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ORIGINAL ARTICLE

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

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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 SCC*mec* 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.

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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.

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. 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

spa-CC[a]	*spa*-CC[c]	Number of *spa* types and representative types[d]	Representative associated CC or STs[e]	Number of isolates	Number of *mecA*-positive isolates	Number of *mecC*-positive isolates	Number of isolates with white phenotype in CA: (*crtM*-positive/*crtM*-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-316/437	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

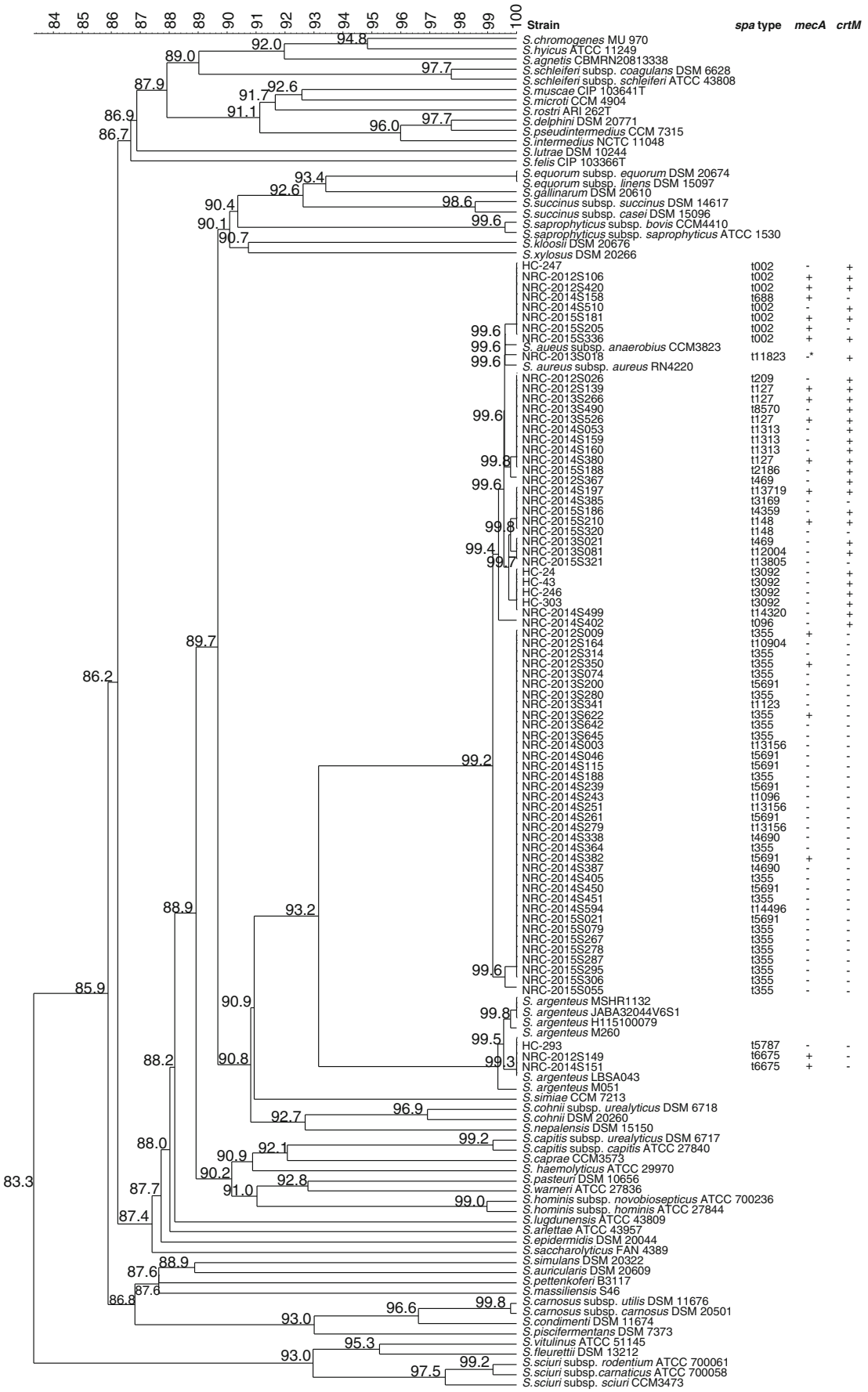
[b] The *S. argenteus* strain type (MSHR1132) has a non-typeable *spa* type lacking the 5' signal, but further analysis of its repeats allowed its repeats pattern to be related to other *spa* types (such as t4411, t6185, t7462, t9505, t11454, t12132 and t12782), which also clustered using relaxed grouping 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/staphtype; 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 ($n=1$)


84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
Strain
spa type
mecA
crtM
S. chromogenes MU 970
S. hyicus ATCC 11249
S. agnetis CBMRN20813338
S. schleiferi subsp. coagulans DSM 6628
S. schleiferi subsp. schleiferi ATCC 43808
S. muscae CIP 103641T
S. microti CCM 4904
S. rostri ARI 262T
S. delphini DSM 20771
S. pseudintermedius CCM 7315
S. intermedius NCTC 11048
S. lutrae DSM 10244
S. felis CIP 103366T
S. equorum subsp. equorum DSM 20674
S. equorum subsp. linens DSM 15097
S. gallinarum DSM 20610
S. succinus subsp. succinus DSM 14617
S. succinus subsp. casei DSM 15096
S. saprophyticus subsp. bovis CCM4410
S. saprophyticus subsp. saprophyticus ATCC 1530
S. kloosii DSM 20676
S. xylosus DSM 20266
HC-247 t002 - +
NRC-2012S106 t002 + +
NRC-2012S420 t002 + +
NRC-2014S158 t688 + -
NRC-2014S510 t002 - +
NRC-2015S181 t002 + +
NRC-2015S205 t002 + -
NRC-2015S336 t002 + +
S. aureus subsp. anaerobius CCM3823
NRC-2013S018 t11823 -* +
S. aureus subsp. aureus RN4220
NRC-2012S026 t209 - +
NRC-2012S139 t127 + +
NRC-2013S266 t127 + +
NRC-2013S490 t8570 - +
NRC-2013S526 t127 + +
NRC-2014S053 t1313 - +
NRC-2014S159 t1313 - +
NRC-2014S160 t1313 - +
NRC-2014S380 t127 + +
NRC-2015S188 t2186 - +
NRC-2012S367 t469 - +
NRC-2014S197 t13719 + +
NRC-2014S385 t3169 - -
NRC-2015S186 t4359 - +
NRC-2015S210 t148 + +
NRC-2015S320 t148 - -
NRC-2013S021 t469 - +
NRC-2013S081 t12004 - +
NRC-2015S321 t13805 - -
HC-24 t3092 - +
HC-43 t3092 - +
HC-246 t3092 - +
HC-303 t3092 - +
NRC-2014S499 t14320 - +
NRC-2014S402 t096 - +
NRC-2012S009 t355 + -
NRC-2012S164 t10904 - -
NRC-2012S314 t355 - -
NRC-2012S350 t355 + -
NRC-2013S074 t355 - -
NRC-2013S200 t5691 - -
NRC-2013S280 t355 - -
NRC-2013S341 t1123 - -
NRC-2013S622 t355 + -
NRC-2013S642 t355 - -
NRC-2013S645 t355 - -
NRC-2014S003 t13156 - -
NRC-2014S046 t5691 - -
NRC-2014S115 t5691 - -
NRC-2014S188 t355 - -
NRC-2014S239 t5691 - -
NRC-2014S243 t1096 - -
NRC-2014S251 t13156 - -
NRC-2014S261 t5691 - -
NRC-2014S279 t13156 - -
NRC-2014S338 t4690 - -
NRC-2014S364 t355 - -
NRC-2014S382 t5691 + -
NRC-2014S387 t4690 - -
NRC-2014S405 t355 - -
NRC-2014S450 t5691 - -
NRC-2014S451 t355 - -
NRC-2014S594 t14496 - -
NRC-2015S021 t5691 - -
NRC-2015S079 t355 - -
NRC-2015S267 t355 - -
NRC-2015S278 t355 - -
NRC-2015S287 t355 - -
NRC-2015S295 t355 - -
NRC-2015S306 t355 - -
NRC-2015S055 t355 - -
S. argenteus MSHR1132
S. argenteus JABA32044V6S1
S. argenteus H115100079
S. argenteus M260
HC-293 t5787 - -
NRC-2012S149 t6675 + -
NRC-2014S151 t6675 + -
S. argenteus LBSA043
S. argenteus M051
S. simiae CCM 7213
S. cohnii subsp. urealyticus DSM 6718
S. cohnii DSM 20260
S. nepalensis DSM 15150
S. capitis subsp. urealyticus DSM 6717
S. capitis subsp. capitis ATCC 27840
S. caprae CCM3573
S. haemolyticus ATCC 29970
S. pasteuri DSM 10656
S. warneri ATCC 27836
S. hominis subsp. novobiosepticus ATCC 700236
S. hominis subsp. hominis ATCC 27844
S. lugdunensis ATCC 43809
S. arlettae ATCC 43957
S. epidermidis DSM 20044
S. saccharolyticus FAN 4389
S. simulans DSM 20322
S. auricularis DSM 20609
S. pettenkoferi B3117
S. massiliensis S46
S. carnosus subsp. utilis DSM 11676
S. carnosus subsp. carnosus DSM 20501
S. condimenti DSM 11674
S. piscifermentans DSM 7373
S. vitulinus ATCC 51145
S. fleurettii DSM 13212
S. sciuri subsp. rodentium ATCC 700061
S. sciuri subsp.carnaticus ATCC 700058
S. sciuri subsp. sciuri CCM3473

◀ **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 Sbírka 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 MRSArg 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 *hlb*-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	*spa* type	ST
HC-293	Screening, 2007	5/unknown	Brussels, kindergarten	PEN/*blaZ*	*sak*, *scn*	t5787	2250
NRC-2012S149	Screening, 2012	67/male	Ghent, hospital	FOX-OXA-PEN/*blaZ*, *mecA* (SCC*mec* IV [2B])	*sak*, *scn*,	t6675	2250
NRC-2014S151	Wound, 2014	83/male	Bonheiden, hospital	FOX-OXA-PEN/*blaZ*, *mecA* (SCC*mec* IV [2B])	*sak*, *scn*, *selk*, *selq*	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.

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Compliance with ethical standards

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

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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.

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