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ORIGINAL ARTICLE

Early microbial colonization of cystic fibrosis patients identified by neonatal screening, with emphasis on *Staphylococcus aureus*

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Abstract

Objectives: To assess bacterial colonization prospectively in patients with cystic fibrosis identified by neonatal screening. To assess susceptibility to antimicrobials and to perform the molecular typing of *Staphylococcus aureus* strains isolated from the oropharynx of patients during the study.

Methods: Twenty-five cystic fibrosis patients receiving regular treatment at the Cystic Fibrosis Outpatient Clinic of Hospital de Clínicas of Universidade Federal do Paraná, Brazil, were included in the study. All patients were identified by trypsin-like immunoreactivity and their diagnosis was confirmed by two or more sweat tests. Oropharyngeal swabs were collected and cultured according to routine methods; bacterial colonies were phenotypically identified and their susceptibility to antimicrobials was tested. *S. aureus* isolates were submitted to molecular typing using pulsed-field gel electrophoresis.

Results: Out of 234 oropharyngeal swabs, *S. aureus* was the most frequently isolated strain (76% of patients, 42% of swabs), followed by *Pseudomonas aeruginosa* (36% of patients, 16% of swabs) and *Haemophilus* spp. (76% of patients; 19% of swabs). Seventy-three isolates were obtained from 19 patients colonized with *S. aureus*, of which 18 were oxacillin-resistant (24.6%), isolated from two patients, with the same electrophoretic profiles as that of the Brazilian clone. The remaining oxacillin-sensitive isolates were distributed into 18 electrophoretic profiles.

Conclusion: There was higher prevalence of *S. aureus*, with earlier isolation than other pathogens. Multi-sensitive isolates were distributed into different clones, characterizing non-transmissibility among community-acquired strains. The isolated oxacillin-resistant *S. aureus* showed identical electrophoretic profiles, probably acquired in hospital. *P. aeruginosa* was not so frequent in the studied population.

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Introduction

Cystic fibrosis (CF) is the most common lethal inherited disease, especially among Caucasians.[1] It is caused by mutations in the CFTR gene, which changes the viscosity of airway mucus and leads to chronic endobronchial infection, which may eventually have a fatal outcome in virtually all patients.[2] The major cause of death in CF is the infection and inflammation caused by *Pseudomonas aeruginosa,* the most common infectious agent.[1] Some authors have raised the hypothesis that infection by *Staphylococcus aureus* provides a favorable environment for the adherence of *P. aeruginosa* to the airways of CF patients.[3] However, there has been a paucity of available studies in Brazil on the colonization and dynamics of *S. aureus* infection in this population.

The inclusion of research about this disease in the neonatal screening program (heel prick test) in the state of Paraná, Brazil, in September 2001 helped to identify several cases that would probably be diagnosed only later, after unsuccessful treatments of recurrent lung infections. The early diagnosis of CF allows starting appropriate treatment immediately and improving the prognosis of patients.[4] The aim of this study was to assess early microbial colonization in CF patients diagnosed by neonatal screening, mainly with the aim of determining the transmissibility of *S. aureus* among patients.

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Methods

The study was approved by the Research Ethics Committee of Hospital de Clínicas da Universidade Federal do Paraná (HC-UFPR). The hospital and all parents or surrogates agreed to participate in the study. Swab samples were collected from the posterior pharynx of children with CF, identified by neonatal screening (trypsin-like immunoreactivity), with diagnostic confirmation by two or more sweat tests.[5] Twenty-five patients regularly treated at the Cystic Fibrosis Outpatient Clinic of HC-UFPR between August 2003 and December 2004 were included in the study. The swabs were collected based on the outpatient's routine according to the clinical outcome of each patient. The age of analyzed patients averaged 14.3 months for males and 15.5 months for females. A descriptive analysis was used for the data (mean, median and confidence interval).

The biological material (swab) was placed in tubes with 1 mL of PBS containing 0.1% of bacteriological gelatin, in an isothermal box.[6] On arrival to the laboratory, the samples were homogenized for 30 s using a vortex shaker and inoculated with a calibrated loop of 10 µL (0.01 mL) into three plates with different culture media, blood agar, supplemented chocolate agar and MacConkey agar, so as to allow a semiquantitative analysis of bacterial growth.[7] The plates were incubated at usual temperature and atmosphere.

The colonies of bacteria classically isolated in this sample of patients, such as *S. aureus*, *P. aeruginosa* and *H. influenzae*, were identified using the routine methods adopted by the Department of Bacteriology of the Division of Clinical Analyses of HC-UFPR.[8,9] The same isolates were submitted to the susceptibility test using the classic disk diffusion method (Kirby Bauer).[10] The bacterial samples of interest were suspended in cryopreservation solution (brain heart infusion broth containing 15% of glycerol) and stored at -80 ºC for complementary tests to be subsequently performed together.[11] The confirmatory tests for identification of *S. aureus* after the reactivation of the stored isolates included hemolysis on sheep blood agar plate, coagulase test (clumping factor), growth on agar containing 7.5% of sodium chloride, mannitol fermentation, colony pigmentation and deoxyribonuclease activity, following standard techniques.[12]

The susceptibility test to determine the minimum inhibitory concentration (MIC) of all *S. aureus* isolates was carried out by dilution on agar, with different concentrations of ciprofloxacin, erythromycin, gentamicin, oxacillin, sulfamethoxazole-trimethoprim and vancomycin.[13] Sixty-five isolates obtained at an interval longer than 15 days for the same patient were referred to the Special Laboratory of Clinical Microbiology (Laboratório Especial de Microbiologia Clínica, LEMC), in São Paulo, for molecular typing using pulsed-field gel electrophoresis (PFGE). A 1% ultrapure agarose gel was used for the electrophoretic run, with a voltage of 6.0 volts/cm, initial pulse of 5.0 s and final pulse of 60 s and a runtime of 23 hours. The technique consists in analyzing the polymorphism of total DNA fragments after digestion by endonucleases that recognize infrequent sites in the bacterial genome.[14]

Results

A total of 234 oropharyngeal swabs were collected from 25 children (48% male and 52% female), with an average of 9.3 swabs per patient. The frequency of medical appointments ranged from 15 days to 3 months, with an average of 53 days. The isolation of respiratory pathogens (*S. aureus, P. aeruginosa, H. influenzae* and *B. cepacia* complex) occurred in 100 swab cultures (42.73%) obtained from the 25 patients (Table 1).

Table 1 - Proportion of positive cultures for bacteria of interest in cystic fibrosis

Bacterium	Patients	Positive cultures/ patient
S. aureus	19 (76%)	39.7%
H. influenzae	19 (76%)	18.7%
S. pneumoniae	16 (64%)	13.2%
P. aeruginosa	9 (36%)	15.6%
B. cepacia	1 (4%)	11.7%

The first pathogens to be isolated were *S. aureus* in 18 patients (72%) and *H. influenzae* in 10 patients (40%); these two pathogens were concomitantly isolated in the first swab culture in four patients (16%). *P. aeruginosa* was the first pathogen to be isolated in only two patients (8%) and, in one of them, *S. aureus* was isolated concomitantly. The average time up to the first isolation of *S. aureus* was 214 days, with a confidence interval of 120 to 309 days. The average time up to the first isolation of *P. aeruginosa* was 358 days, with a confidence interval of 164 to 553 days.

Seventy-three isolates were obtained from 19 patients colonized with *S. aureus*, and 18 (24.6%) of these isolates were oxacillin-resistant (MRSA), obtained from three patients; in two of them, most isolates (13/14 and 4/6) had this characteristic, whereas in the other patient, only one of the three isolates had such characteristic. The largest rate of positive swab cultures per patient was observed for this bacterium (37.23%, range from 10 to 82.35%).

Fifteen isolates were obtained from nine patients colonized by *P. aeruginosa*, all of them multi-sensitive and

non-mucoid. Of the total number of patients, six had been previously colonized by *S. aureus*, and two by other pathogens (one by *H. influenzae* and the other one by *Streptococcus pneumoniae*). No patient showed persistent colonization by *P. aeruginosa*. In the positive cultures for this bacterium, *Haemophilus* spp., *S. aureus* MRSA and oxacillin-sensitive (MSSA) strains were concomitantly observed, whereas no pathogens were found in eight swab cultures. The average of positive cultures per patient was 15.6%, with a confidence interval of 0 to 43.5%.

Haemophilus spp. was present in 19 patients, totaling 33 isolates, but the colonization was not persistent in any of them. The average of positive cultures per patient was 18.7%, with a confidence interval of 0 to 37.5%.

Twenty isolates were obtained from 16 (64%) patients colonized by *S. pneumoniae*, of which 18 were tested for penicillin sensitivity. Thirteen were resistant (72%) and five were sensitive on the oxacillin disk test and on the E-test to penicillin. No patient showed persistent colonization by *S. pneumoniae.*

Two isolates of the *B. cepacia* complex were obtained from the same patient using consecutive samples. Since it was not the aim of the study to carry out the molecular typing of all isolated microorganisms, the genomic subspecies (genomovar) was not determined. The patient was persistently colonized by MRSA, but the subsequent cultures did not reveal this bacterium.

S. maltophilia and *A. xylosoxidans* were not isolated from the analyzed samples.

The biochemical profile (biotype) and the profile of antimicrobial susceptibility (antibiotype) were analyzed in 69 *S. aureus* isolates. Of 18 colonized patients, four had only one isolate each, and were therefore excluded from the preliminary analysis. Among 10 of the remaining patients, more than 50% of the isolates had biotypes and antibiotypes that suggested they belonged to the same clone, whereas four had isolates with different biotype or antibiotype.

Determination of the MIC of all antibiotics tested against *S. aureus* isolates showed that all of them were highly potent, with an MIC_{50} below the cutoff points for sensitivity. Vancomycin was the most active one, with an MIC_{90} below this value, whereas the value of the other antimicrobials tested were above the cutoff point for resistance (Table 2). By analyzing the results, vancomycin proved efficient against all tested strains (100%), while other agents showed susceptibility between 64.2 and 76.1%.

The 65 *S. aureus* isolates submitted to molecular typing using PFGE were analyzed according to the criteria recommended by Tenover et al.,[15] with 21 different electrophoretic profiles. The MRSA isolates were compared with the Brazilian clone of MRSA in the same analysis (Figure 1). The MRSA obtained from two patients (10 from one patient and three from another one) had the same electrophoretic profile as that of the Brazilian clone. Another MRSA isolate, different from the previously mentioned patients, was obtained in only one sample from a third patient, and was disregarded. The remaining isolates, all MSSA, were distributed into the available 18 electrophoretic profiles (Figure 2).

Discussion

The lungs of fibrocystic children are often colonized or infected in early childhood by bacteria such as *S. aureus* and *H. influenzae*, which may cause injury to the epithelium, causing an increase in adherence and definitive replacement by *P. aeruginosa*.[1] These data confirm the bacterial colonization observed in the analyzed sample of patients.

A European study involving 639 CF patients younger than 18 years revealed that continuous antistaphylococcal prophylaxis increased the risk of colonization by *P. aeruginosa*, compared to individuals who received continuous or intermittent antibiotic therapy or no antibiotic therapy at all against *S. aureus*.[16] A multicenter, double-blinded, randomized and placebo-controlled study concluded that, although prolonged cephalexin prophylaxis delayed the acquisition of *S. aureus*, it increased the colonization by *P. aeruginosa* and did not show any clinically significant benefits to young children with CF.[17]

Table 2 - MIC_{50} and MIC_{90} of antibiotics against *S. aureus* strains

Antibiotic	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	% sensitivity
Ciprofloxacin	< 0.5	> 16	73.5
Erythromycin	< 0.25	> 32	68.6
Gentamicin	< 1.0	> 64	64.2
Oxacillin	< 0.5	> 16	75.0
Sulfamethoxazole-trimethoprim	< 19/1.0	> 304/16	76.1
Vancomycin	< 0.5	< 0.5	100.0

MIC = minimum inhibitory concentration.

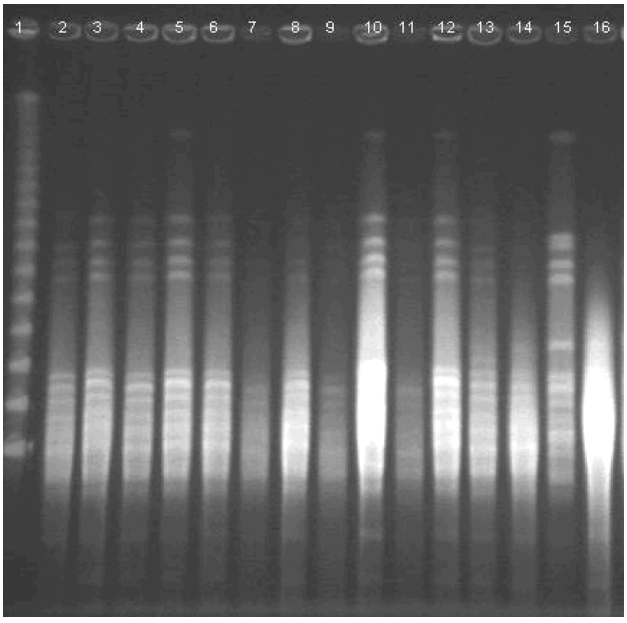

Figure 1 - Electrophoretic profiles of MRSA isolated from three patients and from the Brazilian clone

MRSA = methicillin-resistant *Staphylococcus aureus*.
Line 1: molecular weight marker; Line 2: Brazilian clone of MRSA; Lines 3 to 12: MRSA from patient A (identical with the Brazilian clone); Lines 13 to 15: MRSA from patient B (identical with the Brazilian clone); Line 16: MRSA from patient C (different from the Brazilian clone and from the isolates obtained from patients A and B).

However, chronic airway colonization by *P. aeruginosa* in CF is low in regions where antistaphylococcal therapy is strictly based on necessity rather than being used as prophylaxis.[18] Studies showing the positive effect of treatment against *S. aureus* isolated from oropharyngeal cultures have not been published yet.

The sample of patients analyzed in this study included infants or children aged 1 month to 3 years. The isolation of *S. aureus* in 19 of 25 patients (76% of total) and of *Haemophilus* spp. in the same proportion of individuals, although in a smaller number of samples per patient (18.7 versus 41.8% of patients with *S. aureus*), is consistent with the international data. The patients who were positive for *S. aureus* were treated with two antibiotics for 3 weeks, with monitoring of antibiogram results.

Before the advent of antibiotic therapy, most patients died from *S. aureus* infection.[19] Currently, *P. aeruginosa* is the major causative agent of chronic infection, affecting virtually all patients.[20] Persistence of *S. aureus* has been described in chronic infections among young CF patients for a long time.[21] Even though the present study assessed a population with different age ranges and different types of samples, it can be compared to the study by Kahl et al., who identified unique and persistent clones of *S. aureus* in most analyzed patients.[22] The molecular analysis showed that patients colonized by MSSA kept the same clone throughout, and that the clones were specific to each patient, resulting in non-transmissibility of strains.

The clinical meaning of MRSA infection in CF has not been established yet. A study assessed children with CF for 7 years, whose cultures of respiratory secretions showed the presence of MRSA. The authors concluded that infection by this type of bacterium in children with CF does not significantly affect respiratory function, but has an adverse effect on growth. Patients need intravenous antibiotics in a significantly higher amount and have more abnormal radiological findings than the controls.[3] In this study, only two children had persistent infection by MRSA. Patients had weight curves constantly below the 2.5th percentile, similarly to the results obtained by Miall et al.,[23] although our sample was smaller (two patients *versus* 14 patients of the mentioned study). The two patients with MRSA have been hospitalized several times since birth. One presented with cyanosis and low transcutaneous hemoglobin saturation throughout the follow-up period, whereas the other one had meconium ileus, which had to be surgically treated in the very first months of life.

P. aeruginosa isolates were different from the strains usually found in CF patients: sensitive to most antimicrobials tested and non-mucoid. Burns et al. obtained similar results in a longitudinal study on phenotypic changes in *P. aeruginosa* strains isolated from a cohort of 40 patients during the first 3 years of life.[24]

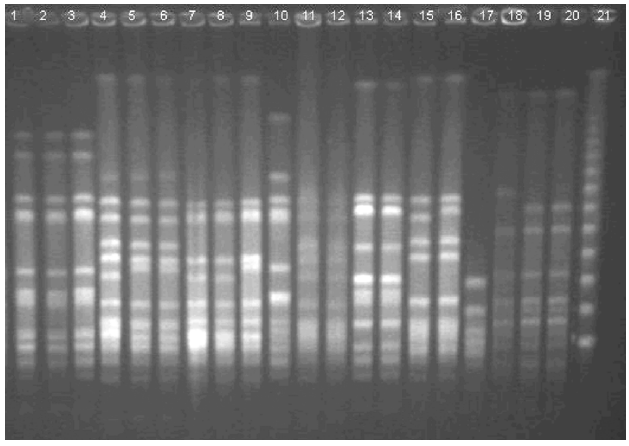

Figure 2 - Electrophoretic profiles of MSSA isolated from six patients

MSSA = methicillin-susceptible *Staphylococcus aureus*.
Lines 1 to 3: isolates from patient D (identical); Lines 4 to 6: isolates from patient E (the first one closely related to the other two, identical); Lines 7 to 9: isolates from patient F (the first two are identical, and the third one closely related to the previous ones); Lines 10 to 12: isolates from patient G (the first one possibly related to the subsequent two, identical); Lines 13 and 16: isolates from patient H (the first two and the last two are identical, characterizing two different clones); Lines 17 to 20: isolates from patient I (the first one possibly related to the subsequent three, closely related); Line 21: molecular weight marker.

Despite the secondary role of *S. pneumoniae* in infection of CF patients, the high rate of penicillin-resistant isolates indicates a process of selection of mutants by the extensive use of antibiotics. No patient was persistently colonized by *S. pneumoniae*, but the high prevalence of this pathogen in the cultured samples highlights the need to vaccinate this group of children.

Emerging bacteria, such as *S. maltophilia* and *A. xylosoxidans*, were not isolated from the analyzed population. Pathogens belonging to the *B. cepacia* complex were not frequent.

The results demonstrate that *S. aureus* was the first pathogen to be isolated, the most commonly occurring bacterium and the one with the largest number of positive cultures per patient. MSSA isolates were sensitive to ciprofloxacin, erythromycin, gentamicin, oxacillin, sulfamethoxazole-trimethoprim and vancomycin, but vancomycin was the most active agent, with 100% sensitivity. The frequency of MRSA is worrying, due to the possible dissemination to other CF patients. MRSA strains were probably acquired through cross-infection in the hospital environment, since they had an identical electrophoretic profile with that of the clone described by Sader in 1994, which has been detected in most Brazilian hospitals.[25] Sensitive *S. aureus* strains were polyclonal and specific to each patient, showing that they were probably acquired from within the patient's own social circle. *P. aeruginosa* non-persistent isolates with non-mucoid and multi-sensitive phenotypes indicate that infection is intermittent and not chronic. The lower sensitivity of oropharyngeal cultures may have contributed to the low rate of bacterial isolation, showing that it is necessary to develop more sensitive detection methods, such as specific antibody tests[26] or polymerase chain reaction.[27]

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