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Intravenous antibiotics given for 2 weeks do not eradicate persistent *Staphylococcus aureus* clones in cystic fibrosis patients

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Abstract

Staphylococcus aureus is the most commonly isolated pathogen in respiratory tract secretions from young patients with cystic fibrosis (CF), and several treatment strategies are used to control the infection. However, it is not known whether intensified treatment with antimicrobial agents causes eradication of *S. aureus* clones. We retrospectively determined the impact of intravenous (IV) antimicrobial agents on the suppression and eradication of *S. aureus* clones. One thousand and sixty-one *S. aureus* isolates cultured from 2526 samples from 130 CF patients during a 2-year study period were subjected to *spa* typing. Intervals between positive samples and the occurrence of clone replacements were calculated in relation to courses of IV antimicrobial agents. Of 65 patients chronically infected with *S. aureus*, 37 received 139 courses of IV antimicrobial agents with activity against *S. aureus* (mean duration, 15 days; range, 6–31 days). Administration of IV antibiotics increased the time to the next sample with growth of *S. aureus*: the mean interval between two positive samples was 68 days if IV treatment had been administered, in contrast to 49 days if no IV treatment had been administered (p 0.003). When *S. aureus* recurred in sputum after IV treatment, the isolate belonged to a different clone in 33 of 114 (29%) intervals, in comparison with 68 of 232 (29%) intervals where IV treatment had not been prescribed (OR 0.98, 95% CI 0.60–1.61). In conclusion, we show that 2 weeks of IV antimicrobial treatment can significantly suppress chronic staphylococcal infection in CF, but is not associated with the eradication of persistent bacterial clones.

Keywords: Antistaphylococcal therapy, colonization, fusidic acid, persistent bacterial clones, *spa* typing
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Introduction

Cystic fibrosis (CF) is a multi-organ disease characterized by dysfunction of exocrine glands. The most serious threat to this group of patients is progressive lung destruction elicited by a vicious cycle of bacterial colonization, infection, and inflammation [1]. *Staphylococcus aureus* is the most commonly isolated pathogen in respiratory tract secretions from young CF patients, often being replaced later by *Pseudomonas aeruginosa* [2–4]. The high prevalence of *S. aureus* in respiratory tract secretions from CF patients has led to different strategies for antimicrobial therapy, including monthly surveillance cultures combined with culture-directed antimicrobial therapy [5], the use of long-term antistaphylococcal therapy [6,7], and treatment depending on clinical symptoms [8].

When *S. aureus* is repeatedly cultured from a patient's specimens for a prolonged period of time, the patient is categorized as chronically infected. Cultivation of *S. aureus* from chronically infected patients may terminate for reasons that are incompletely understood; it may be related to competition between *S. aureus* and a new pathogen, such as *P. aeruginosa*, for the ecological niche in the lungs [9].

Studies examining persistence and strain replacement in CF patients have shown that the same clone of *S. aureus* may be recovered from individual patients for years; however, recovery of interspersed, solitary clones is common [10–12]. These studies differ in patient characteristics, typing methods, and sampling frequencies, and do not provide data on the antibiotic regimens employed or assess the impact of antimicrobial therapy.

It is not known whether intensified treatment with antimicrobial agents causes eradication of *S. aureus* clones and subsequent re-infection by new clones. To elucidate this, we characterized the colonization dynamics of *S. aureus* by *spa* typing isolates from a frequently sampled cohort of CF patients attending a single centre with a relatively strict antibiotic prescription policy.

Materials and Methods

Patients and sampling of respiratory tract specimens

Samples were collected from CF patients attending the CF centre at Aarhus University Hospital from January 2009 to December 2010. Most patients were seen monthly, and cultures were performed on sputum or, if the patient was unable to produce sputum, on airway secretions obtained by laryngeal aspiration. A total of 2526 respiratory tract samples from 154 patients, 87 males and 67 females, with an age range from 0 to 44 years were investigated (range, 1–33 samples per patient).

Identification and antimicrobial susceptibility testing of *S. aureus*

Samples were semiquantitatively plated on 5% horse blood agar, chocolate agar, MacConkey agar, Sabouraud agar (Statens Serum Institut (SSI), Copenhagen, Denmark), and *Burkholderia cepacia* selective agar (bioMérieux, Paris, France). A tentative identification of *S. aureus* was confirmed by use of an agglutination kit (Slidex Staph-PLUS; bioMérieux, France); colonies with atypical reactions in the agglutination kit were identified by the tube coagulase test (SSI), or by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonics, Bremen, Germany). Antibiotic susceptibility testing of all strains was performed by the disk diffusion technique according to the Swedish Reference Group for Antibiotics guidelines (www.srga.org) until November 2009, and thereafter according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (www.eucast.org). All isolates of *S. aureus* were stored at −80°C in 10% glycerol for subsequent typing.

spa and *agr* typing

The polymorphic X region of the staphylococcal protein A gene (*spa*) was amplified with the primers spa-1113f and spa-1514r, as previously described [13]. Sanger sequencing of PCR amplicons was performed at an external facility (GATC Biotech AB, Konstanz, Germany) by use of the forward primer. *spa* types were assigned by use of Ridom StaphType software (Ridom, Würzburg, Germany) [14]. Electropherograms were visually inspected if a change in *spa* type was encountered, and sequencing was repeated on both strands if the shift could be questioned. As shifts in *spa* types can represent mutational change rather than clone replacement [13], application of the BURP (Based Upon Repeat Patterns) algorithm implemented by the Ridom software was used to combine *spa* types into BURP clusters [15]. Accessory gene regulator (*agr*) typing was performed by amplifying the *agr* alleles I, II, III, and IV, as previously described [16].

Definition of chronic infection, clones, and short-term clone replacement

Chronic infection was defined as the isolation of the bacterium in question from more than half of the cultures during a calendar year, provided that the patient had been affiliated with the centre throughout the year and delivered four or more samples (modified 'Leeds criteria' for chronic infection with *P. aeruginosa*) [17]. *S. aureus* isolates from individual patients were considered to be clonally related if they belonged to the same BURP cluster. Solitary clones were recovered only once from individual patients during the study period, whereas persistent clones were detected two or more times. A short-term clone replacement was defined as the culture of an *S. aureus spa* type that differed from the previous isolate from the same patient (after adjustment by the BURP algorithm).

Antibiotic prescription policy and antistaphylococcal treatment regimens

Antibiotic treatment of *S. aureus* in the two Danish CF centres is based on monthly surveillance cultures, and the policy entails patients with positive cultures of *S. aureus* being treated regardless of symptoms [5,18]. The standard regimen consists of 2 weeks of oral treatment with dicloxacillin and fusidic acid, which is modified according to antimicrobial susceptibility testing. In the case of severe clinical deterioration attributed to staphylococcal infection, the patient is treated with intravenous (IV) dicloxacillin or cefuroxime for 2 weeks; the regimen is initiated in the outpatient clinic, and thereafter administered at home. In recent years, a number of chronically infected patients have been treated with IV cefuroxime at fixed

intervals (usually four courses/year), rather than with multiple oral antistaphylococcal treatments.

IV antimicrobial agents were administered as recommended by the Leeds Regional Adult and Paediatric Cystic Fibrosis Units (www.cysticfibrosismedicine.com). Antistaphylococcal activity of antimicrobial agents was categorized as sufficient or insufficient according to EUCAST guidelines (www.eucast.org).

Statistical analysis

Mean time to next sample and time to next sample with growth of *S. aureus* in patient groups were compared by using a paired *t*-test, and statistical significance was taken as $p < 0.05$. An OR with 95% CI was calculated to evaluate the association between IV antimicrobial therapy and probability of clone replacement.

Results

S. aureus spa types and susceptibility to antimicrobial agents

Of 2526 samples from 154 patients obtained during a period of 2 years, 1047 samples (41%) from 130 patients (84%) yielded growth of *S. aureus* (range, 1–23 samples per patient). From five samples, two different *S. aureus* isolates were identified by different susceptibility patterns and stored, whereas 26 isolates had not been stored. We found 32 samples with more than one *spa* type, resulting in 67 *spa* types from the 32 samples. In total, 1061 *S. aureus* isolates from 1021 samples from 130 patients were characterized by *spa* typing; from these, 129 different *spa* types were detected. Up to ten different *spa* types were detected from the same patient during the 2-year study period. Of the 130 patients, 35 (27%) harboured only a single *spa* type, 31 (24%) harboured two *spa* types, 31 (24%) harboured three *spa* types, and 33 (25%) harboured four or more *spa* types. When identical *spa* types from different patients were classified separately, a total of 353 patient *spa* types were detected. Table 1 shows the frequencies of the most prevalent *spa* types found in the present study, and the frequencies with which the same *spa* types were reported to the Ridom SpaServer (http://spa.ridom.de/frequencies.html). The most prevalent *spa* type at our centre was t230, which was identified in 26 of 130 patients and recovered from 101 of 1021 samples (9.9%), whereas it is rarely reported to the Ridom SpaServer. The 26 *S. aureus* t230 isolates belonged to three different *agr* groups (results not shown), indicating that this *spa* type combines several clones. *Spa* type t002, which was the second most commonly detected *spa* type at our centre, had a similar prevalence in our study population as that reported to the Ridom SpaServer.

TABLE 1. Frequencies of the most prevalent *spa* types in cystic fibrosis sputa in the present study and as reported to the Ridom SpaServer

		Frequency	
Spa type	No. of patients	Study (%)[a]	SpaServer (%)[b]
t230	26	7.4	0.35
t002	20	5.7	6.41
t127	16	4.5	1.85
t012	15	4.3	1.69
t084	15	4.3	1.81
t065	13	3.7	0.53
t021	10	2.8	1.01

[a]In relation to the total number of patient *spa* types ($n = 353$).
[b]Ridom SpaServer (www.spaserver.ridom.de), accessed 27 October 2012.

Application of the BURP algorithm to isolates from individual patients resulted in the merging of 353 patient *spa* types into 331 patient BURP clusters, referred to as patient clones. The results of antimicrobial susceptibility testing for the 331 patient clones are shown in Table 2. Resistance to the routinely prescribed antimicrobial agents dicloxacillin and fusidic acid was infrequent. A single patient harboured methicillin-resistant *S. aureus* (MRSA) during the 2-year study period; several clones were identified from this patient, belonging to the porcine-associated clonal complexes 5 and 8 [19]. MRSA carriage in this patient was terminated by 5 weeks of treatment with oral rifampicin plus fusidic acid; in addition, the patient ceased his occupational-related exposure to pigs.

Population dynamics of *S. aureus* in different patient categories

Ten patients had either four or five sputum samples positive for *S. aureus* during the 2-year study period; the microbiological findings and antibiotic therapy of these patients are summarized in Fig. 1. Antimicrobial therapy includes treatments given in the absence of positive cultures, or directed

TABLE 2. Antimicrobial susceptibility rates of the 331 *Staphylococcus aureus* patient clones included in the present study

	Susceptibility	
Antimicrobial agent	S (%)	R (%)
Benzylpenicillin	13.8	86.2
Erythromycin	96.0	4.0
Cefoxitin[a]	98.8	1.2
Tetracycline	97.9	2.1
Clindamycin	97.1	2.5
Fusidic acid	87.6	12.4
Rifampicin	73.2	25.5
Linezolid	99.7	0.3
Moxifloxacin	96.3	3.8
Norfloxacin	94.1	5.9

[a]Susceptibility to cefoxitin is interpreted as susceptibility to all penicillinase-stable β-lactams, including methicillin.

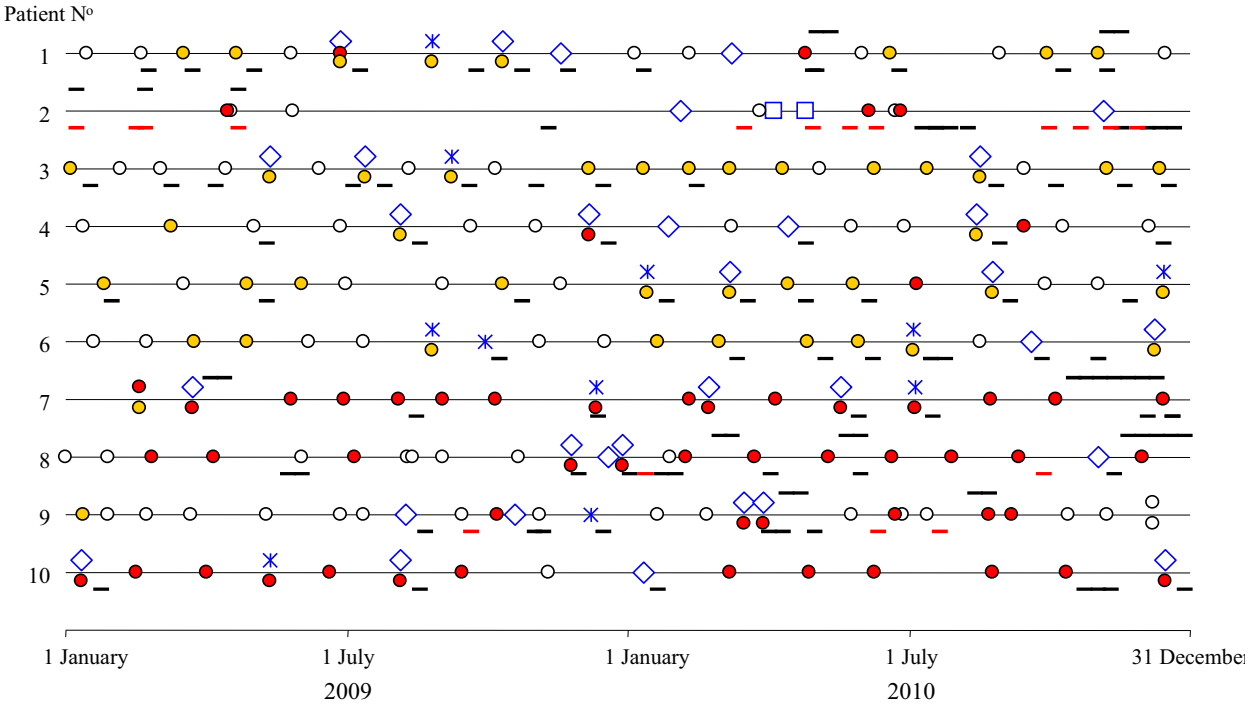

FIG. 1. Population dynamics of *Staphylococcus aureus*, other bacterial pathogens detected, and antimicrobial agents prescribed to ten patients with four or five sputum samples with *S. aureus* (six females and four males; age range, 2–41 years). Of 184 respiratory samples, 46 were positive for *S. aureus*. Blue diamonds and squares represent first and second persistent clones of *S. aureus*, respectively; a second persistent clone was observed in one patient; solitary clones are designated by blue asterisks. Yellow circle: *Haemophilus nfluenzae*. Red circles: Gram-negative non-fermenting rods (*Pseudomonas aeruginosa*, *Achromobacter* species, *Burkholderia cepacia* complex, or *Stenotrophomonas maltophilia*). White circles: negative samples. Patients received 81 courses of oral antimicrobial agents (black bars), 17 courses of intravenous antimicrobial agents (red bars) and 12 courses of inhaled antimicrobial agents (bars above patient lines) with activity against *S. aureus*.

against other microorganisms (but with activity against *S. aureus*, such as amoxycillin–clavulanic acid for *Haemophilus influenzae*). The majority of isolates (35 of 46) belonged to persistent clones, and 11 were solitary clones.

Ten patients had ≥17 sputum samples positive for *S. aureus* during the 2-year study period; the microbiological findings and antibiotic therapy of these patients are summarized in Fig. 2. Of 187 typed isolates, 131 (70%) belonged to the dominant clone of the patient, 35 (19%) belonged to other persistent clones, and 21 (11%) were solitary clones.

IV antimicrobial therapy and recurrence of *S. aureus* in chronically infected patients

Fifty-one patients were chronically infected in 2009, and 54 patients were chronically infected in 2010; 25 patients were chronically infected in only 1 year, and 40 patients were chronically infected in both years. These 105 chronically infected patient-years were selected for longitudinal analysis of colonization dynamics. Table 3 lists the 139 courses of IV antimicrobial agents with activity against *S. aureus*, which were given to 37 chronically infected patients; the mean duration of treatment was 15 days (range, 6–31 days). We identified 371 intervals between two samples with growth of *S. aureus*, and in 118 of these intervals one or more courses of IV antibiotics with activity against *S. aureus* had been administered (Table 4). The mean interval between two samples with growth of *S. aureus* was 49 days if no IV treatment had been given, but was 68 days when IV treatment had been administered (p 0.003). Thirteen of the patients were also chronically infected with Gram-negative non-fermenting rods, which could be the primary target for the IV antimicrobial therapy. Exclusion of Gram-negative non-fermenting rod patients from analysis resulted in a similar estimate; for this subgroup, administration of IV antibiotics increased the interval between two positive samples from 50 to 69 days (Table 4).

In accordance with local guidelines, patients were rarely sampled during antimicrobial therapy. Prescription of IV treatment was associated with a mean delay of 7 days in time to next sample obtained for culture, which may partly explain the increase in interval between two positive samples (calculations not shown).

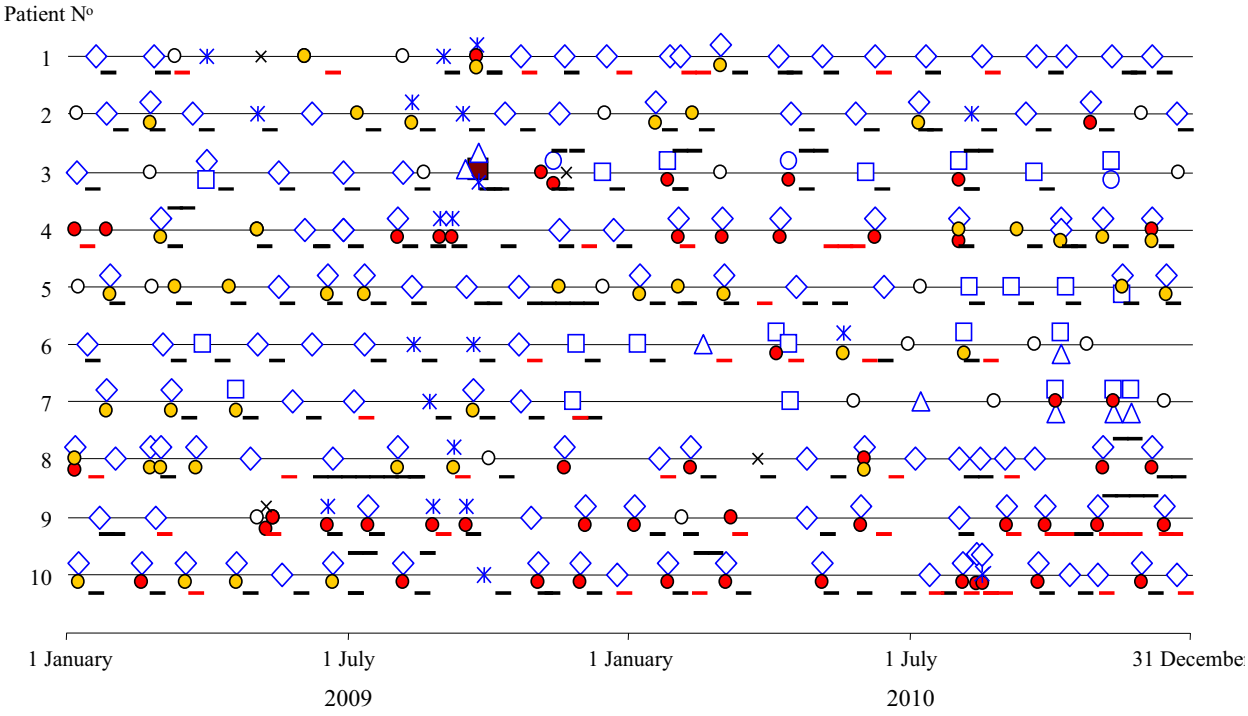

FIG. 2. Population dynamics of *Staphylococcus aureus*, other bacterial pathogens detected, and antimicrobial agents prescribed to ten patients with ≥17 sputum samples positive for *S. aureus* (six females and four males; age range, 4–19 years). Of 217 respiratory samples, 181 were positive for *S. aureus*. For interpretation of symbols, see Fig. 1. Blue triangles and circles represent third and fourth persistent clones of *S. aureus*, observed for three and one patients, respectively; small black crosses designate four non-stored *S. aureus* isolates. Brown square: methicillin-resistant *S. aureus* (patient no. 3). Patients received 152 courses of oral antibiotics, 45 courses of intravenous antibiotics and ten courses of inhaled antibiotics with activity against *S. aureus*.

TABLE 3. Antistaphylococcal intravenous antimicrobial agents administered in 139 courses to 37 chronically infected patients

Antimicrobial agent	Courses
Cefuroxime	47
Piperacillin–tazobactam	42
Ceftriaxone	26
Meropenem	12
Dicloxacillin	6
Sulphamethoxazole–trimethoprim	3
Ertapenem	1
Tigecycline	1
Imipenem	1

IV antimicrobial therapy and probability of short-term clone replacement

We assessed the probability of short-term clone replacement in chronically infected patients. Analysis was restricted to samples with a single *spa* type, disregarding ten samples that had not been stored and 16 samples containing more than one *spa* type. A total of 666 isolates from 65 patients (range, 2–22 isolates per patient obtained during either 12 or 24 months) could be categorized as 175 patient clones; of these, 92 were solitary clones, and 83 were persistent clones. A short-term clone replacement was defined as the sequential isolation of *S. aureus* of different *spa* types after adjustment by the BURP algorithm. In total, 186 of 602 stored pairs of sequential isolates of *S. aureus* were different, corresponding to an overall short-term clone

TABLE 4. Interval lengths between samples with *Staphylococcus aureus* in all chronically infected patients receiving intravenous (IV) antimicrobial therapy (n = 33) and in chronically infected patients without concomitant Gram-negative non-fermenting rods (GNNFs) (n = 24)

Interval	No. of intervals	Mean length of intervals Days (SD)	p-value
All chronically infected patients			
All intervals	371	53 (18)	
IV intervals	118	68 (35)	0.003
Non-IV intervals	253	49 (27)	
Chronically infected patients without GNNFs[a]			
All intervals	282	53 (18)	
IV intervals	85	69 (37)	0.023
Non-IV intervals	197	50 (29)	

SD, standard deviation.
p-values refer to comparisons of IV intervals with non-IV intervals.
[a]Excluded patients were chronically infected with: *Achromobacter* species (4), *Stenotrophomonas maltophilia* (4), *Pseudomonas aeruginosa* (2), *Burkholderia cepacia* complex (1), *Stenotrophomonas maltophilia* plus *P. aeruginosa* (1), and *B. cepacia* complex plus *P. aeruginosa* (1).

replacement frequency of 31%. In 33 patients who had received IV therapy in the intervals between two samples with growth of *S. aureus*, 101 of 346 (29%) stored pairs of sequential isolates were different. No difference was observed between 114 intervals where IV treatment had been administered (33 short-term clone replacements = 29%) and 232 intervals where no IV treatment had been administered (68 short-term clone replacements = 29%) (OR 0.98, 95% CI 0.60–1.61).

Discussion

We used *spa* typing to describe the population dynamics of *S. aureus* in CF in a setting where intensified treatment of *S. aureus* with IV antibiotics is implemented. We found *spa* type t230 to be the most common type among our patients, being detected in 26 of 130 patients and constituting 7.4% of all patient *spa* types. Previous studies on a restricted number of samples have reported *spa* types such as t008, t091 and t189 to be over-represented in CF samples [12,20]. Accumulation of particular *spa* types in CF could be attributable to disease-specific factors, to selection of clones with special virulence factors, or to the introduction of epidemic clones of the species and subsequent spread within and between clinics. Our isolates of *S. aureus* t230 belonged to three different *agr* groups, but we did not use additional typing methods to rule out minor clusters of transmission in our population. Further studies are needed to substantiate the association of particular clones of *S. aureus* with CF.

We found chronic infection to be associated with persistent clones, in agreement with previous studies [10–12]. However, most patients with infrequent cultures of *S. aureus* were likewise colonized with a dominant clone. In these patients, the persistent clone could only occasionally be detected, and was scattered between sputum samples without growth of *S. aureus* (Fig. 1), indicating that, in such patients, *S. aureus* colonizes the airways in low amounts.

A recent publication showed that eradication of *S. aureus* from the airways of CF patients was achieved by antibiotic therapy [18]. The authors defined a successful treatment as the absence of the *S. aureus* clone at the next visit to the clinic, even if re-infection with another *spa* type had occurred. The authors did not follow the patients for longer periods after they finished antibiotic therapy, and recurrence of the clone at a later time-point was not considered. Therefore, long-term eradication or clone displacements were not documented, and the observations could represent suppression, rather than eradication, of persistent *S. aureus* clones.

From the beginning of centralized CF treatment in Denmark in the 1960s, the antimicrobial treatment strategy for *S. aureus* has been based on monthly surveillance cultures [5]. A patient with *S. aureus* is treated regardless of symptoms, the standard regimen being 2 weeks of oral dicloxacillin plus fusidic acid or rifampicin. Despite this intensive use of antimicrobial agents, only one patient was colonized with MRSA during the study period, and overall resistance to fusidic acid and rifampicin was 12% and 26%, respectively. Rifampicin was preferred over fusidic acid for some years at our centre, but this practice was discontinued in 2006 when rifampicin resistance rates exceeded 50% (data not shown). The rifampicin resistance rate may still be elevated, owing to low turnover of clones, but increased use of fusidic acid has not yet led to high resistance. Resistance to fusidic acid is probably induced in *S. aureus* at a lower rate, and fusidic acid should therefore be preferred over rifampicin for combination with a β-lactamase-stable penicillin.

The composite microbiology and intricate treatment strategy makes it difficult to assess the effectiveness of antistaphylococcal antimicrobial chemotherapy in CF patients. The number of samples with growth of Gram-negative organisms surpassed the number of *S. aureus* cultures for all but one of the ten patients with only four or five detections of *S. aureus* during the 2-year study period (Fig. 1). A positive culture of *S. aureus* elicited antimicrobial therapy in almost all cases; however, most treatments with antimicrobial agents with activity against *S. aureus* were prescribed because of culture of Gram-negative organisms, or because of clinical deterioration in the absence of positive sputum cultures (Fig. 1). For patients with multiple detections of *S. aureus*, the number of cultures with *S. aureus* was greater than the number of cultures with Gram-negative organisms (Fig. 2); nevertheless, antimicrobial therapy with activity against *S. aureus* was sometimes primarily directed against other bacteria, or the regimen was modified to cover a larger spectrum of bacteria, as can be inferred from Table 3.

The retrospective nature of this study inevitably involves a potential for bias, and the conclusions should ideally be confirmed by randomized trials. With this important limitation in mind, the routine sampling of our patients on a monthly basis enabled us to quantitate the bacterial suppression exerted by therapeutic regimens. We calculated the mean length of the interval between cultures with growth of *S. aureus* for chronically infected patients, and observed a significant extension if IV antimicrobial agents with activity against *S. aureus* had been prescribed. The mean length of intervals between positive samples was 49 days in the absence of IV treatment, but 68 days after administration of IV antimicrobial agents. When *S. aureus* did recur in patient sputa, the probability of clone replacement was not increased after IV therapy. Thus, the significant extension in time to next culture with growth of *S. aureus* was a result of suppression, rather than eradication, of the bacteria. The number of lasting clone replacements was too low to

permit analysis, but the absence of a short-term effect makes a lasting effect unlikely. As all patients were treated, we could not estimate the effectiveness of the standard oral treatment for *S. aureus*, but we found persistent clones to be common, even in patients with infrequent detection of *S. aureus* (Fig. 1). It is reasonable to assume that the 2-week oral treatment for *S. aureus* likewise confers suppression, rather than eradication, of the infection. Although antimicrobial treatment is justified on clinical grounds, our data do not support an eradication strategy for *S. aureus* clones after establishment of chronic infection [18].

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Author Contributions

The study was conceived and planned by C. Andersen, B. C. Kahl, and N. Nørskov-Lauritsen. C. Andersen performed the microbiological characterization, and C. Andersen and H. V. Olesen extracted antimicrobial treatments from patient records. C. Andersen and N. Nørskov-Lauritsen analysed data and wrote the first draft of the manuscript, which was revised critically for important intellectual content by B. C. Kahl, H. V. Olesen, and S. Jensen-Fangel. All authors approved the final version of the manuscript.

Transparency Declaration

The authors declare no conflicts of interest.

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