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Epidemiology of *Staphylococcus aureus* in patients with cystic fibrosis

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SUMMARY

Seven hundred and thirty-four isolates of *Staphylococcus aureus*, recovered from the sputum of 238 cystic fibrosis patients in six French hospitals, were characterized by esterase electrophoretic typing, capsular polysaccharide serotyping and phage typing and tested against 14 antibiotics for sensitivity. Thirty-four esterase electrophoretic types were found with a genotypic diversity coefficient of 0·91. Five hundred and forty-eight (78·7%) isolates produced capsular polysaccharide and 350 (50·3%) were type 8. Four hundred and sixty isolates (66·6%) were phage typable and 202 (28·2%) were lysed by group III bacteriophages. No esterase electrophoretic type, capsular type or phage type was specific to cystic fibrosis. Isolates belonged to a wide range of types, similar to strains acquired outside hospitals. Eighty-five patients had three or more consecutive isolates over at least 6 months. The ability of *S. aureus* to persist for long periods of time has been demonstrated in 73% of them. Methicillin-resistance was encountered among 73 strains (9·8%) which were also multiresistant. Two hundred and eighty-nine (39·9%) strains were sensitive to all antibiotics tested except to penicillin. Pristinamycin and co-trimoxazole were the most effective antibiotics. These results could contribute to the elaboration of a rational approach to the prophylaxis and therapy of respiratory staphylococcal infections in cystic fibrosis patients.

INTRODUCTION

Staphylococcus aureus is the second most persistent pathogen recovered from the respiratory tract of infected cystic fibrosis patients, especially those who are under

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the age of 10 years [1–4]. Colonization by *S. aureus* is usually followed by *Pseudomonas aeruginosa* the most persistent pathogen. Multiresistant strains of *S. aureus* have been shown to be infrequent in these patients [5, 6]. Despite the availability of effective antistaphylococcal therapy, recurrent infections are common [5, 7, 8]. Little information is available on the epidemiology of *S. aureus* in cystic fibrosis patients and to our knowledge it has not been clearly demonstrated whether the same strain or different strains are responsible for repeated bronchopulmonary infections [6, 9, 10].

Electrophoretic techniques are powerful tools for detecting genetic variations of enzymes. Variations in esterase electrophoretic patterns have made a significant contribution to the epidemiological analysis of *S. aureus* [11–13]. Capsular polysaccharides have been identified in clinical isolates of *S. aureus* [14–16] and studies have shown the predominance of capsular types 5 and 8 among clinical isolates in both the United States and Europe [14–17]. A similar predominance has also been found in cystic fibrosis patients [18]. Although phage typing can be limited by the occurrence of *S. aureus* strains that are not typable, this method has been traditionally used for epidemiological studies, and offers a high degree of discrimination [19].

We have used esterase electrophoretic typing, capsular typing and phage typing to investigate the epidemiology of *S. aureus* strains responsible for recurrent bronchopulmonary infections in cystic fibrosis patients. Sensitivity against a broad spectrum of antibiotics known to be active against staphylococci was also investigated.

MATERIALS AND METHODS

Bacterial strains

A total of 734 strains of *S. aureus* was collected from the clinical laboratories of six French hospitals [(Giens (A), Tours (B), Lyon (C), Paris (D and E) and Besançon (F)] between January 1984 and December 1991. The organisms were isolated from the sputum of 238 cystic fibrosis patients suffering from chronic or intermittent infections. Eighty-five patients provided three or more consecutive isolates (up to 28) recovered over a period of at least 6 months (up to 3 years).

Esterase electrophoretic typing

The electrophoretic mobility patterns of esterases A, B and C were investigated as previously described [12, 20] with the following modifications. Strains were grown overnight at 37 °C in 25 ml brain heart infusion broth (Sanofi, Diagnostics Pasteur, France). The bacteria were collected by centrifugation. The pellets were washed in 0·075 M Tris 0·06 M glycine buffer, pH 8·7 and suspended in 2·0 ml of the same buffer. They were lysed by incubation with 100 μg lysostaphin (Sigma Chemical Co.) at 37 °C for 1 h, with vigorous shaking and centrifuged at 20000 g for 10 min at 4 °C. The extracts were analysed by polyacrylamide-agarose gel electrophoresis on a 5% acrylamide and 0·8% agarose gel in a 0·01 M Tris, 0·35 M glycine buffer, pH 8·7. Electrophoresis was performed at a constant value of 7 V. cm^{-1} until the dye marker had run 11 cm. Esterases were stained in the gel with α-naphthyl propionate as substrate.

Capsular polysaccharide typing

Capsular polysaccharide types 5 and 8 were detected as previously described [21]. Briefly, bacteria were grown overnight at 37 °C on Columbia Agar slants (Difco Laboratories, Detroit, MI), suspended in phosphate buffered saline and autoclaved at 121 °C for 1 h. Debris was removed by centrifugation and the polysaccharides in the supernatant were detected by inhibition enzyme-linked immunosorbent assay using purified capsular polysaccharide 8 or 5 and the corresponding monoclonal antibodies. Strains lacking both type 5 and type 8 capsular polysaccharides were designated as non-typable.

Phage typing

The international set of 23 phage types (group I: 29, 52, 52A, 79 and 80; group II: 3A, 3C, 55 and 71; group III: 6, 42E, 47, 53, 54, 75, 77, 83A, 84 and 85; and miscellaneous 81, 94, 95 and 96) was applied using standard methods [19]. An additional experimental phage, 54A, was also tested. Isolates were typed at the routine test dilution and at 100-fold higher concentration. Only reactions showing major lysis were considered. Two isolates are considered to have different phage types when one is lysed by at least two phages which produce no lysis of the other.

Antibiotic sensitivity

Strains were examined for antibiotic sensitivity by disk diffusion test on Muller Hinton agar (Sanofi, Diagnostics Pasteur, France), according to the guidelines of the antibiogram committee of the French Society for Microbiology [24]. The antibiotics concerned were penicillin, oxacillin (for the detection of methicillin sensitivity), gentamicin, tobramycin, kanamycin, tetracycline, erythromycin, clindamycin, pristinamycin, pefloxacin, co-trimoxazole, fosfomycin, fusidic acid and rifampicin. Penicillinase activity was detected with the cefinase test (BioMérieux, France).

Genotypic diversity

The genotypic diversity for a zymotype was estimated by the formula $h = 1-\Sigma x_i^2 [n/(n-1)]$ where x_i is the frequency of the ith zymotype and n the number of zymotypes [22, 23].

Statistical analysis

Statistical significance was determined with the chi-square test with Yates correction when indicated.

RESULTS

Esterase electrophoretic typing

All the 734 isolates tested were typable by this method, and 34 distinct combinations of electromorphs for esterases A, B and C (zymotypes) were found. In addition to 19 zymotypes described previously [12, 13, 25], 14 new zymotypes were distinguished: 4a, 10c, 11b, 14a, 14b, 22, 23, 24, 24a, 25, 26, 27, 28, 33. Only one isolate was used for analysis for patients having repeated isolates of the same

Table 1. *Hospital zymotype distribution*

Zymotypes	Number of strains in each hospital*						Total strains	
	A	B	C	D	E	F	No.	%
1		2	1	1			4	1·10
2	7	1	3	3			14	3·85
4a	1	1					2	0·55
5	16	6	4	4			30	8·26
6	4	1	1	6	1		13	3·58
7	22	11	11	7		1	52	14·32
7a	1						1	0·27
9a	2	2	3		1		8	2·20
10a	20	3	5	8		1	37	10·19
10c	1						1	0·27
11	20	6	4	3			33	9·09
11a			1				1	0·27
11b	1						1	0·27
12		1	1				2	0·55
14	25	10	10	7	1	2	55	15·15
14a	5	2	1	1		1	10	2·75
14b				1			1	0·27
15	2	1	1	2	1		7	1·92
16	10	6		3			19	5·23
16a	11	8	7	3	1	2	32	8·81
17a	1						1	0·27
17b	1	2		1			4	1·10
19	1		1				2	0·55
20				1			1	0·27
21		1					1	0·27
22		1					1	0·27
23	2	2	2	2	1		9	2·47
24	1	1		1			3	0·82
24a	2						2	0·55
25	2			1			3	0·82
26	1						1	0·27
27	3	3	1	2			9	2·47
28			1				1	0·27
33	2						2	0·55

* One strain only was considered for patient having repeated isolates of the same zymotype.

zymotype (Table 1). This provided a total of 363 strains. The overall genotypic diversity coefficient was 0·91. The most frequent types, 14, 7, 10a, 11, 16a and 5 accounted for 15, 14, 10, 9, 9 and 8% of the strains, respectively. The frequencies of the zymotypes did not differ significantly between hospitals ($P > 0·2$), except for zymotype 6 which was more frequent among the strains isolated from patients in hospital D ($P < 0·01$) (Table 1). Five patients provided two strains with different zymotypes in a single sample: this occurred once for four patients and three times for one patient.

Capsular typing

A total of 696 isolates was studied, of which 350 (50·3%) were capsular type 8. 198 (28·4%) were capsular type 5 and 148 (21·3%) were non-typable.

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Table 2. *Relationship between phage types and zymotypes*

Zymotypes	Number of strains in each groups of bacteriophages							
	I	II	III	94/96	95	81	Mix G*	NT†
1							1	4
2	1	17	1					
4a			1		7			
5	1		50		1		5	12
6			18			1	1	5
7		29	2		3		3	41
7a			1					
9a			14					2
10a		2	13		23		2	14
10c					1			
11			59				10	18
11a			2					
11b			1					
12			1					1
14	28		18		1	9	9	50
14a	4	1	1			1		35
15		18						3
16	2		3			1		13
16a				62				3
17b		2						3
19			4					
21		1						
22			1					1
23	1	1	2					11
24								3
24a								3
25			6					
26			2					
27		8						6
28			2					
33	1							3
Total of strains	38	79	202	62	36	12	31	231
% strains	5·5	11·4	29·2	9	5·2	1·7	4·5	33·4

* Mix G. strains lysed by phages from more than one group.
† NT. non-typable.

Phage typing

Of the 691 isolates typed, 460 (66·6%) were phage typable (Table 2). Two hundred and two (29·2%) isolates were lysed by group III bacteriophages and 79 (11·4%) by group II bacteriophages. Bacteriophages 81, 94/96, 95 and group I bacteriophages lysed fewer than 10% of the isolates each. A small percentage (3·6%) of the isolates were lysed by phages belonging to several lytic groups.

Correlation of the three typing methods

Two strains with identical zymotype that were typable by capsular typing never had different capsular types. For example, all isolates of zymotypes 14, 10a and 7 were capsular type 8 and all isolates of zymotypes 16a, 11 and 5 were

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Table 3. *Correlation between capsular types and esterase electrophoretic types*

Capsular types	Zymotypes
5	1, 5, 6, 11, 11a, 12, 15, 16a
8	2, 4a, 7, 9a, 10a, 14, 14a, 16, 17b, 23, 27, 33

Table 4. *Distribution of zymotypes in the three groups of patients*

	Number of strains		
Zymotypes	Group 1*	Group 2	Group 3
1	—	—	1
2	1	—	4
4a	1	—	1
5	8	—	7
6	2	—	1
7	5	2	7
9a	2	1	3
10a	5	1	7
11	7	2	8
14	12	3	12
14a	2	—	1
15	2	—	—
16	1	1	2
16a	5	1	7
17b	—	—	1
19	—	1	1
22	—	1	—
23	1	1	2
24	—	—	1
26	—	1	—
27	1	—	2
33	—	—	1

* Group 1 and group 2: patients with persistent strains, group 3: patients with varying strains.

capsular type 5 (Table 3). Two strains phage-typable with identical zymotype could differ in their phage type, likewise two strains with identical phage type could differ in their zymotype (Table 2).

Persistence of S. aureus *strains*

Consecutive isolates were recovered from 26 patients over periods of 6–12 months, from 29 patients over periods of 12–18 months, and from 30 patients over periods greater than 18 months. A minimum of three consecutive isolates was analysed per patient.

As all the strains were typable by the esterase electrophoretic technique and as the genotypic diversity obtained was satisfactory, the zymotype was used to divide the patients into three groups. One group of 55 patients had persistent strains of the same zymotype. Fifteen different zymotypes were identified (Table 4). Some patients had strains that persisted for over 2 years. The second group of seven patients included four patients whose strain type changed, each type persisted for 3–16 months, and three patients who had two strains of different types that persisted throughout the study. Eleven zymotypes were identified

Table 5. *Distribution of the 85 patients with 3 or more consecutive isolates over at least 6 months in the 6 hospitals*

	Hospital (number of patients)					
Patients*	A	B	C	D	E	F
Group 1	22	9	16	5	2	1
Group 2	2	2	2	0	1	0
Group 3	10	5	7	1	0	0

* Group 1 and group 2: patients with persistent strains, group 3: patients with varying strains.

(Table 4). Twenty-three patients in the third group had very variable strain populations with 19 different zymotypes (Table 4). Three to five strain types were recovered from each patient. However, according to capsular type and zymotype a single strain was found twice in the consecutive isolates from six patients and three times in the consecutive isolates from two patients. Seven patients had the strains with identical capsular type and zymotype that were either not phage typable or differed in phage type. Only one patient had a phage typable strain isolated at the end of the study that was identical by all three markers (capsular type 5, zymotype 11 and phage type 83A/85) to a strain isolated at the beginning of the study.

The number of patients in all the hospitals having persistent strains (groups 1 and 2) was greater than the number of patients with very variable strains (group 3) (Table 5).

There was no statistical difference between the distribution of the major zymotypes (zymotypes 14, 7, 10a, 11, 16a, 5) among the persistent strains of groups 1 and 2, among the variable strains of group 3, or within the total population of strains studied ($P < 0{\cdot}50$) (Tables 1 and 4).

The phage type of the consecutive isolates from patients in groups 1 and 2 with persistent infections due to a single strain according to capsular type and zymotype were analysed (Tables 6 and 7). Thirty-six patients had consecutive isolates with the same phage type throughout the study (Table 6). The consecutive isolates from 13 patients had different phage types (Table 7) but 9 patients had consecutive isolates lysed by phages belonging to the same group III bacteriophage. The isolates from only four patients were lysed consecutively by phages of different groups (group I, group III and 81) (Table 7). Twenty patients had consecutive isolates which were not typable by phages.

Antibiotic sensitivity

The results of antibiotic sensitivity testing are given in Tables 8 and 9. The majority of strains was resistant to penicillin. However, penicillinase activity was not detected in 168 (22·8%) strains. Seventy-three strains (9·8%) were resistant to oxacillin (methicillin). Besides penicillin, the highest rates of resistance were found against erythromycin (18·1%), kanamycin (14·5%) and tetracycline (12·3%). Two hundred and eighty-nine (39·3%) strains were sensitive to all antibiotics tested except penicillin. Six hundred and twenty-eight (85·5%) strains were sensitive to all aminoglycosides. Methicillin-resistant strains were resistant

Table 6. *Characteristics of 36 persistent strains with stable phage types*

Numbers of patients	Hospital	Capsular type	Zymotype	Lytic group	Phage type
1	B	8	2	II	3c/55/71
1	B	8	4a	95	95
1	D	5	5	III	75
1	D	NT	5	III	6/47/54/75/83A/85
1	A	NT	5	III	83A/85
1	B	NT	5	III	42E/47/54
2	C	8	7	II	71
1	B	8	7	II	55
2	C	8	9a	III	83A/85
3	C, D	8	10a	95	95
3	A	5	11	III	83A
1	C	5	11	III	42/47
5	A, C, D	8	14	I	29
1	A	8	14	I	52
1	C	8	14	81	81
1	C	8	14	III	75/85/54
2	B, E	5	15	II	3A
6	A, B, C, E	5	16a	94/96	94/96
1	A	8	19	III	75/83A/85
1	A	8	27	II	3A

Table 7. *Characteristics of 13 persistent strains with variable phage types*

Number of patients	Hospital	Capsular type	Zymotype	Lytic group
2	A, C	5	5	III
2	A, E	5	6	III
1	A	8	10a	III
3	A, B, C	5	11	III
1	A	8	14	III
1	A	5	11	I+III
1	C	8	14	I+III
1	A	8	14a	I+III+81
1	A	8	16	81+III

to many other antibiotics useful for treatment: about 90% were resistant to all aminoglycosides, 82·5% to erythromycin, 42% to clindamycin and 49% to pefloxacin. Resistance to co-trimoxazole, fusidic acid and rifampicin (9, 12 and 12% respectively) was higher in comparison to methicillin-sensitive strains (0·3, 2·4 and 2·7% respectively). Resistance of the methicillin-sensitive strains against erythromycin was about 10% and pefloxacin 5% and gentamicin was the most active aminoglycoside with only 2% of resistant strains.

According to the hospital, resistance to some antibiotics (e.g. oxacillin, kanamycin, erythromycin) varied greatly, mainly due to persistent strains of the same type (as described earlier), showing an identical antibiotic pattern. For instance, in hospital E, resistance to oxacillin reached 37·5% of the strains: however, of the 32 isolates tested, 12 isolates of the same type (zymotype 6), recovered from the same patient during 23 months, were resistant to oxacillin.

Resistance to the antibiotics tested was roughly higher for persistent strains of Group 1 patients in comparison to strains of Group 3 patients.

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Table 8. *Sensitivity to 14 antimicrobial agents of 734 isolates of* S. aureus

Hospital	No. of isolates	No. of patients	% of strains resistant to P*	Ox	Ka	To	Ge	Te	Er	Cl	Pr	Pe	Co	Fo	Fu	Ri
A	316	109	77·2	9·8	14·5	10·5	9·2	12·3	18·1	8·7	0	9·41	1	3·7	3·1	3·4
B	145	32	58·7	12·1	18	18	17	15·7	17·4	5·6	0	11·8	0	6·2	0·3	2
C	135	39	93·7	0	1·4	0	0	4·20	1·2	1·4	0	0	0	0	4·9	0
D	79	49	88	4·8	9·6	5·6	4·8	8	15·2	8·8	0	14·4	2·4	2·4	8	12
E	32	4	100	19·7	19·7	16·7	9·1	NT	31·8	24·2	0	9·1	4·5	4·5	4·5	4·5
F	27	5	78·1	37·5	12·5	0	0	43·8	43·8	43·8	0	15·6	0	3·2	0	0
Total	734	238	92·6	0	55·6	0	0	0	7·4	0	0	0	0	0	0	0

* P, Penicillin; Ox, Oxacillin; Ka, Kanamycin; To, Tobramycin; Ge, Gentamicin; Te, Tetracycline; Er, Erythromycin; Cl, Clindamycin; Pr, Pristinamycin; Pe, Pefloxacin; Co, Co-trimoxazole; Fo, Fosfomycin; Fu, Fusidic acid; Ri, Rifampicin; NT, not tested.

Table 9. *Antibiotic sensitivity of the* S. aureus *isolates among the three groups of patients*

Group	No. of isolates	No. of patients	% of strains resistant to P*	Ox	Ka	To	Ge	Te	Er	Cl	Pr	Pe	Co	Fo	Fu	Ri
Group 1	336	55	83·9	11	17·9	11·6	10·1	12·7	22·4	9·9	0	10·4	0·3	3·6	4·5	4·2
Group 2	60	7	63·3	6·7	6·7	6·7	6·7	26·7	8·3	6·7	0	23·3	1·7	1·7	3·3	6·7
Group 3	95	23	80	6·3	10·5	9·5	8·4	10·6	13·7	4·2	0	5·3	1·1	5·3	0	3·2

* P, Penicillin; Ox, Oxacillin; Ka, Kanamycin; To, Tobramycin; Ge, Gentamicin; Te, Tetracycline; Er, Erythromycin; Cl, Clindamycin; Pr, Pristinamycin; Pe, Pefloxacin; Co, Co-trimoxazole; Fo, Fosfomycin; Fu, Fusidic acid; Ri, Rifampicin.

DISCUSSION

Respiratory infections play a major role in reducing pulmonary function in cystic fibrosis patients. *Staphylococcus aureus* alone is the initial bacterial pathogen but it later becomes associated with *P. aeruginosa* [2, 3]. The use of epidemiological markers to type bacteria is important for conducting surveys of infections and can help to distinguish between reinfection and persistent infection and it can also be used to detect cross infections and type the strains associated with a disease [9–11, 26].

The 734 isolates of *S. aureus* analysed in the present study displayed 34 different zymotypes. No specific zymotype was associated with cystic fibrosis: zymotype 14, which is the common zymotype, accounted for only 15% of the strains. The frequencies of the major zymotypes were also not significantly different ($P > 0{\cdot}5$) from the frequencies of the same zymotypes in strains that were not from cystic fibrosis patients [12, 25]. The overall genotypic diversity for esterases was greater than in previous studies [12, 25]. Statistical analysis showed no difference in the frequencies of the zymotypes from each of the hospitals, except for zymotype 6, which was encountered more frequently in hospital D. Zymotype 6 is closely associated with methicillin-resistance [12, 13, 25], indeed zymotype 6 accounts for 68–77% of the methicillin-resistant French strains. Only three methicillin-sensitive strains of zymotype 6 have been found; those strains were penicillin-sensitive but carried the same resistance to aminoglycosides and tetracycline as most of the French methicillin-resistant strains [12]. Strains of zymotype 6 are considered to be responsible for epidemic hospital-acquired infections [11, 12]. In the present study, the zymotype 6 strains found were all methicillin-resistant. Many of the strains were non-typable by phage (33·4%). As described previously [9], group III was the major lytic group and no specific phage type of *S. aureus* was associated with cystic fibrosis. The *S. aureus* strains isolated from cystic fibrosis patients belong to a wide range of types, and apart from the zymotype 6 strains, are similar to strains acquired outside hospitals [6].

Most of the isolates (79%) had capsular polysaccharide types 8 or 5, and 64% of them had a capsular polysaccharide type 8. Similar results were obtained by Albus and co-workers for *S. aureus* strains isolated from cystic fibrosis patients and from healthy individuals [18]. There was a good correlation between capsular type and zymotype in the present study, corroborating the findings for other clinical isolates of *S. aureus* [25, 26].

Esterase electrophoretic typing indicated that *S. aureus* persisted for long periods in 73% of the patients (group 1 and 2) followed for at least 6 months. All the patients had a single persistent strain at any one time, except for three of them who had two persistent strains at the same time. Although phage typing is often unsatisfactory because its typability is lower than other typing methods [6, 9, 26], a combination of phage type, capsular type and zymotype reliably delineated persistent strains in 36 patients. Indeed, the phage type did not change in those strains throughout the study. However, consecutive isolates that were the same type according to zymotype and capsular type, had different phage types in 13 patients and were not typable in 20 patients. Whether those later consecutive isolates are identical strains remains to be clarified by another typing technique.

such as DNA fingerprinting of chromosomal DNA by pulsed-field gel electrophoresis [26]. Goering and colleagues [9] studied 34 cystic fibrosis patients with *S. aureus* positive cultures, and found that 6 patients had persistent infection (for up to 10 months) on the basis of plasmid and phage type. The present results also indicate that strains of *S. aureus* can persist for long periods.

The ability of *S. aureus* to interchange has been shown in only 27% of the patients (group 3). However, a strain isolated from one patient at the beginning of the study reappeared at the end of the study with three markers identical. Strains reappeared in seven other group 3 patients based on capsular type and esterase electrophoretic type. Goering and colleagues [9] observed cycles of carriage, loss, and reinfection in 2 of the 6 patients with persistent infection. It is also possible that double colonization prevented detection of the persistent strains. The isolates should be examined by genome typing to clarify these points.

Although it has not been clearly demonstrated that the capsular antigens of *S. aureus* induce protective immunity [26], the observation that a high percentage of strains isolated from cystic fibrosis patients has a type 8 or 5 capsular polysaccharide is encouraging for the development of an effective capsular vaccine to prevent repetitive bronchopulmonary infections.

Bauernfeind [5] reported that only netilmicin and glycopeptides were active against all the 179 strains isolated from 107 cystic fibrosis patients. Activity of the other antibiotics tested, including the most frequently used as co-trimoxazole, was impaired by resistance. Of the 179 strains, 11·7% were methicillin-resistant. In our study about 10% of the strains were methicillin-resistant. Resistance to the antibiotics tested varied greatly from one hospital to another, therefore the number of antibiotics available for therapy and prophylaxy depends on each hospital. However, 40% of the strains remained fully sensitive to all antibiotics tested except to penicillin. Pristinamycin was active on all the isolates and only 1% of the isolates were resistant to co-trimoxazole, an antibiotic widely used for treatment in these hospitals. Persistent strains of group 1 patients appeared to be more resistant to antibiotics than the varying strains of group 3 patients; however, clinical correlations are needed to provide a better understanding of the persistence of the strains.

The results of this study could contribute to the elaboration of a rational approach to the prophylaxis and therapy of staphylococcal infections in cystic fibrosis patients.

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