


Low occurrence of the new species *Staphylococcus argenteus* in a *Staphylococcus aureus* collection of human isolates from Belgium

M. A. Argudín¹  · M. Dodémont¹ · S. Vandendriessche² · S. Rottiers¹ · C. Tribes¹ · S. Roisin¹ · R. de Mendonça¹ · C. Nonhoff¹ · A. Deplano¹ · O. Denis¹

Received: 29 February 2016 / Accepted: 18 March 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract *Staphylococcus argenteus* is a novel *Staphylococcus* species closely related to *Staphylococcus aureus* that has been recently described. In this study, we investigated the proportion and the characteristics of *S. argenteus* recovered from humans in Belgium. *S. aureus* human isolates collected in Belgium from 2006 to 2015 ($n=1,903$) were retrospectively characterised via the presence of non-pigmented colonies on chocolate agar, *spa* typing and *rpoB* sequencing to determine if some of them were in fact *S. argenteus*. Out of 73 strains non-pigmented on chocolate plates, 3 isolates (0.16 %) showed *rpoB* sequences, in addition to *spa* and sequence types (ST2250/t5787, ST2250/t6675, ST3240/t6675), related to *S. argenteus*. Two of them were methicillin-resistant, harbouring a SCCmec type IV. The three *S. argenteus* isolates carried genes (*sak*, *scn*) of the immune evasion cluster. This first Belgian nationwide analysis showed a low occurrence of *S. argenteus*. Further studies should be conducted to identify the distribution range and the clinical impact of this new species.

Introduction

Staphylococcus argenteus is a novel *Staphylococcus* species that has recently been described [1]. *Staphylococcus argenteus* isolates have been ascribed to phylogenetically distinct *Staphylococcus aureus* lineages (clonal complex [CC] 75, CC2198, CC2483, CC1594) [2–5]. This species is characterised by a lack of the genes that produce the carotenoid pigment staphyloxanthin [6] and therefore shows a non-pigmented phenotype on chocolate agar plates [1, 6]. *S. argenteus* displays a reduced virulence compared with *S. aureus* [7], but isolates carrying Panton–Valentine leukocidin (PVL) have been described [8, 9]. Although this new species may be widely distributed, its geographical distribution range remains unknown. Technical difficulties in performing multilocus sequence typing (MLST) using conventional primers on these isolates may contribute to underreporting [3]. However, other phylogenetic analyses such as the *rpoB*, *gap*, *sodA*, *tuf* and *hsp60* sequence identification are able to successfully identify *S. argenteus* [3]. In this work, we described the first Belgian nationwide analysis on human *S. argenteus* occurrence.

Electronic supplementary material The online version of this article (doi:10.1007/s10096-016-2632-x) contains supplementary material, which is available to authorised users

✉ M. A. Argudín
argudinmaria@gmail.com

Materials and methods

Strain collection

Two collections of *S. aureus* strains ($n=1,903$) were retrospectively analysed for the presence of *S. argenteus*. Most isolates ($n=1,650$) were sent by Belgian microbiology laboratories from 2012 to 2015 to the National Reference Centre—*Staphylococcus aureus* (NRC), Brussels. Belgian microbiology laboratories were invited to refer *S. aureus* isolates to the NRC in the following cases:

¹ National Reference Centre-*Staphylococcus aureus*, Department of Microbiology, Hôpital Erasme, Université Libre de Bruxelles, Route de Lennik 808, 1070 Brussels, Belgium

² Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium

1. Diagnostic problems including identification and susceptibility testing
2. Exotoxin gene detection
3. Outbreak investigation

The remaining isolates ($n=253$) corresponded to nasopharyngeal samples recovered from healthy children attending kindergartens from Brussels between 2006 and 2008 [10].

Identification and molecular typing

All isolates were analysed by *16S/mecA/nuc*, *16S/mecC* PCR and *spa* typing, as previously described [10, 11]. The *spa* types were determined with Ridom StaphType software (www.ridom.de/staphtype) and analysed by the based upon repeat pattern (BURP) algorithm using default parameters (types shorter than five repeats were excluded, and *spa* types were grouped into the same group if cost was less or equal to four) and non-restrictive conditions (types shorter than five repeats were included, and *spa* types were grouped into the same group if cost was less or equal to six). All isolates were inoculated onto Chocolate agar PolyViteX (bioMérieux, France) for 48 h at 35 °C. Isolates with non-pigmented (white) phenotype on chocolate agar plates were considered to be possible *S. argenteus* [6] and were further analysed for the presence of the dehydrosqualene synthase gene *crtM* involved in staphyloxanthin production (Table S1) and subjected to *rpoB* typing using primers and conditions previously described [12]. *rpoB* sequences were analysed with the Bionumerics 6.5 software (Applied Mathematics, Belgium). A similarity dendrogram was constructed using the multiple sequence alignment and the unweighted pair group method with arithmetic averages (UPGMA). *rpoB* sequences from *S. argenteus*, *S. aureus* and other staphylococci (<http://www.ncbi.nlm.nih.gov/>) were used as controls. MLST of *S. argenteus* isolates was performed, as previously described [13].

Antimicrobial resistance

Antimicrobial susceptibility of *S. argenteus* isolates was determined using the disk diffusion method and interpreted according to CLSI breakpoints for *S. aureus* [14] for the following antibiotics: penicillin, oxacillin, cefoxitin, gentamicin, kanamycin, tobramycin, fusidic acid, trimethoprim/sulfamethoxazole, ciprofloxacin, minocycline, tetracycline, rifampicin, erythromycin, clindamycin, mupirocin, chloramphenicol and linezolid. Vancomycin susceptibility was determined using the E-test (bioMérieux) and interpreted according to CLSI [14].

Antimicrobial resistance and detection of virulence determinants

S. argenteus isolates were tested for the presence of genes encoding β -lactamase (*blaZ*), PVL, toxic shock syndrome toxin 1 (TSST-1), exfoliatins (*eta*, *etb*), staphylokinase (*sak*), staphylococcal complement inhibitor (*scn*), chemotaxis inhibitory protein (*chp*) and enterotoxins (*sea* to *see*, *seg* to *selu*; Table S1). Staphylococcal chromosomal cassette *mec* (SCC*mec*) typing was determined for methicillin-resistant *S. argenteus* (MRSArg) isolates (Table S1).

Results

All 1,903 isolates collected at the NCR were positive for *nuc* and *16S* genes, 48.0 % ($n=913$) were *mecA*-positive and 0.3 % ($n=6$) were *mecC*-positive. The 1,903 isolates belonged to 445 different *spa* types. The BURP analysis using default parameters (Table 1) grouped the 1,903 isolates into 20 groups, whereas 124 isolates were excluded or designated as singletons. Most groups could be related to typical *S. aureus* lineages (Table 1). The BURP analysis using relaxed grouping parameters (Table 1) allowed the isolates to be clustered into three groups, including a cluster grouping of *spa* types related to *S. argenteus* CC2483 ($n=3$).

Most isolates coloured grey to yellow on chocolate agar plates ($n=1,830$), and only 73 (3.8 %) isolates were non-pigmented. Twenty-nine out of the 73 non-pigmented isolates (28.8 %) carried *crtM* (Fig. 1). Non-pigmented isolates were subjected to *rpoB* typing. Three *crtM*-negative isolates with *spa* types t5787 or t6675 that grouped in the *spa*-CC related to *S. argenteus* CC2483 showed *rpoB* sequences that grouped at 99.5 % similarity with sequences of *S. argenteus* control strains. The remaining non-pigmented isolates ($n=70$) carried diverse *spa* types ($n=26$), and their *rpoB* sequences grouped at 99.2 % similarity with sequences of *S. aureus* control isolates. The clusters grouping *S. argenteus* and *S. aureus* *rpoB* sequences were associated at 93.2 % similarity. Other staphylococci *rpoB* sequences used as controls showed less similarity than *S. argenteus* and *S. aureus* sequences (<90.9 %).

Overall, only three isolates (0.16 %) corresponded to *S. argenteus* isolates by *rpoB* typing. The three isolates were collected from two hospitals and one kindergarten located in different Belgian cities (Table 2). The three *S. argenteus* isolates belonged to CC2883 (two isolates were ST2250, and the third was ST3240). All isolates showed resistance to penicillin and carried the *blaZ* gene. The two hospital-associated isolates were MRSArg-SCC*mec* IV. The three isolates were fully susceptible to the remaining antimicrobials tested. Regarding virulence factors, one isolate carried enterotoxin-like genes (*selk*, *selq*), and all carried the immune evasion cluster (IEC) *sak* and *scn* genes.

Table 1 Groups defined with the based upon repeat pattern (BURP) clustering

<i>spa</i> -CC ^a	<i>spa</i> -CC ^c	Number of <i>spa</i> types and representative types ^d	Representative associated CC or STs ^e	Number of isolates	Number of <i>mecA</i> -positive isolates	Number of <i>mecC</i> -positive isolates	Number of isolates with white phenotype in CA: (<i>crtM</i> -positive/ <i>crtM</i> -negative) ^f
CC-021	CC-002	42 (t002)	CC5	276	192	0	8 (6/2)
CC-021	CC-005	17 (t005, t223)	CC22	62	18	0	0
CC-021	CC-008	40 (t008, t024)	CC8	318	257	0	0
CC-021	CC-021	43 (t012, t021)	CC30	168	33	0	1 (1/0)
CC-021	CC-078	15 (t056, t078)	ST26, ST101	23	0	0	2 (2/0)
CC-021	CC-159	15 (t159, t645)	CC121	60	1	3	1 (1/0)
CC-021	CC-160	3 (t160)	CC12	6	1	0	0
CC-021	CC-166	16 (t166)	ST34	65	1	0	0
CC-021	CC-021	10 (t437)	CC59	38	31	0	0
CC-021	CC-355	6 (t355)	CC152/377	40	4	0	30 (0/30)
CC-021	CC-364	5 (t364, t5739)	ST611	32	0	0	0
CC-021	CC-740	151 (t011, t044, t084, t127, t740)	CC1, CC45, CC80, CC398	632	323	0	8 (6/2)
CC-1458	CC-1458	3 (t1458)	Unknown	6	0	0	0
CC-021	CC-3092	6 (t148)	CC72	26	15	0	9 (7/2)
CC-021	CC-4690	3 (t4690)	Unknown	12	0	0	5
CC-021	CCWF-16	2 (t1721, t3743)	Unknown	2	0	0	0
CC-021	CCWF-17	2 (t1811, t4699)	ST93	2	2	0	0
CC-021	CCWF-18	2 (t773, t14835)	ST34	4	0	0	0
CC-021	CCWF-19	2 (t937, t3096)	ST291	5	0	0	0
CC-021	CCWF-20	2 (t100, t8570)	CC9	2	0	0	1 (1/0)
CC-021	Singletons	25	Not applicable	51	11	0	5 (5/0)
CC-021	Excluded	27	Not applicable	60	22	0	0
CCWF-3 ^b	Singletons	2 (t5787, t6675)	CC2483	3	2	0	3 (0/3)
Singletons	Singletons	6	Not applicable	10	0	3	0
Total	Not applicable	445	Not applicable	1903	913	6	73 (29/44)

CA chocolate agar plates, CCWF closely related *spa* types without founder

^a BURP analysis with non-restrictive conditions: types shorter than five repeats were included, and *spa* types were grouped into the same group if the cost was less or equal to six

^b The *S. argenteus* strain type (MSHR1132) has a non-typeable *spa* type lacking the 5' signal, but further analysis of its repeats allowed its repeats pattern to be related to other *spa* types (such as t4411, t6185, t7462, t9505, t11454, t12132 and t12782), which also clustered using relaxed grouping parameters with t5078 and t6675 (data not shown)

^c BURP analysis performed using the default parameters: types shorter than five repeats were excluded, and *spa* types were grouped into the same group (closely related *spa* types: *spa*-CC) if the cost was less or equal to four

^d The most prevalent *spa* types of each *spa*-CC are given in parenthesis

^e Representative associated clonal complex (CC) or sequence type (STs) (www.ridom.de/staphype; <http://saureus.mlst.net>)

^f The *crtM*-positive non-pigmented isolates carried *spa* types: t002 (*n* = 6), t096 (*n* = 1), t127 (*n* = 4), t148 (*n* = 1), t209 (*n* = 1), t469 (*n* = 2), t1313 (*n* = 3), t2186 (*n* = 1), t3092 (*n* = 4), t4359 (*n* = 1), t8570 (*n* = 1), t11823 (*n* = 1), t12004 (*n* = 1), t13719 (*n* = 1) or t14320 (*n* = 1). The *crtM*-negative non-pigmented isolates carried *spa* types: t002 (*n* = 1), t148 (*n* = 1), t355 (*n* = 19), t688 (*n* = 1), t1096 (*n* = 1), t1123 (*n* = 1), t3169 (*n* = 1), t4690 (*n* = 2), t5691 (*n* = 8), t5787 (*n* = 1), t6675 (*n* = 2), t10904 (*n* = 1), t13156 (*n* = 3), t13805 (*n* = 1) or t14496 (*n* = 1)

Fig. 1 Unweighted pair group method with arithmetic averages (UPGMA) tree based on partial RNA polymerase B (*rpoB*) gene sequences showing the phylogenetic relationship among different species and subspecies of the genus *Staphylococcus* ($n=59$) and National Reference Centre—*Staphylococcus aureus* (NRC) and healthy children (HC) collection isolates, which showed a non-pigmented phenotype on agar chocolate plates ($n=73$). The partial *rpoB* gene sequences corresponded to a 485-bp fragment between nucleotides 1444 and 1928, as previously described [12]. The scale indicates the similarity on the basis of the multiple sequence alignment. *S. argenteus* control isolates were sequenced by Tong et al. [1], whereas the remaining control isolates corresponded to sequences of strains deposited from diverse culture collections. *spa* types, and the presence (+) or absence (−) of the *mecA* and *crtM* genes, are indicated at the right of the NRC and HC collection isolates. The asterisk indicates that the strain NCR-2013S018 carried the *mecC* gene. ATCC American Type Culture Collection, Manassas, USA; DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; FRI Food Research Institute, University of Wisconsin, Madison, USA; CCM Česká Sběrka Mikroorganismů, Brno, Czech Republic

Discussion

In this study, 0.16 % of the Belgian human *S. aureus* isolates investigated were identified as *S. argenteus*. This low occurrence should be considered carefully as two different populations (clinical samples and healthy carriers) were analysed. In this sense, only 0.12 % of the human clinical isolates investigated corresponded to *S. argenteus*. In Australia, *S. argenteus* has been reported to be the predominant community-acquired methicillin-resistant lineage in Aboriginal communities, with a prevalence of 71 % [2]. However, recent studies have reported *S. argenteus* at a low occurrence in hospital settings from Australia [8], Fiji [15], Thailand [5, 16] and Trinidad and Tobago [13]. *S. argenteus* carriage has also been reported in Cambodia [17] and French Guyana [4]. In Europe, *S. argenteus* studies are scarce, and this species has only been reported in two patients in France, who had an epidemiological link with Mayotte (Indian Ocean) [9]. In our study, one out of the three *S. argenteus* isolates had an epidemiological link

with the Philippines (Table 2). The three Belgian isolates were related to carriage state ($n=2$) or to soft-tissue infections ($n=1$), like most *S. argenteus* isolates described so far [4, 15]. However, some *S. argenteus* isolates have been related to invasive infections [5, 16].

The screening of non-pigmented isolates on chocolate agar plates appeared to be a useful tool for the identification of *S. argenteus* isolates [6]. However, some *S. aureus* isolates were also non-pigmented. *S. aureus* defective in earlier enzymes of the staphyloxanthin pathway or with mutations in genes involved in regulation can show differences in the pigment production [18]. Therefore, the chocolate agar screening should be confirmed by other genetic analyses to accurately identify *S. argenteus*.

The three *S. argenteus* isolates described in this study carried *spa* types (t5078, t6675) that have previously been related to CC2483 (<http://saureus.mlst.net>). The *spa* types t127, t376 and t1635 have been associated with *S. argenteus* CC1594 in the literature [4]. In this study, we found some non-pigmented t127 isolates ($n=4$), but they carried *crtM*, and were ascribed to *S. aureus* by using *rpoB* typing. Thus, *spa* typing does not seem to be a discriminatory typing technique for *S. argenteus* isolates. Moreover, non-typeable types have been reported in few *S. argenteus* isolates (<http://saureus.mlst.net>). Further revision of the *spa* typing scheme is needed to improve this technique for *S. argenteus* isolates.

In this study, the two CC2483 MRSA_{Arg} isolates carried SCC_{mec} type IV, which has previously been related to *S. argenteus* isolates of the CC75 [2]. The *S. argenteus* isolates described in this study carried IEC genes and/or enterotoxin-like genes (*selk*, *selq*), which have been related to *hly*-converting prophages in *S. aureus*. These results, together with the recent characterisation of PVL-positive strains [8, 9, 16], underline the capacity of this new bacterium to acquire typical *S. aureus* virulence factors.

As far as we know, our study represents the first large study of this new species in a European country. Although its occurrence in Belgium seems low, it is notable that the first isolate

Table 2 Characteristics of *S. argenteus* strains from Belgium, 2012–2015

Strain ^a	Source, isolation year	Carrier/patient age (years/sex)	City, centre	Antimicrobial resistance phenotype/genotype ^b	Virulence genes	<i>spa</i> type	ST
HC-293	Screening, 2007	5/unknown	Brussels, kindergarten	PEN/ <i>bla</i> _Z	<i>sak</i> , <i>scn</i>	t5787	2250
NRC-2012S149	Screening, 2012	67/male	Ghent, hospital	FOX-OXA-PEN/ <i>bla</i> _Z , <i>mecA</i> (SCC _{mec} IV [2B])	<i>sak</i> , <i>scn</i> ,	t6675	2250
NRC-2014S151	Wound, 2014	83/male	Bonheiden, hospital	FOX-OXA-PEN/ <i>bla</i> _Z , <i>mecA</i> (SCC _{mec} IV [2B])	<i>sak</i> , <i>scn</i> , <i>selk</i> , <i>selq</i>	t6675	3240 ^c

FOX cefoxitin, HC healthy carrier, NRC National Reference Centre—*Staphylococcus aureus*, OXA oxacillin, PEN penicillin, ST sequence type

^a The strain recovered in 2012 belonged to a patient with a wound associated with adenopathy, who had travelled to the Philippines before being hospitalised. No more information about the remaining isolates was available

^b The type of SCC_{mec} was determined by the combination of *ccr* complex type and *mec* complex class

^c The ST3240 corresponded to a new single locus variant of ST2250

was recovered in 2007. Further studies are needed to determine the geographical distribution of this new species.

Acknowledgements We thank our microbiologist colleagues for sending their staphylococci strains to the NRC.

Compliance with ethical standards

Nucleotide accession numbers The *rpoB* sequences generated in this study were deposited in GenBank: accession numbers KU555518 to KU555590).

Funding This study was supported by internal funding.

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval For this type of study formal consent is not required.

References

1. Tong SY, Schaumburg F, Ellington MJ et al (2015) Novel staphylococcal species that form part of a *Staphylococcus aureus*-related complex: the non-pigmented *Staphylococcus argenteus* sp. nov. and the non-human primate-associated *Staphylococcus schweitzeri* sp. nov. *Int J Syst Evol Microbiol* 65:15–22
2. McDonald M, Dougall A, Holt D et al (2006) Use of a single-nucleotide polymorphism genotyping system to demonstrate the unique epidemiology of methicillin-resistant *Staphylococcus aureus* in remote aboriginal communities. *J Clin Microbiol* 44: 3720–3727
3. Ng JW, Holt DC, Lilliebridge RA et al (2009) Phylogenetically distinct *Staphylococcus aureus* lineage prevalent among indigenous communities in northern Australia. *J Clin Microbiol* 47:2295–2300
4. Ruimy R, Angebault C, Djossou F et al (2010) Are host genetics the predominant determinant of persistent nasal *Staphylococcus aureus* carriage in humans? *J Infect Dis* 202:924–934
5. Thaipadungpanit J, Amornchai P, Nickerson EK et al (2015) Clinical and molecular epidemiology of *Staphylococcus argenteus* infections in Thailand. *J Clin Microbiol* 53:1005–1008
6. Holt DC, Holden MT, Tong SY et al (2011) A very early-branching *Staphylococcus aureus* lineage lacking the carotenoid pigment staphyloxanthin. *Genome Biol Evol* 3:881–895
7. Tong SY, Sharma-Kuinkel BK, Thaden JT et al (2013) Virulence of endemic nonpigmented northern Australian *Staphylococcus aureus* clone (clonal complex 75, *S. argenteus*) is not augmented by staphyloxanthin. *J Infect Dis* 208:520–527
8. Tong SY, Lilliebridge RA, Bishop EJ et al (2010) Clinical correlates of Pantone-Valentine leukocidin (PVL), PVL isoforms, and clonal complex in the *Staphylococcus aureus* population of Northern Australia. *J Infect Dis* 202:760–769
9. Dupieux C, Blonde R, Bouchiat C et al (2015) Community-acquired infections due to *Staphylococcus argenteus* lineage isolates harbouring the Pantone-Valentine leukocidin, France, 2014. *Euro Surveill* 20:21154
10. Blumental S, Deplano A, Jourdain S et al (2013) Dynamic pattern and genotypic diversity of *Staphylococcus aureus* nasopharyngeal carriage in healthy pre-school children. *J Antimicrob Chemother* 68:1517–1523
11. Deplano A, Vandendriessche S, Nonhoff C et al (2014) Genetic diversity among methicillin-resistant *Staphylococcus aureus* isolates carrying the *mecC* gene in Belgium. *J Antimicrob Chemother* 69:1457–1460
12. Mellman A, Becker K, von Eiff C et al (2006) Sequencing and staphylococci identification. *Emerg Infect Dis* 12:333–336
13. Monecke S, Stieber B, Roberts R et al (2014) Population structure of *Staphylococcus aureus* from Trinidad & Tobago. *PLoS One* 9: e89120
14. Clinical and Laboratory Standards Institute (2014) Performance standards for antimicrobial susceptibility testing: twenty-fourth informational supplement M100-S24. CLSI, Wayne, Pennsylvania, USA
15. Jenney A, Holt D, Ritika R et al (2014) The clinical and molecular epidemiology of *Staphylococcus aureus* infections in Fiji. *BMC Infect Dis* 14:160
16. Chantratita N, Wikraiphat C, Tandhavanant S et al (2016) Comparison of community-onset *Staphylococcus argenteus* and *Staphylococcus aureus* sepsis in Thailand: a prospective multicentre observational study. *Clin Microbiol Infect*. doi:10.1016/j.cmi.2016.01.008
17. Ruimy R, Armand-Lefevre L, Barbier F et al (2009) Comparisons between geographically diverse samples of carried *Staphylococcus aureus*. *J Bacteriol* 191:5577–5583
18. Fey PD, Endres JL, Yajjala VK et al (2013) A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* 4:e00537-12