

## National survey of molecular epidemiology of *Staphylococcus aureus* colonization in Belgian cystic fibrosis patients

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**Objectives:** Epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) is poorly defined in cystic fibrosis (CF) patients, and *S. aureus* detection may be hampered by the presence of small colony variants (SCVs). We conducted a multicentre survey to determine the prevalence of *S. aureus* and MRSA colonization in Belgian CF patients and characterize the phenotype and clonal distribution of their staphylococcal strains.

**Methods:** *S. aureus* isolated from CF patients attending nine CF centres were collected. Oxacillin resistance was detected by oxacillin agar screen and *mecA* PCR. Antibiotic susceptibility was tested by microdilution. MRSA strains were genotyped by PFGE and SCCmec typing and compared with hospital-associated MRSA strains.

**Results:** Laboratories used a diversity of sputum culture procedures, many of which appeared substandard. *S. aureus* was isolated from 275/627 (44%) CF patients (20% to 72% by centre). The prevalence of SCV colonization was 4%, but SCVs were almost exclusively recovered from patients in two centres performing an SCV search. Phenotypically, 14% of *S. aureus* isolates were oxacillin-resistant: 79%

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carried *mecA* and 19% were SCVs lacking *mecA*. The mean prevalence of 'true' MRSA colonization was 5% (0% to 17% by centre). By PFGE typing, 67% of CF-associated MRSA were related to five epidemic clones widespread in Belgian hospitals.

**Conclusions:** This first survey of *S. aureus* colonization in the Belgian CF population indicated a diversity in local prevalence rates and in proportion of oxacillin-resistant and SCV phenotypes, probably related to variation in bacteriological methods. These findings underscore the need for standard *S. aureus* detection methods and MRSA control policies in Belgian CF centres.

Keywords: oxacillin, MRSA, small colony variants

## Introduction

*Staphylococcus aureus* is frequently isolated from bronchial secretions of cystic fibrosis (CF) patients. It is of particular concern in paediatric patients, before *Pseudomonas aeruginosa* has established its colonization. The prevalence of *S. aureus* colonization in the CF population is over 50%, with a maximum in the 13–17-year-old group.<sup>1</sup> Similar prevalence is reported among adult CF patients in some centres.<sup>1,2</sup> Appropriate microbiology practices are essential for accurate aetiological diagnosis in CF lung infections. The use of selective culture media enhances the recovery of various pathogens from CF sputum.<sup>2,3</sup> In the USA, prevalence of *S. aureus* varies with age and use of selective culture media. From 40% in infants, the prevalence reaches a peak of 58% in adolescents to decrease to 38% to 46% in adults with an altered respiratory function, without and with an *S. aureus*-specific culture medium respectively.<sup>2,3</sup> The American CF Foundation has published updated recommendations for microbiological workup of CF sputum, and it has recently been shown that since then, the number of centres in the USA using appropriate media allowing for *S. aureus* detection in CF sputum has increased from 65% to 82%.<sup>3,4</sup> In Belgium, official centres are accredited for CF patient healthcare since 1999, but microbiological practice by the laboratories supporting these centres is not standardized.

Small colony variant (SCV) phenotypes of *S. aureus* have been associated with chronic infection and are a common finding in CF patients.<sup>5,6</sup> SCVs are slowly growing organisms that exhibit a small, non-pigmented, non-haemolytic colony morphotype. They are usually dependent on various substrates (mainly thymidine, haemin and menadione) supplementation for growth and are more resistant to antibiotics such as aminoglycosides and co-trimoxazole.<sup>5,6</sup> Epidemiological studies in CF patients have documented a 10% prevalence of SCV *S. aureus* strains from two centres in the USA and a 33% prevalence in one centre in Germany.<sup>7,8</sup>

In several regions of the world, infection with methicillin-resistant *S. aureus* (MRSA) is reported with increasing frequency in children and is no longer confined to patients with specific risks factors.<sup>9,10</sup> In Europe and the USA, the proportion of MRSA among *S. aureus* recovered from CF patients varies from 8% to 23%.<sup>11–14</sup> The clinical impact of MRSA carriage remains uncertain in CF patients. In a retrospective case–control study, children with MRSA infection received more courses of intravenous antibiotic therapy and had poorer growth than patients without MRSA. Chest X-rays were worse at baseline in the MRSA-infected patients, and no effect of this infection could be shown on lung function.<sup>15</sup> Long-term persistence of *S. aureus* in CF lungs has been demonstrated.<sup>16,17</sup> In adult CF

patients, MRSA colonization has been shown to be transient in some cases or persist for years in other patients.<sup>18,19</sup> The present study was undertaken to determine the prevalence and microbiological characteristics of colonization with methicillin-susceptible *S. aureus* (MSSA) and MRSA in Belgian CF patients receiving care in specialized treatment centres and one rehabilitation facility.

## Patients and methods

### Study design

We conducted a 7 month multicentre prospective survey from June until December 2001. Eleven specialized CF care facilities were invited to participate. Nine were included, among which were six of the eight accredited CF centres, two other hospitals caring for CF patients and one rehabilitation centre. At the time of study, the National CF Registry counted 788 patients who attended a CF centre three or four times a year. Sputum samples are usually collected for microbiological analysis on each visit. All participating laboratories were asked to refer *S. aureus* isolates from consecutive CF patients attending each clinic to the coordinating laboratory. The isolates were sent on trypticase soy agar transport medium, except for the rehabilitation centre, which was invited to send primary sputum cultures on mannitol salt agar medium (Becton Dickinson, Cockeysville, MD, USA). One isolate of *S. aureus* was collected per patient, unless different phenotypes (morphological aspect or antibiotic resistance profile) were recovered on the same or consecutive sputum samples. Participating laboratories also registered the total number of sputum cultures performed in CF patients and the number of CF patients for which at least one respiratory culture was obtained during the study period. They were asked to describe their standard operating procedures for CF sputum microbiological testing.

### Characterization of *S. aureus* strains

**Identification and oxacillin susceptibility testing.** *S. aureus* isolates were referred to the National Reference Laboratory for Staphylococci, where identification was confirmed by coagulase test in human plasma and latex agglutination test (Pastorex Staph-Plus, Sanofi Pasteur). SCVs of *S. aureus* were defined as small greyish or non-pigmented colonies, non-haemolytic and slowly growing on blood agar media.<sup>20</sup> Susceptibility to oxacillin was tested according to the CLSI (formerly the NCCLS) on oxacillin screen agar (BBL, Becton Dickinson) with 6 mg/L oxacillin for 24 h at 35°C.<sup>21</sup> Identification of *S. aureus* and oxacillin resistance was confirmed by triplex PCR, allowing for the detection of fragments of 16S rRNA (*Staphylococcus spp.* specific region), *nuc* (coding for *S. aureus* thermonuclease) and *mecA* (coding for the PBP2a) genes.<sup>22</sup>

## *S. aureus* colonization in CF patients

**Antimicrobial susceptibility testing.** MICs were determined using broth microdilution testing according to the CLSI for eight antibiotics: oxacillin, vancomycin, teicoplanin, amikacin, gentamicin, ciprofloxacin, co-trimoxazole and linezolid.<sup>21</sup> Mueller–Hinton (MH) broth was used for MIC determination of *S. aureus* isolates with normal colony phenotype, and MH broth supplemented with 2% NaCl was used for oxacillin susceptibility testing. Isolates with an SCV phenotype were tested in MH broth supplemented with thymidine (100 mg/L), haemin (1 mg/L) and menadione (1 mg/L) with a prolonged incubation of 48 h at 35°C. Endpoints were read after 18, 24 and 48 h of incubation.

**Molecular typing.** MRSA strains, defined as *S. aureus* isolates harbouring the *mecA* gene, were genotyped by PFGE of genomic macrorestriction fragments (using *Sma*I).<sup>23</sup> Patterns were normalized and analysed with the BioNumerics software (Applied Maths, Kortrijk, Belgium), in comparison with a database of >1000 normalized patterns from a collection of MRSA isolates from hospital surveys in Belgium and other European countries during the period 1987–2001. Pattern matching was based on the Dice coefficient for similarity analysis. The unweighted pair group method using arithmetic average (UPGMA) was used to establish the dendrogram grouping of PFGE types. PFGE profiles of CF patients isolates were compared with 12 epidemic clones previously described from the national surveillance of Belgian hospitals.<sup>23</sup> PFGE profiles were classified according to the published criteria.<sup>24</sup> MRSA were further characterized by determining the type of SCC*mec* cassette using a multiplex PCR.<sup>25</sup>

### Statistical analysis

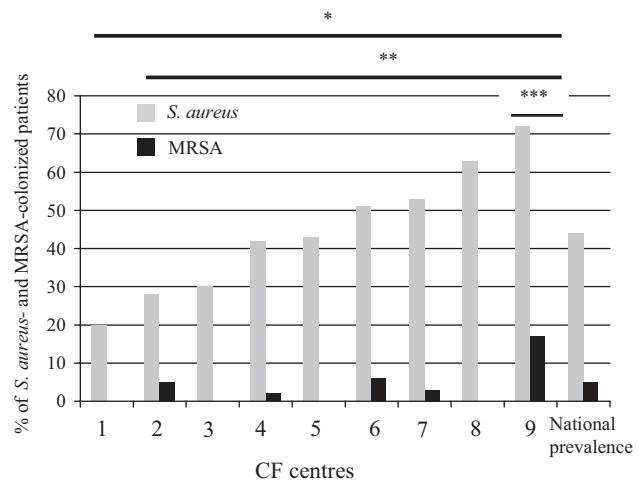
Analyses were performed using SPSS version 13.0 and EpiInfo version 3.3.2. We used the Pearson  $\chi^2$  and Fisher's exact test for expected count less than 5. The Mann–Whitney test was used for median comparison (non-Gaussian distributions).

## Results

### *S. aureus* prevalence and characteristics

During the study, 627 CF patients had at least one sputum culture performed in one of the participating centres (80% of all CF patients in the national registry). The review of laboratory procedures for processing CF sputum for culture indicated that all but three laboratories were plating CF sputum on *S. aureus*-specific media: mannitol salt agar from different manufacturers was used in five centres (Oxoid in Centres 5, 6 and 7, Becton Dickinson in Centre 4 and Difco in Centre 9). An enriched Gram-positive-specific medium containing colistin and aztreonam was used by one laboratory. *S. aureus* was isolated from 275 (44%) patients with a median age of 17 years (ranging from 3 months to 49 years). The prevalence of *S. aureus* colonization ranged from 20% to 72% by centre (Figure 1) (heterogeneity  $\chi^2 = 42$  and 43,  $P < 0.000001$ ). In the centre where *S. aureus* was isolated in 72% of the CF patients, the median age was 22 years, whereas it was 13 and 18 years in the two centres with the lowest prevalence of *S. aureus* (20% and 28%, respectively) (Table 1). All isolates were referred to the coordinating laboratory, except for 33 isolates from Centre 5, which were not available.

A total of 424 strains from 242 patients were received by the reference laboratory. Phenotypic and genotypic identification of



**Figure 1.** *S. aureus* and MRSA prevalence (rate of *S. aureus* and MRSA in CF patients who had at least one sputum sample collected during the study period) by CF centre ( $n = 9$ ) and national mean prevalence. Comparison of *S. aureus* prevalence: prevalence varied widely among the centres: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.0001$ .

*S. aureus* was confirmed for these 424 strains, and the *mecA* status was determined in order to exclude duplicate isolates from the same patient. These were defined as follow: (i) strains with identical colony morphology, i.e. normal phenotype or SCV phenotype and (ii) strains with identical *mecA* status, i.e. MRSA or MSSA.

A total of 268 *S. aureus* strains were included for further study. A single strain was analysed from 219 patients and multiple strains with distinct morpho/resistance type (49 strains) were characterized from 23 patients. The coagulase test on human plasma was positive in 261/268 (97.4%) strains and latex agglutination in 265 (98.9%) strains. Species identification was confirmed by PCR detection of the *nuc* gene in 268 (100%) isolates and the 16S rRNA gene in 265 (98.9%). Identification of the three remaining strains, which presented an SCV phenotype, was confirmed by sequence determination of the 16S rRNA gene amplified with a different DNA polymerase.

### Small colony variants

SCV *S. aureus* isolates were recovered from 25/594 (4.2%) CF patients, with wide variation between the centres (Table 1). Only four centres found SCV isolates, but only three were performing a systematic search for SCVs in CF patients' sputa. The majority of SCVs were isolated in a single laboratory which processed specimens from two centres. These SCVs were recovered on mannitol salt agar from Becton Dickinson in Centre 4 and Difco in Centre 9. The median age of patients carrying SCV *S. aureus* strains was 21 years (ranging from 9 to 38 years), when compared with 16 years (ranging from 0 to 49 years) for patients with normal phenotype *S. aureus* ( $P < 0.001$ ).

### Antibiotic susceptibility

MICs were determined for 259 (96.6%) strains. MIC results indicated that all isolates were susceptible to vancomycin, teicoplanin and linezolid, 91% were susceptible to gentamicin, 89%

**Table 1.** Prevalence of *S. aureus*, MRSA and *S. aureus* SCV colonization by centre

Centre	Mean sputum samples per patient	Number of CF patients with culture	Median age (range in years)	Number of CF patients (%) with		
				<i>S. aureus</i> <sup>a</sup>	MRSA	SCV
1	3.8	20	13 (7–19)	4 (20)	0	0
2	3.4	116	18 (4–40)	33 (28)	6 (5)	0
3	3.6	37	16 (5–39)	11 (30)	0	0
4	5.7	82	15 (5–35)	34 (42)	2 (2)	7 (10)
5	3.1	131	13 (8–27)	23 + 33 <sup>b</sup> (43)	2 (NA)	0 (NA)
6	3.7	81	20 (6–49)	41 (51)	5 (6)	2 (3)
7	2.2	91	15 (0–34)	48 (53)	3 (3)	4 (4)
8	4.3	16	07 (2–11)	10 (63)	0	0
9	5.2	53	22 (10–40)	38 (72)	9 (17)	12 (23)
National	3.75	594 + 33 <sup>b</sup>	17 (0–49)	242 + 33 <sup>b</sup> (44)	27 (5)	25 (4)

NA, not available.

<sup>a</sup>All *S. aureus*, including MRSA and SCV.

<sup>b</sup>Thirty-three patients whose strains were not available for study.

to amikacin and co-trimoxazole and 58% to ciprofloxacin (Table 2).

On the basis of MIC testing, 35 (14%) of the isolates were oxacillin-resistant. Among these, 11% (27 strains) were MRSA and 3% (8 strains, including 7 SCVs) did not carry *mecA* but had oxacillin MIC  $\geq 4$  mg/L. On the other hand, 20% of MRSA isolates did not grow on oxacillin screen agar: this was seen for 3/5 MRSA isolates with SCV phenotype when compared with 3/24 of those with normal phenotype ( $P < 0.05$ ). SCV strains were more likely to be resistant to co-trimoxazole (88%) and oxacillin (48%) than strains with normal phenotype (1.7% and 11%, respectively;  $P < 0.00001$ ).

'True' MRSA were recovered from 5% of the CF patients included in the study (Figure 1). The MRSA prevalence by centre varied significantly (heterogeneity  $\chi^2 = 21.62$ ,  $P = 0.003$ ), with no MRSA recovered from CF patients in three centres and 17% in one centre. Similarly, the proportion of MRSA among *S. aureus* was significantly different in the nine centres (crude OR by the maximum likelihood method: 0.321; (95% CI: 0.131–0.746;  $P = 0.026$ ). This difference almost lost statistical significance once adjusted for age ( $< 19$  years old and

$\geq 19$  years old). Adjusted OR was 0.415 (0.165–0.997) ( $P = 0.045$ ), indicating that age was a confounding factor. The median age of MRSA-colonized patients was significantly higher than that of MSSA-colonized patients (22 versus 17 years, respectively;  $P < 0.005$ ). Moreover, there was a positive correlation between the median age of the patients in a given centre and the proportion of MRSA recovered from these patients ( $r^2 = 0.611$ ,  $P = 0.02$ ).

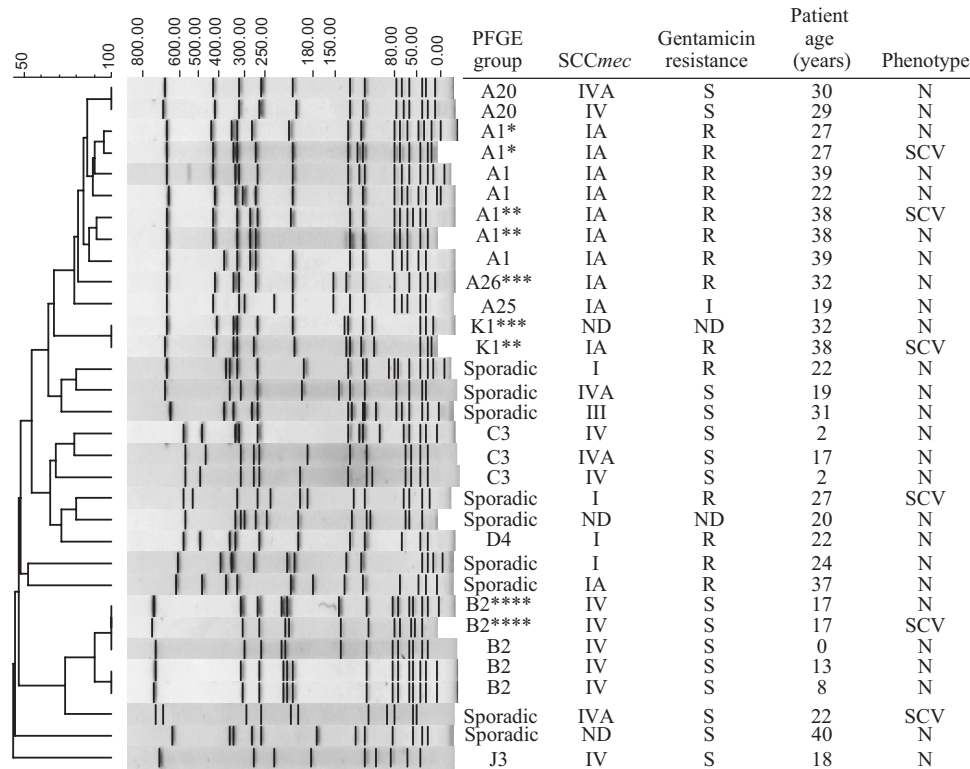
The majority of CF patients colonized with MRSA (67%) were carrying a strain belonging to one of the epidemic clones that are disseminated in Belgian hospitals (Clones A, B, C, D, J). Nine patients carried Clone A MRSA, four Clone B2 and three Clone C3, whereas Clones D and J were recovered from one patient each (Figure 2). Patients colonized with Clone A were older (median age of 30 years) than the patients harbouring Group B strains (median age 10.5 years;  $P = 0.003$ ). There were four clusters of two patients carrying the same strain (two pairs of patients cared for in the same centre and two from distinct centres). SCC*mec* types were correlated with the PFGE types and the gentamicin resistance profile (Figure 2).

**Table 2.** MICs for 259 *S. aureus* isolates from 242 CF patients (patients with both normal and SCV phenotype or MRSA and MSSA had two strains tested) determined by broth microdilution methods

Antibiotic	Number of isolates with MIC value (mg/L)											
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Oxacillin	44	49	60	43	18	10	3	4	3	3	4	18
Vancomycin	16	70	108	53	12	0	0	0	0	0	0	0
Teicoplanin	189	38	15	10	1	6	0	0	0	0	0	0
Gentamicin	97	53	45	29	10	0	1	0	0	0	5	19
Amikacin	0	2	3	8	34	78	60	36	11	15	4	8
Ciprofloxacin	47	30	27	20	27	22	15	22	12	15	9	13
Co-trimoxazole	133	57	26	8	5	1	2	2	2	1	1	20
Linezolid	14	27	100	94	22	1	0	0	0	0	0	0

One strain of MSSA with normal phenotype was not tested for co-trimoxazole and linezolid.

## S. aureus colonization in CF patients



**Figure 2.** PFGE profiles of 32 MRSA strains from 27 CF patients, similarity percentage (Dice coefficient) between the profiles and group, type and subtype according to SCCmec type, gentamicin resistance, patient age and phenotype. ND, not determined; R, resistant; I, intermediate; S, susceptible; N, normal phenotype; SCV, SCV phenotype. \*, \*\*, \*\*\* and \*\*\*\* indicate MRSA isolates from the same patients, respectively, when patients were carrying both normal and SCV phenotypes (\*, \*\* and \*\*\*\*) and in \*\* and \*\*\*, distinct MRSA clones.

## Discussion

The mean prevalence of *S. aureus* carriage (44%) among paediatric and adult CF patients in Belgium was slightly lower than that reported for Europe.<sup>1</sup> Although we expected to find more paediatric than adult patients colonized with *S. aureus*, no association was observed between the median age of the patients and the prevalence of *S. aureus* in a given centre. There was evidence for bias towards underdetection of *S. aureus* in the centres which did not use specific *S. aureus* selective isolation medium as recommended by the CF Foundation.<sup>3</sup> Microbiological workup of sputum from CF patients constitutes a challenge, because specific media must be used to enhance recovery of slow growing or variant organisms like *S. aureus* SCVs. In patients colonized with *P. aeruginosa*, this pathogen can overgrow *S. aureus* and thereby interfere with detection if selective media are not used. Investigators have shown that *S. aureus* DNA could be identified in CF sputum in patients whose culture was negative.<sup>26</sup> Furthermore, the frequency of sputum sampling per patient could also influence the *S. aureus* recovery, as the organism may be intermittently isolated. However, we did not find a correlation between the number of sputum samples per patient and the *S. aureus* carriage rate.

SCV *S. aureus* have been associated with persistent infections and are typically recovered from CF patients who are recurrently treated with co-trimoxazole or other antibiotics.<sup>8,20,27</sup> SCV *S. aureus* prevalence in this study was lower than that previously reported from CF patients in other countries.<sup>7,8</sup> However, this is probably due to underdetection, as only three centres were

actively searching for SCVs in respiratory specimens from CF patients. Most centres failed to detect any SCVs, presumably because they were not using an appropriate technique. SCVs may be more common in older patients, as they have had a persistent infection for a longer period of time and have received more antibiotic courses. Nevertheless, one study found an SCV phenotype in 32% of the *S. aureus* from CF children.<sup>28</sup>

The MRSA prevalence observed in Belgian CF patients was low (5%), but showed wide variation by centre. This can be partly explained by differences in sensitivity of the methods used for *S. aureus* detection. Oxacillin agar screen was not a suitable medium for detection of SCV MRSA. As shown by others, PCR detection of *mecA* remains the most sensitive diagnostic tool for SCV MRSA detection.<sup>29</sup> However, the proportion of MRSA among *S. aureus* isolates recovered from CF patients also varied greatly by centre. It is likely that the variations in MRSA prevalence rates were related to the incidence of nosocomial acquisition in the hospital where the CF centre is located and to local infection control practices. Another risk factor for MRSA colonization was age. Older patients were at an increased risk of carrying MRSA. Older patients are more likely to have spent more time in hospital, thereby increasing their probability of becoming colonized by hospital-acquired MRSA. The finding that most MRSA isolated from the CF population in this study belonged to predominant Belgian nosocomial clones is consistent with this source of contamination, as proposed by other investigators.<sup>19</sup> Additional evidence to support this hypothesis is the observation that older MRSA-colonized patients harboured preferentially gentamicin-resistant Clone A1-SCCmec I strains,

which were found in the majority of Belgian hospitals during the 1980s and early 1990s, whereas younger CF patients typically carried gentamicin-susceptible Clone B2-SCC*mec* IV MRSA, which has gradually emerged in the late 1990s.<sup>24</sup> However, the distribution of these epidemic clones was different in CF patients when compared with the general population. Group A1-SCC*mec* I strains accounted for 19% of all MRSA in CF patients, whereas this clone represented 3% of the Belgian MRSA during the same period.<sup>24</sup> PFGE type A1 MRSA probably persisted for many years in the CF patients' lungs, as has been shown for *S. aureus* in CF patients' lungs.<sup>16</sup>

Multiple antibiotic-resistant *S. aureus* was not a common occurrence in this study. Oxacillin resistance was due to *mecA* in 11% of the *S. aureus* strains of the CF patients and to other non-investigated mechanisms in 3% of the cases. Oxacillin resistance was high among SCV strains, which had not been reported in other studies.<sup>8,28</sup> The strains were tested in non-standard conditions using a supplemented MH broth, providing the nutrient supplementation that their auxotrophism requires. No standard technique exists for *S. aureus* SCV susceptibility testing and, as enriched media are used, revertants might be tested in some cases. However, in *S. aureus* isolated from CF patients, selection of mutants with novel resistance mechanisms has been linked to the expression of a modified PBP3 with a decreased affinity for oxacillin in one patient<sup>30</sup> and for macrolides by ribosomal modification due to mutations in the 23S rRNA gene.<sup>31</sup>

In conclusion, we observed a similar prevalence of MRSA and MSSA colonization in CF patients treated in specialized centres in Belgium to that reported elsewhere in Europe and North America. It is likely that true prevalence was underestimated because of the use of suboptimal laboratory methods. In particular, *S. aureus* SCVs were not sought nor detected in many laboratories. Further studies would be necessary to ascertain their incidence and study their clinical impact on CF patients. Our findings underline the need for improvement and harmonization of microbiological procedures for diagnosis of lung infection in CF patient care centres in Belgium.

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## Transparency declarations

None to declare.

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## *S. aureus* colonization in CF patients

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