MOLECULAR IDENTIFICATION AND TYPING

Persistence of *Staphylococcus aureus* strains among cystic fibrosis patients over extended periods of time

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Pulsed-field gel electrophoresis (PFGE) of *Sma*I macrorestriction fragments of chromosomal DNA was used to confirm the persistence of methicillin-sensitive *Staphylococcus aureus* isolates in the sputum of 25 cystic fibrosis patients in five French hospitals. Three-to-eight consecutive isolates, with the same esterase electrophoretic type isolated from each patient over a period of 12–28 months, were analysed. Consecutive isolates with indistinguishable PFGE profiles were found in 12 patients (48%) and consecutive isolates with similar PFGE profiles showing minor differences of one-to-four fragments (similarity coefficient $\geq 84\%$) were found in 11 patients. Consecutive isolates with different PFGE profiles were obtained from only two patients, but the profiles found in each patient were more closely related to each other than to other profiles. The results were in agreement with esterase electrophoretic typing for 23 patients, and we considered that those patients were infected with a single persistent strain. For any given patient, variations in antibiotype and phage types of consecutive isolates were not associated with major genotypic variations. PFGE is useful in confirming the persistence of *S. aureus* strains in cystic fibrosis patients over long periods.

Introduction

Patients with cystic fibrosis suffer from recurrent and chronic endobronchial bacterial infections which play a major role in deteriorating pulmonary function. *Staphylococcus aureus* is the initial bacterial pathogen, but it later becomes associated with *Pseudomonas aeruginosa* [1, 2]. Multiresistant strains of *S. aureus* are not frequent in these patients [3, 4] and early aggressive treatment is essential to prevent lung tissue damage [5].

Three phenotypic markers – esterase electrophoretic typing, capsular typing and phage typing – were used recently to demonstrate that a majority of cystic fibrosis patients (73%) developed recurrent infections with a persistent strain [4]. However, the discriminatory power of capsular typing was low (only two capsular types were identified), and the value of phage typing was impaired by a significant number of non-typable strains. Although the discriminatory power of esterase electrophoretic typing was found to be satisfactory, this technique only explores a small part of the genome [6]. Schlichting *et al.* [7] showed that esterase electrophoretic typing is less powerful for distinguishing between strains of *S. aureus* than fingerprinting of chromosomal DNA by pulsed-field

gel electrophoresis (PFGE). Thus, the finding that the isolates obtained during consecutive infections from a given patient were indistinguishable should be confirmed by a molecular technique. Pulsed-field gel electrophoresis has been used successfully to type various bacteria [8–12] and has been recommended as the typing method of choice for *S. aureus* by several groups of investigators [13–16]. Quantitative determination of the macrorestriction pattern of similarity allows the estimation of inter-strain genetic relatedness which can be useful for tracing the evolution of strains over extended periods of time.

The present report describes the typing by PFGE of methicillin-sensitive isolates obtained over periods of at least 12 months from 25 cystic fibrosis patients with recurrent broncho-pulmonary infections due to a single persistent strain according to esterase electrophoretic typing. The stability over time of the persistent strain was examined by detecting changes in the macrorestriction patterns. The molecular results were also correlated with phage types and antibiotype.

Materials and methods

Bacterial strains

A total of 97 isolates of *S. aureus* isolated over 12–28 months from the sputum of 25 cystic fibrosis patients

was examined. The patients were attending five French hospitals, situated in different areas, between January 1984 and December 1991. The isolates are part of a collection previously characterised by esterase electrophoretic typing and phage typing [4]. For each patient, three-to-eight consecutive isolates of the same esterase electrophoretic type were analysed. The characteristics of the isolates are shown in Table 1.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the standard disk diffusion method on Muller-Hinton Agar (Sanofi, Diagnostics Pasteur, France) according to the guidelines of the AntibioGram Committee of the French Society for Microbiology [17]. The antibiotics tested were (μ g): penicillin (6), oxacillin (5), neomycin (30), streptomycin (10), gentamicin (15), tobramycin (10), kanamycin (30), tetracycline (30), erythromycin (15), clindamycin (2), pristinamycin (15), pefloxacin (5), sulphonamide (200), trimethoprim (5), fosfomycin (50), fusidic acid (10) and rifampicin (30).

Genome typing

PFGE was performed after restriction endonuclease digestion of whole chromosomal DNA with *Sma*I (Boehringer) as described by Schlichting *et al.* [7]. The restriction fragments were separated by a contour-clamped homogeneous electric field (CHEF-DRII system, BioRad) in $0.5 \times$ Tris-borate EDTA buffer at 12°C and 200 V for 19 h with switch times of 1–45 s.

Analysis of DNA relatedness

A similarity index was determined for each pair of strains by the coefficient of Dice [18]. Clustering correlation coefficients were calculated by the unweighted pair group method with arithmetic averages with Gel Compar 3.1 software (Applied Maths, Kortrijk, Belgium).

Results

*Analysis of the *S. aureus* strains*

Isolates of the same esterase electrophoretic type were compared in adjacent lanes of the same gel. Electrophoresis of *Sma*I digests gave patterns of 13–16 fragments of 28–700 kb (Fig. 1). Patients were assigned to one of three groups based on whether the *S. aureus* isolates, isolated consecutively from the patients, had indistinguishable, similar or different macrorestriction profiles by PFGE. All the consecutive isolates from each of 12 patients of the first group had identical macrorestriction profiles (Table 1). This demonstrated that each patient was infected by a single persistent strain throughout the study. The consecutive isolates

from four patients (nos. 1, 2, 3 and 7) showed no variation in their phage type or antibiotic type. Those from the other eight patients showed variations in either antibiotic type, phage type or both. The 11 patients in the second group had consecutive isolates with similar macrorestriction profiles, showing minor differences of one-to-four fragments and similarity coefficients $\geq 84\%$ (Fig. 2). These profiles were considered to be subtypes of a major genome type. Major genome types were designated with numerals and each of their variant subtypes by a letter suffix (Table 1). Isolates from the same patient differing by four bands were considered to be related because each one differed from a third isolate by only one, two or three bands. Group 2 included six patients (nos. 13, 15, 19, 20, 21 and 22) whose isolates were represented by two-to-four successive different subtypes of a major genome type. Among the isolates from the four other patients, (nos. 14, 16, 17 and 18) a subtype was recovered that was identical at the beginning and at the end of the study. Isolates of patient 23 were represented by two subtypes which were isolated several times at random during the study. The patients of the second group were considered to be infected by a persistent strain that had undergone minor DNA re-arrangements over time. The consecutive isolates from three patients (nos. 13, 17 and 18) had identical phage types and antibiotic types. Those from the other eight patients showed variations in phage type or antibiotic type or both. The median period over which the isolates were studied was similar for the first and second groups: 15.3 months (range 12–20 months) for group 1 and 16.3 months (range 12–28 months) for group 2.

The third group contained only two patients who had consecutive isolates showing different macrorestriction patterns. The first patient (no. 24) was consecutively infected by isolates of two different major genome types, having two subtypes. One of the three isolates of genome type 5 was isolated at the beginning of the study, and the two others at the end of the study. Genome types 4 and 5 differed from each other by five-to-seven fragments, had similarity coefficients of 76–81%, and were in the same cluster of the dendrogram (Fig. 3). The second patient (patient 25) was infected by isolates of two major genome types, 9 and 10, one having two subtypes. However, the two genome types, 9, 10 differed from one another by eight fragments, had similarity coefficients of 69% and were genetically closer to each other than to the other genome types described in this study (Table 1 and Fig. 3). The three isolates had identical antibiotic types and the isolate of genome type 10 was phage non-typable.

Correlation between the markers

As previously described [7], results revealed that isolates with identical genome type did not differ in esterase electrophoretic type. Isolates with identical

esterase type belonged to different genome types on several occasions which were found clustered in the same branch of the dendrogram (Fig. 3). For example, isolates of esterase electrophoretic type 5 were represented by genome types 6, 7 and 8. The phage type differed even when the genome type was identical; however in most cases, phage-typable isolates of the same genome type were lysed by phages belonging to the same group (Table 1).

Comparison of isolates from different patients

Similar or identical genome types were found among isolates from different patients. This happened three times (genome types 6, 13 and 14) in patients attending the same hospital and five times (genome types 1, 2, 4, 9 and 14) in patients attending different hospitals, without any evidence of contact. Isolates from different patients with identical genome type often had the same antibiotic and the same phage type. Thus, five patients (nos. 3, 16, 17, 18 and 25) who attended five different hospitals, were infected by isolates of genome type 4 that were resistant to penicillin only and were of phage type 94/96. However, isolates from different patients with identical genome type sometimes had different phenotypes: isolates of genome type 13 were resistant to penicillin and pefloxacin for patient no. 9, but were resistant to penicillin only for patient no. 22 (Table 1).

Discussion

The use of epidemiological markers to type bacteria is important for conducting surveys of infections, and can help to distinguish between re-infection and persistent infection. They can also be used to detect cross-infection [8, 9, 13, 19–21]. Esterase electrophoresis was helpful in delineating persistent strains of *S. aureus* in cystic fibrosis patients with chronic or recurrent infections [4]. However, further molecular investigations were needed to confirm the identity of the strains. The data obtained previously from esterase polymorphism and DNA macrorestriction fragment polymorphism by PFGE were convergent [7]. In addition, several authors have found that restriction profiles obtained by PFGE are better for discriminating among epidemiologically unrelated isolates of methicillin-resistant *S. aureus* than other molecular techniques [15, 16]. Therefore, PFGE was used to type persistent strains isolated from 25 cystic fibrosis patients over periods of at least 12 months.

The consecutive isolates from the first group of 12 patients (48%), had identical macrorestriction patterns. These patients were presumably infected by one persistent strain with a genome that was stable for at least 12 months. However, as only one macrorestriction enzyme (*Sma*I), was used to characterise the isolates, some mutations or DNA re-arrangements

Table 1. Characteristics of the 97 *S. aureus* strains isolated from 25 cystic fibrosis patients

Patient	Hospital	Follow-up period	ET	PT	GT	Antibiotic resistance
Group 1						
1	C	12	23	NT	1a	–
			23	NT	1a	–
			23	NT	1a	–
2	F	12	14a	NT	3	Pe, S, N, K
			14a	NT	3	Pe, S, N, K
			14a	NT	3	Pe, S, N, K
			14a	NT	3	Pe, S, N, K
			14a	NT	3	Pe, S, N, K
			14a	NT	3	Pe, S, N, K
3	C	20	16a	94/96	4a	Pe
			16a	94/96	4a	Pe
			16a	94/96	4a	Pe
			16a	94/96	4a	Pe
4	A	19	5	6/42E/47/54/75/77/83A/54A	6d	–
			5	47/54/83A	6d	Pe, L
			5	54/83A	6d	Pe, L
5	A	12	7	71	9a	–
			7	71	9a	–
			7	71/3A	9a	–
6	C	18	7	71	9c	Pe
			7	71	9c	Pe
			7	55/71/3A	9c	Pe
			7	55/71	9c	Pe
7	B	16	4a	95	11	–
			4a	95	11	–
			4a	95	11	–
			4a	95	11	–
8	A	20	10a	54/75/54A	12	–
			10a	47/54/83A	12	–
			10a	42E	12	–

Table 1. (continued)

Patient	Hospital	Follow-up period	ET	PT	GT	Antibiotic resistance
9	C	18	9a	83A/85	13a	Pe, Pf
			9a	85	13a	Pe, Pf
			9a	83A	13a	Pe, Pf
10	A	13	11	29/52/47/54/83A/85	14c	Pe
			11	29/52A/83A	14c	Pe
			11	83A	14c	Pe, Pf
11	A	12	11	83A/85/52A	14b	Pe, Fo
			11	42E	14b	Pe
			11	83A	14b	Pe
12	B	24	11	53/85	14b	E, L
			11	47/53/54	14b	St, E, L
			11	53/54/77/84/85	14b	E, L
Group 2						
13	E	19	23	NT	1b	Pe
			23	NT	1a	Pe
			23	NT	1a	Pe
			23	NT	1a	Pe
			23	NT	1a	Pe
			23	NT	1a	Pe
			23	NT	1a	Pe
14	C	12	14	81	2a	—
			14	81	2d	Pe, Fu
			14	81	2a	Pe
15	B	26	14	NT	2a	Pe
			14	NT	2b	Pe
			14	NT	2c	Pe, Fu
16	F	12	16a	94/96	4a	Pe
			16a	94/96	4d	Pe
			16a	94/96	4a	Pe, E
17	A	12	16a	94/96	4a	Pe
			16a	94/96	4c	Pe
			16a	94/96	4a	Pe
18	E	17	16a	94/96	4a	Pe
			16a	94/96	4b	Pe
			16a	94/96	4a	Pe
			16a	94/96	4a	Pe
19	A	18	5	83A	6a	Pe, E, L
			5	83A	6c	Pe, E, L
			5	83A/85	6b	Pe, E, L
			5	83A	6e	Pe
20	B	12	5	42E/47/54	7a	Pe
			5	47/54	7a	—
			5	42E/47/54	7b	—
21	B	28	5	NT	8b	Pe, T, Fu
			5	NT	8c	Pe, Fu
			5	NT	8a	Pe, Fu
			5	NT	8a	Pe, Fu
22	C	12	9a	83A/85	13a	Pe
			9a	83A/85	13a	Pe
			9a	53/83A	13b	Pe
23	C	12	11	47/54/75/83A/85/54A	14a	Pe
			11	47/54/83A/85/54A	14a	Pe, R, Fu
			11	83A	14b	Pe, R, Fu
			11	42E/47/54/75/83A/85/54A	14a	Pe, R, Fu
			11	83A	14b	Pe, R
			11	42E/47/54/75/85/54A	14a	Pe, R, Fu
			11	47/75/83A/54A	14b	Pe, R, Fu
			11	NT	14a	Pe, R
Group 3						
24	B	12	16a	94/96	5a	—
			16a	94/96	4a	—
			16a	94/96	4b	Pe
			16a	94/96	4a	Pe
			16a	94/96	5b	Pe
			16a	94/96	5b	—
25	B	17	7	55	9b	Pe
			7	55	9a	Pe
			7	NT	10	Pe

ET, esterase electrophoretic type; PT, phage type; GT, genome type; NT, non-typable; P, penicillin; E, erythromycin; L, lincomycin; Pf, pefloxacin; Fu, fusidic acid; T, tetracycline; Fo, fosfomycin; R, rifampicin; S, streptomycin; N, neomycin; K, kanamycin; —, absence of resistance to the antibiotics tested; Group 1, patients with isolates having indistinguishable genome types; Group 2, patients with isolates having similar genome types; Group 3, patients with isolates having different genome types.

Fig. 1. Major restriction genome types determined by *Sma*I analysis among *S. aureus* isolates from 25 cystic fibrosis patients. Lanes 1–9a, genome types 1–9; 9b, variant of genome type 9; 10–14, genome types 10–14; T, molecular size markers, lambda DNA concatenate.

Fig. 2. PFGE patterns after *Sma*I digestion of consecutive *S. aureus* isolates from six patients illustrating the minor variations in the patterns of the persistent strains of each patient. Lanes 1–3, genome types 4a, 4c, 4a (patient 17); 4–6, genome types 2a, 2d, 2a (patient 14); 7, genome type 13a (patient 9); 8 and 9, genome types 13a, 13b (patient 22); 10–12, genome types 2a, 2b, 2c (patient 15); 13–15, genome types 6a, 6c, 6b (patient 19); T, molecular size markers, lambda DNA concatenate.

may not have been seen [9, 22]. The 11 patients in the second group were infected by closely related isolates; there were minor variations in their macrorestriction patterns, which resulted in one-to-four fragment differences according to the patient. As with *P. aeruginosa* [9], several genetic events in *S. aureus* persistent strains may occur at random during chronic or recurrent infections in cystic fibrosis patients, and generate variant macrorestriction patterns differing in

total by more than three fragments, among the consecutive isolates. From each of five patients, the same subtypes of a genome type could be isolated two or more times, mostly at random during the study, suggesting that these patients were infected by several subtypes derived from a single strain. This might have been confirmed by studying several colonies of *S. aureus* from the culture of the clinical sample, instead of one. Genetic variations in a persistent strain did not

Fig. 3. Dendrogram showing estimates of the percentage similarities between macrorestriction DNA type (genotype) of isolates of *S. aureus*.

depend on the length of colonisation of a patient, because mean times over which the isolates were obtained were similar in the patients from groups 1 and 2. Variations in PFGE macrorestriction patterns in pairs of *S. aureus* isolates, collected 4 weeks apart, from three cystic fibrosis patients attending a summer camp in Germany were also noticed by Schlichting *et al.* [7], as well as the colonisation of one patient by two subtypes of a single genome type. Hartstein *et al.*, comparing sequential pairs of methicillin-resistant *S. aureus* isolates collected over 30–228 days from patients, found that among the putative identical isolates, 80% had identical PFGE types and 20% had related PFGE type with minor differences, most likely caused by a single genetic event [23]. The greater frequency of PFGE differences in sequential

isolates in the present study may be because more than two isolates per patient were examined over a longer period.

Only two patients had consecutive isolates with different genome types. However, the genome types in each patient were more related to each other than to the other genome types described in the study. The coefficients of similarity were 69% and $\geq 76\%$ respectively. Furthermore, the isolates from each of the patients were of a similar phenotype. In such a study it is difficult to conclude whether each episode of infection was due to the acquisition of a different strain or to a co-infection of related strains derived from a single common strain that had undergone several genetic events over time. With the exception of the

two latter patients, the results obtained with PFGE were in good agreement with those obtained with esterase electrophoretic typing, indicating that 23 patients were infected by a persistent strain. PFGE is more discriminatory than esterase electrophoretic typing for identifying persistent strains: three different genome types were identified in the four patients that had a persistent strain of esterase electrophoretic type 5.

In this study, antibiotic resistance typing was not a useful tool in delineating persistent strains. Few patients had isolates with a stable antibiotype which is rarely encountered among methicillin-sensitive strains. Antibiotic subtypes were dissociated from genotypic variations. Thus two isolates with identical macrorestriction patterns varied in their antibiotype and two isolates with identical antibiotypes had minor variations in their macrorestriction patterns. Resistance typing may be a useful initial typing tool for epidemiological investigation; however, as noticed by Struelens *et al.* [13], the reproducibility of the technique may be impaired by the instability of the resistance traits *in vivo* or *in vitro*, and thus discrimination between strains can be overestimated. This could have been the case in the present study, where the isolates obtained over long periods of time could have acquired or lost antibiotic determinants *in vivo* as well as *in vitro* during subculture or storage [24].

Phage types are usually considered to be different if they differ by the presence or absence of two or more phage susceptibilities [25]. According to this criterion, the consecutive isolates of a given major genome type from six patients had different phage types. However, the isolates from each of the six patients were lysed by phages belonging to the same group. As for antibiotype, variations in phage type were not associated with observable genotypic variations. Bacteriophage typing has been the reference epidemiological typing method for *S. aureus*, but problems with reproducibility have been described by several authors [24, 26] and recently it has been proposed that phage typing should be replaced by PFGE for typing *S. aureus* isolates when investigating outbreaks [27]. In the present study, consecutive isolates from a given patient were generally phage typed, at different times. The use of different phage stocks, and hence different technical conditions, may explain variations in phage type.

Schlichting *et al.* noticed that there were identical methicillin-sensitive *S. aureus* genome types in groups of patients originating from France or Germany [7]. Strains with similar or identical PFGE patterns were also found in patients from diverse French geographic regions in this study. Genome types 9, 15, 2, 4 and 1 were found among patients at different hospitals, without any evidence of patient contact. Strains with identical genome types could be distinguished by their

phage type or antibiotype in some patients, but strains were identical by all three markers in other patients. The use of several macrorestriction enzymes, or the use of alternative molecular typing systems could, perhaps, help to differentiate strains more precisely, unless this feature is due to a slow genetic development of methicillin-sensitive *S. aureus*. If that is the case, the epidemiology of methicillin-sensitive *S. aureus* may be quite complicated, particularly when analysis of cross-infection is needed. Limited genetic diversity within a species or subtype has been described for *Neisseria meningitidis* and for *Escherichia coli* O157:H7 [28, 29]. As in other studies [7, 13, 21], PFGE has been useful for typing isolates of *S. aureus* and confirming the persistence of strains over long periods in cystic fibrosis patients. However a combination of several typing methods, including a phenotypic method such as phage typing, may still be needed for *S. aureus* epidemiological studies, as well as detailed investigations of the background of the isolates, to avoid misleading conclusions [21].

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