pathogen with notable clinical and microbiological characteristics that stand out among other CNS. Several infections due to *S. lugdunensis* have been reported, including native valve endocarditis, skin and soft tissue infections (SSTIs), bloodstream infections, peritonitis, and urinary tract and central nervous system infections [1]. The high level of pathogenicity indicates the presence of various virulence factors.

Despite its aggressive nature, *S. lugdunensis*, unlike most CNS, has remained remarkably susceptible to a wide range of antimicrobial agents, regardless of the source of infection [2]. In accordance to the exhibited susceptibility, *S. lugdunensis* has generally been susceptible to oxacillin and polymerase chain reaction (PCR) screening for *mecA* has frequently yielded negative results. Resistance to beta-lactams due to the production of beta-lactamase has been reported [3], as well as *ermC* gene carriage, which mediates resistance to macrolides and lincosamides [4, 5].

A variety of microorganisms have the ability to form biofilm, a microbial-derived sessile community characterised by irreversibly attached cells to a substratum, interface or to each other. Bacterial cells are embedded in a matrix of extracellular

polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription [6]. In staphylococci, biofilm formation is mediated by the production of polysaccharide intercellular adhesin (PIA), encoded by the *ica* operon or other well-characterised compounds [7, 8]. Biofilm formation is a predominant virulence mechanism employed by *S. lugdunensis*, whose genome harbours homologues of the *ica* operon. Biofilm development allows for the deep-sited cells to become more resistant to administered antibiotics and the body's natural mechanisms, interfering with attempts by the host immune system to clear the infection [1].

The ability of *S. lugdunensis* to cause endocarditis and prosthetic device-associated infections (PDAIs) suggests that the organism has the potential to interact with host tissues and proteins that may coat foreign surfaces after implantation. A protein has been identified that specifically binds von Willebrand factor (vWf) [9]. vWf is a blood plasma glycoprotein produced by endothelial cells and platelets involved in coagulation, via binding to platelets and subendothelial collagen after vascular injury and stabilising factor VIII of the clotting cascade [10]. The vWf-binding protein of *S. lugdunensis* (vWbl) is a 2,060-amino-acid protein encoded by the *vwbl* gene [9]. *S. lugdunensis* isolates also possess the *fbl* gene, which encodes a surface-located fibrinogen-binding adhesin, referred as the Fbl protein. It mediates binding to the fibrinogen γ-chain and has close sequence and organisational similarity to clumping factor A of *S. aureus* [11]. It has been reported that *fbl* may serve as a species-specific target for *S. lugdunensis* identification by means of a simple PCR protocol [12].

S. lugdunensis also possess the *slush* locus, which encodes for haemolytic peptides with delta-toxin-like activity. The *S. lugdunensis* synergistic haemolysin (SLUSH) is encoded by a locus outside the *agr* region and comprises three very similar 43-residue peptides with synergistic haemolytic activity with β-toxin [13]. The *atlL* gene is responsible for the production of an autolysin involved in cell separation, stress-induced autolysis and contributes to bacterial pathogenesis [14].

The aim of the present study was to investigate the biofilm-forming ability, antimicrobial resistance patterns and genetic background of *S. lugdunensis* recovered from infection sites of patients hospitalised in two hospitals in Greece during a six-year period (2008–2013). Clonal distribution and the frequency of virulence factor-encoding genes were determined.

Materials and methods

Patients and hospitals

A collection of 38 *S. lugdunensis* isolates recovered from different inpatients hospitalised in a tertiary-care University General Hospital in Patras (UGHP: 37 isolates) and the Pentelis Paediatric Hospital in Athens (PPHA: one isolate), Greece, during a six-year period (2008–2013) were selected to be further analysed. Thirty-six *S. lugdunensis* were isolated from adults, one from the Neonatal Intensive Care Unit of the UGHP and one from the PPHA that admits patients under the age of 14 years old. The Ethics Committee of the University Hospital of Patras approved this study and waived the need for informed consent (approval no.: 316).

Phenotypic identification and antibiotic susceptibility testing

CNS were identified to the species level using the VITEK 2 Advanced Expert System (bioMérieux, Marcy l'Etoile, France). Susceptibility to cefoxitin (FOX), ampicillin (AMP), rifampicin (RIF), erythromycin (E), clindamycin (CC), kanamycin (KAN), tetracycline (TE), gentamicin (GM), ciprofloxacin (CIP), fusidic acid (FA) and sulphamethoxazole/trimethoprim (SXT) was tested by the disc diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [15]. Macrolide–lincosamide–streptogramin B (MLSB) resistance phenotype was investigated by the combined erythromycin and clindamycin disc method (D-test) [15]. The minimum inhibitory concentrations (MICs) of oxacillin (OX), vancomycin (VA), teicoplanin (TEC), linezolid (LNZ) and daptomycin (DAP) were determined by the Etest (bioMérieux). Isolates exhibiting a resistance phenotype to at least three different classes of antimicrobials were considered multidrug-resistant. Beta-lactamase production was tested by the nitrocefin assay (Becton Dickinson, Franklin Lakes, NJ, USA). Biofilm formation was tested by the quantitative microtitre plate assay using the reference *S. epidermidis* ATCC35984 (RP62A), slime producing/*ica*-positive strain, and ATCC12228, slime-negative/*ica*-negative strain, as positive and negative controls, respectively [16].

Molecular analysis

Amplification of the gene encoding the fibronectin-binding protein (*fbl*) was performed for the verification of species identification [12]. The presence of *mecA* [17], one gene of the *ica* operon (*icaA*) [18], the synergistic haemolysin (*slush*) [19], the von-Willebrand binding factor (*vwbl*) [19] and the ribosomal methylase gene *ermC* [5], encoding resistance to erythromycin, was investigated by PCRs with specific primers.

For PCR-based detection of the gene encoding the major cell wall autolysin (*atlL*), a pair of gene-specific primers was synthesised. The primers were as follows: 5′-CCATCAACAC CTACAAATCC-3′ as the forward primer and 5′-CAGCCA GATTTACCATTCAC-3′ as the reverse primer, amplifying a 449-bp region. Thermal cycling conditions included an initial

denaturation step (10 min at 94 °C), followed by 35 cycles of amplification (denaturation for 30 s at 95 °C, annealing for 30 s at 56 °C and extension for 30 s at 72 °C). The reaction was terminated with a 10-min final extension step at 72 °C. PCR products were analysed by electrophoresis into 1 % agarose gels.

Clonal identification

The strains were classified into pulsotypes by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA *Sma*I digests [20], named by capital letters. A dendrogram comparing the molecular weights of DNA fragments was performed by FPQuest software version 4.5 (Bio-Rad Laboratories, Inc.). According to criteria established by Miragaia et al., patterns differing by less than 79 % (corresponding to a difference of less than seven bands) were considered to belong to the same PFGE type [21].

Results

A collection of 38 *S. lugdunensis* clinical isolates was studied. The majority of strains (22/38, 57.9 %) derived from SSTIs (wounds, abscesses and skin infections), nine strains (23.7 %) from deep-sited infections (DSIs, including three bacteraemias, osteomyelitis tissue samples, synovial and peritoneal fluids) and seven strains (18.4 %) from PDAIs (intravenous and peritoneal catheters) (Table 1). Two *S. lugdunensis* strains were recovered from children.

All 38 isolates were *fbl*-positive, cefoxitin- and oxacillin-susceptible (MICs≤1.5 μg/mL), and did not carry the *mecA* gene. Moreover, all isolates were susceptible to linezolid (MICs≤1 μg/mL), daptomycin (MICs≤0.75 μg/mL), vancomycin (MICs≤2 μg/mL), teicoplanin (MICs≤2 μg/mL), gentamicin, kanamycin, ciprofloxacin and rifampicin. The higher resistance rates were detected for ampicillin (19/38, 50 %), erythromycin (7/38, 18.4 %) and clindamycin (7/38, 18.4 %). Only four strains (10.5 %) were multi-resistant, whereas all of them belonged to the same PFGE type (C). Nineteen isolates produced beta-lactamase (Table 2). Seven erythromycin- and clindamycin-resistant strains expressing constitutive resistance carried the *ermC* gene.

In total, 14/38 isolates (36.8 %) produced biofilm, whereas 26/38 (68.4 %) carried the *ica* operon. Among biofilm-producers, *ica* operon carriage was detected in 10/14 isolates (71.4 %). Of the 24 remaining biofilm-negative *S. lugdunensis*, 16 (66.7 %) were also found to carry the *ica* operon. The four biofilm-positive *ica*-deficient isolates belonged to the same PFGE type, C, and carried the *atlL* gene. Biofilm formation and *atlL* gene carriage were more frequent in isolates from PDAIs, whereas *vwbl* and *slush* genes were more frequent in isolates from DSIs (Table 1).

PFGE typing revealed a low level of genetic diversity; the 38 *S. lugdunensis* strains were classified into seven pulsotypes. Thirty-one isolates (81.6 %) were classified into two major clones, C and D, consisting of 22 and nine strains, respectively. A dendrogram showing the main *S. lugdunensis* pulsotypes is presented in Fig. 1. All *S. lugdunensis* recovered from bacteraemias (three strains) belonged to the main PFGE type, C. Pulsotype D strains were recovered from SSTIs and DSIs (Table 1). Strains belonging to clone C prevailed in biofilm formation and in *ermC* carriage as compared to pulsotype D (Table 2).

Among the virulence genes detected, *fbl* and *atlL* predominated in this study. All *S. lugdunensis* carried *fbl*, as expected, whereas *atlL* was identified in 36/38 isolates (94.7 %, Table 1). Thirty-one (81.6 %) *S. lugdunensis* carried *vwbl*, while *slush* was detected in only 15 strains (39.5 %, Table 1). Type D strains carried more frequently *vwbl* (88.9 %) and *slush* (77.8 %, Table 2). Six out of nine type D and four out of 22 type C strains carried both *vwbl* and *slush* genes. Five out of six type D strains carrying both genes were recovered from SSTIs, whereas type C strains were isolated from variable infection sites. PFGE type C was identified in both hospitals (UGHP and PPHA), located in different areas of the country.

Table 1 Characteristics of *S. lugdunensis* isolates in relation to infection sites

	PDAIs, n=7 (%)	SSTIs, n=22 (%)	DSIs, n=9 (%)	Total, N=38
Clones	5C, M, G	11C, 7D, 2K, M, E	6C, 2D, F	38
Biofilm formation	4 (57.1)	8 (36.4)	2 (22.2)	14
fbl	7 (100)	22 (100)	9 (100)	38
atlL	7 (100)	21 (95.5)	8 (88.9)	36
vwbl	5 (71.4)	18 (81.8)	8 (88.9)	31
ica	3 (42.9)	20 (90.9)	3 (33.3)	26
slush	2 (28.6)	9 (40.9)	4 (44.4)	15
ermC	2 (28.6)	5 (22.7)	–	7

PDAIs prosthetic device-associated infections; *SSTIs* skin and soft tissue infections; *DSIs* deep-sited infections

Table 2 Characteristics of the main *S. lugdunensis* PFGE types

PFGE types	Type C, *n*=22 (%)	Type D, *n*=9 (%)	Others, *n*=7 (%)
Methicillin resistance	–	–	–
Ampicillin resistance	11 (50)	4 (44.4)	4 (57.1)
Erythromycin resistance	5 (22.7)	1 (11.1)	1 (14.3)
Clindamycin resistance	5 (22.7)	1 (11.1)	1 (14.3)
Fusidic acid resistance	3 (13.6)	–	1 (14.3)
Tetracycline resistance	2 (9.1)	–	–
Sulphamethoxazole/trimethoprim resistance	1 (4.5)	–	–
Biofilm formation	9 (40.9)	2 (22.2)	3 (42.9)
Beta-lactamase	11 (50)	4 (44.4)	4 (57.1)
fbl	22 (100)	9 (100)	7 (100)
atlL	22 (100)	9 (100)	5 (71.4)
vwbl	16 (72.7)	8 (88.9)	7 (100)
ica	15 (68.2)	6 (66.7)	5 (71.4)
slush	5 (22.7)	7 (77.8)	3 (42.9)
ermC	5 (22.7)	1 (11.1)	1 (14.3)

Discussion

S. lugdunensis is found as a skin commensal in healthy individuals, but has also been implicated in invasive diseases, especially native and prosthetic cardiac valve endocarditis, meningitis, peritonitis and SSTIs [1]. In our study, 38 *S. lugdunensis* were isolated during a six-year period in two Greek hospitals. Only three patients had bacteraemia, whereas skin and soft tissue were the main infection sites.

Various studies have demonstrated increased resistance of CNS to antimicrobials [22]. On the contrary, *S. lugdunensis* remains remarkably susceptible to most antistaphylococcal agents. An analysis of 28 *S. lugdunensis* from patients with bacteraemia in Switzerland concluded that most strains (82 %) were penicillin-susceptible and none was oxacillin- or multi-resistant [23]. Likewise, all isolates in our collection were susceptible to cefoxitin and oxacillin (methicillin-susceptible *S. lugdunensis*). Moreover, no isolate resistant to gentamicin, kanamycin, ciprofloxacin, rifampicin, vancomycin, teicoplanin, linezolid and daptomycin was found. Despite the high level of susceptibility, 50 % of the studied population was resistant to ampicillin, whereas four type C strains were multi-resistant without correlation to any outbreak; three of them were isolated from skin infections in 2010 and one from an intravenous catheter in 2013. Two multi-resistant strains were recovered from patients hospitalised in the Nephrology Department, one from the Vascular Surgery Clinic and one from the Outpatients Department (UGHP). In contrast to other CNS, *S. lugdunensis* produces beta-lactamase in a relatively low rate (19/38, 50 %), confirming the low level of resistance. The carriage of *ermC* was also not prevalent (7/38, 18.4 %), confirming the high level of susceptibility to macrolides.

A major factor in the pathogenesis of staphylococcal infections is biofilm formation [6]. As compared to the planktonic phase, biofilm confers to staphylococci increased mechanical, metabolic, immune and antibiotic resistance. Soon after the description of *S. lugdunensis*, Lambe et al. showed that a

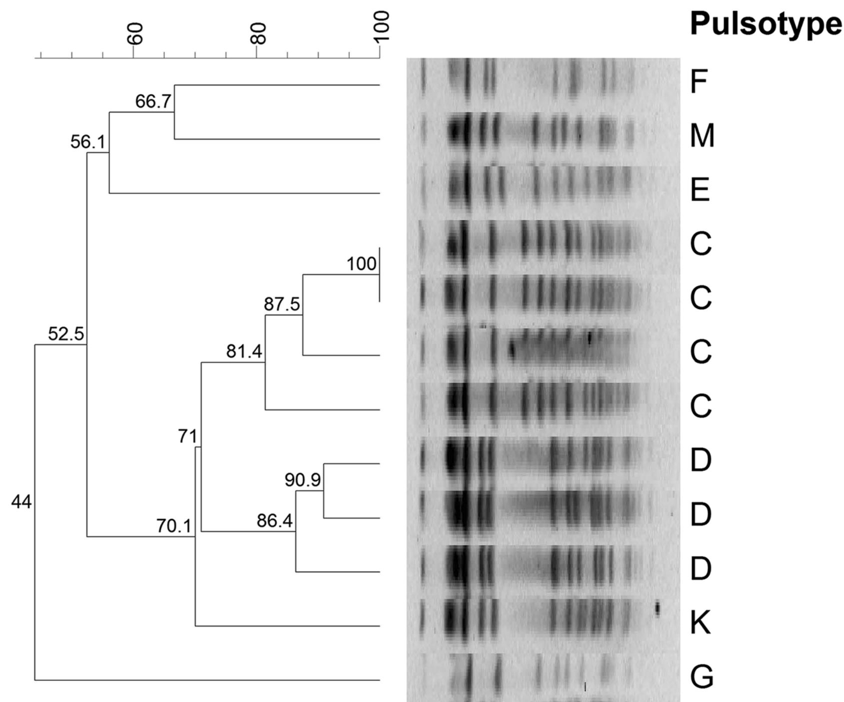

Fig. 1 Dendrogram of *S. lugdunensis* isolates after the digestion of DNA with *Sma*I and PFGE. The scale bar shows percentages of similarity

glycocalyx, described as a negatively charged polysaccharide matrix surrounding bacterial microcolonies, could be visualised by transmission electron microscopy when cells were stained with ruthenium red, a stain with affinity for polyanionic structures [24]. In the present study, 14 out of 38 isolates (36.8 %) produced biofilm. *S. lugdunensis* isolated from PDAIs formed biofilm in a higher rate as compared to other infection sites (Table 1), confirming that the presence of a foreign surface is important for the initial bacterial attachment, which is the first step in biofilm formation and the pathogenesis of the infection. A locus with homology to the *S. aureus* and *S. epidermidis ica* operon has been identified in *S. lugdunensis*, but no direct relation between *ica* carriage and biofilm formation was determined in our collection. It has been reported that *S. lugdunensis* forms biofilm, but its biofilm extracellular matrix is predominantly proteinaceous and independent of poly-N-acetylglucosamine production, which is encoded by *ica* [25]. As published by Pereira et al., investigating biofilm production in 23 *S. lugdunensis* clinical isolates, even though all of them carried *ica*, only 14 strains were biofilm-positive and in a detachment assay, using proteolytic enzymes to analyse biofilm composition, they showed protein-mediated biofilm structure [26]. Similarly, proteinaceous biofilm composition was identified in the present study, in which among the 26 *ica*-positive strains, only ten produced biofilm, whereas the remaining four biofilm-producers carried *atlL*.

PFGE analysis of *S. lugdunensis* strains revealed that, although they were recovered from different patients and treated in different wards and hospitals, two main clones (C and D) predominated during this six-year period. The three bacteraemic as well as the four multi-resistant isolates belonged to PFGE type C. In a study published by Hellbacher et al., 39 *S. lugdunensis* were classified into nine pulsotypes, and the majority (56 %) belonged to one main PFGE type [27]. In our collection, the main pulsotype, C, also comprised the majority of strains (22/38, 57.9 %), indicating a low degree of genetic diversity. This may indicate that the *S. lugdunensis* genome is highly conserved or that specific clones are more likely to cause invasive infections. Clone C predominated, regardless of the site of infection. The nine isolates that comprised pulsotype D (23.7 %) originated from SSTIs and DSIs, but not PDAIs.

As reported by Chatzigeorgiou et al., *fbl* may serve as a species-specific target for *S. lugdunensis* identification by means of a simple PCR protocol [12]. In our study, the VITEK 2 Advanced Expert System (bioMérieux) used to phenotypically identify CNS species correctly detected all *S. lugdunensis* proven by the *fbl* PCR assay.

An important virulence factor in our collection was found to be *atlL*, which was detected in the majority (94.7 %) of isolates, causing mainly PDAIs and SSTIs, including all four biofilm-positive/*ica*-deficient *S. lugdunensis*. AtlL is a major autolysin involved in *S. lugdunensis* cell division. Quantitative PCR of *atlL* gene expression indicates that it is transcribed throughout all phases of growth (early exponential, mid-exponential and stationary growth phases), with an increased level (5.5-fold) at the early exponential stage, which is consistent with the hypothesis of an involvement in cell division [28]. Gibert et al., investigating the role of AtlL by an *atlL*-inactivated *S. lugdunensis* mutant, concluded that this protein could act as an autolysin/adhesin, conferring initial bacterial attachment and release of extracellular DNA, being, thus, a major component in PIA-independent proteinaceous *S. lugdunensis* biofilms [14].

The majority of *S. lugdunensis* studied (81.6 %) also carried *vwbl*, which encodes the putative cell surface protein, vWbl. The purified protein has an overall organisation typical of staphylococcal cell surface proteins, with an N-terminal signal peptide and a C-terminal cell wall sorting signal. A high prevalence of *vwbl* was also reported by Nilsson et al., where Southern blot analysis showed that *vwbl* was present in all 12 *S. lugdunensis* strains tested [9].

Several staphylococcal species express a synergistic activity, which potentiates haemolysis by β-haemolysin. Haemolysis of red blood cells is an important pathogenicity factor. In *S. lugdunensis*, this activity results from the production of three small peptides coded by the *slush* locus [13]. In our study collection, *slush* was present in 15 isolates (39.5 %), whereas the analysis of 58 *S. lugdunensis* clinical isolates by Szabados et al., detected it in 29 isolates (50 %) [19]. The presence of other genes encoding haemolysins should also be considered.

In conclusion, *S. lugdunensis* stands out between other CNS and has been fairly well described as 'a wolf in sheep's clothing' [1]. Despite its susceptibility to antimicrobials, clonal expansion, carriage of virulence factors such as *fbl*, *ica*, *atlL*, *vwbl* and *slush*, combined with biofilm-producing ability, render this species an important pathogen that should not be ignored in clinical practice.

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Conflict of interest Nothing to declare.

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