ORIGINAL ARTICLE

Retrospective analysis of microorganisms isolated from cystic fibrosis patients in Southern Italy, 2002-2010

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Key words

Cystic Fibrosis • Bacteria • Yeasts • Moulds

Summary

Objective. *This study aim was to determine the prevalence of microorganisms in the respiratory tract of patients with cystic fibrosis (CF) admitted to the CF Reference Centre in Southern Italy between 2002-2010.*
Methods. *Microbiology assessment of samples (sputum and tracheal aspirates) collected from patients with pulmonary exacerbation admitted to hospital was carried out. All patients were registered in a database and clinical and microbiological data were retrospectively analysed.*
Results. *Overall, 188 patients were included and a total of 1217 samples were analysed. The most common microorganisms were Staphylococcus aureus (78.7% of the patients) and Pseudomonas aeruginosa (58%), followed by Candida albicans (19.1%), Haemophilus influenzae (13.3%) and Aspergillus fumigatus (9.6%).*
Conclusion. *Compared to similar studies performed in other European countries, our microbiological data, especially the low occurrence of filamentous fungi, suggest a specific local epidemiology, probably related to some uncommon CFTR mutations, which are specific to Southern Italy.*

Introduction

Cystic Fibrosis (CF) is the most prevalent autosomal-recessive hereditary disease in the Caucasian population. The disease is due to mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. There are about 1800 *CFTR* mutations. Most of them are worldwide distributed, while some are purely regional or "familial" and some others are unique to certain ethnic groups. The CF median birth incidence is 1:3500 in Europe, whereas the overall birth prevalence in the United States is about 1 in 3,700. It is estimated that 1 in 2,500 White births are affected in comparison to 1 in 13,500 Hispanics, 1 in 15,100 African Americans, and 1 in 31,000 to more than 100,000 Asians [1-3]. In the past, CF used to be a predominantly paediatric disease with the onset of symptoms in childhood and a typically unfavourable prognosis within the first decade of life. Recently, the epidemiological profile has changed with almost half of these patients being now adults, reflecting a clear improvement in patient management [4]. Moreover, genetic testing methods are able to detect couples at risk, and many families with a CF child use prenatal diagnosis to prevent the birth of other affected children.
CF disease is characterized by chronic endobronchial infections and a progressive obstructive lung disease. As a consequence of *CFTR* mutations, the production of dehydrated bronchial mucus promotes the colonization of the airways by microorganisms and makes them less sensitive to defence mechanisms, as well as to antibiotic therapy. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are still the most frequently detected bacteria in CF patients, but the prevalence of *Burkholderia cepacia* has risen in recent years, resulting in increasing rates of morbidity and mortality [5]. Less frequently, moulds have been also reported to colonize the respiratory tract, sometimes leading to various respiratory infections [6, 7]. The most common is the allergic bronchopulmonary aspergillosis (ABPA). *Aspergillus fumigatus* has also been described as a possible cause of lung deterioration in the absence of ABPA [8], while *Scedosporium apiospermum* has been described as a cause of a pulmonary or a disseminated disease [9, 10].
The Italian Registry of Cystic Fibrosis (IRCF) has been active in Rome since January 1, 1988. Its aims are to collect patient demographic data, to increase knowledge of the disease, and to monitor epidemiological trends. This registry indicates that the CF incidence in Italy is 1:4238 live births, with wide variations among regions. 43% of patients are older than 18 years, 23.5% were diagnosed within the first year of life, and 16.7% after the age of 18 years. The CF incidence in the Southern Italy is not reported [11]. On January 1, 1994 a new Italian Law (Law

No.548) established a network of specialized Reference Centres across regions aiming at preventing, diagnosing and treating complications, as well as coordinating medical, social and research aspects.
The aim of the present study was to determine the prevalence of microorganisms (bacteria and fungi) in the respiratory tract of the patients admitted to the CF Reference Centre of South Italy between 2002 and 2010.

Materials and methods

The CF Reference Centre of Southern Italy is located in a large University Hospital in Apulia. It admits 260 patients per year in follow up (with about 12 new diagnosis per year) and hosts clinics, laboratories, facilities and services, all meeting the standards of care for CF patients.
The follow-up of CF patients at this Centre requires periodic medical check-ups with a frequency appropriate for the severity of the disease (at least every 4 months) and admission programs aimed at early detection of complications of this disease (i.e. diabetes mellitus, chronic liver disease). A microbiological analysis of respiratory secretions is carried out only during the pulmonary exacerbation.

Study design

The clinical diagnosis was established on the basis of the results of 2 positive sweat chloride tests (> 60mEq/L) by Gibson and Cooke [12] procedure, and the identification of two CF-disease causing mutations in trans [13, 14].
Since 2002, all CF patients with a microbiological analysis of sputum or tracheal aspiration positive for bacteria and/or fungi were included in our study and entered in a database (Microsoft Access 2003). Categorical variables were expressed as proportions or percentages, and numerical data as the mean $\pm$ SD and range. Bivariate analyses were performed using Student's t-test, with P-value < 0.05 considered significant. Statistical analysis was performed using the software SAS system version 9.2.

Laboratory procedures

For bacteriological cultures, sputum samples and tracheal aspirations were homogenized with N-acetyl-cysteine (Carlo Erba reagent SpA, Milano, Italy) and inoculated in parallel on the following media: Columbia with 5% sheep blood agar (CNA, bioMérieux, Marcy l'Etoile, France), Haemophilus Chocolate agar 2 with Bacitracin (HAE2, bioMérieux), MacConkey agar (Becton-Dickinson, Heidelberg, Germany), Oxidation/Fermentation-Polymyxin-Bacitracin-Lactose agar (OFPBL, Becton-Dickinson) and Mannitol Salt Agar (Becton-Dickinson). All plates were incubated for 24-72 hours at 37°C.
For mycological cultures, samples were inoculated on two Sabouraud-chloramphenicol-dextrose agar plates (SC2, bioMérieux) which were incubated at 30°C and 37°C, respectively and examined daily up to 10 days.

The bacteria and yeasts identifications were performed using VITEK-2 System (bioMérieux). Filamentous fungi were identified on the basis of their macroscopic and microscopic morphological features, according to standard descriptions.

Results

From 2002-2010, a total of 1,217 samples from 188 CF patients admitted to CF Reference Centre were analysed.
Table I summarizes the demographic, clinical and genetic data of these patients. The male:female ratio was 86:102 (45.7% vs 54.3%), ages ranged from 1 month to 68 years old, and a mean age at date of CF diagnosis of 17.9 ± 12.8 years. In particular 70 out of 188 patients (37.2%) were less than 10 years old; in addition, females resulted more frequently affected than males in the first (62.9% vs 37.1%) and in the fourth (68% vs 32%) decade of life.

Tab. I. Demographic, clinical and genetic data from 188 enrolled CF patients admitted to the CF Reference Centre and during the study period (2002-2010).

	Patients (n = 188)
Males/Females	86/102 (45.7% vs 54.3%)
Age range (years)	0-68
Mean age at date of CF diagnosis (years)	17.9 ± 12.8
Pancreatic insufficiency	114 (60.6%)
Diabetes mellitus	12 (6.4%)
FEV1 (% predicted)	63.68 ± 28.22
BMI (Z score)	19.51 ± 4.06
F508del / F508 del	38 (20.2%)
F508del / Other mutation	83 (44.1%)
Other / Other mutation	63 (33.5%)
Genotype incomplete or unknown	4 (2.1%)

Unless otherwise stated, data are presented as mean ± SD. FEV1: forced expiratory volume in 1 s; BMI: body mass index.

Thirty-eight out of 188 patients (20.2%) were found homozygous for the F508del mutation. Among other patients, 83 (44.1%) were found heterozygous for F508del mutation, 63 (33.5%) both homozygous and compound heterozygous for other mutations, 4 (2.1%) had CF chromosomes not yet genetically identified.
Other found mutations were N1303K (12.8%) and G542X (7.4%). Uncommon mutations, specific to CF chromosomes from Southern Italy [15], were 4382delA (9.6%), 1259insA (5.3%), 852del22 (5.3%), G1349D (4.8%), D579G and R1158X (3.2%), I502T (2.7%) and L1077P (2.1%). Overall, 52 different mutations in various frequency and genetic combination, were found in our study. Interestingly, 127 (67.5%) and 57 (30.3%) patients had *severe* and *mild CFTR* mutations, respectively.
Table II shows the bacteria isolated from clinical samples collected from 188 patients. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most frequent

Tab. II. Overall bacterial species isolated from CF patients admitted to CF Reference Centre during the study period (2002-2010).

Isolates	Positive patients (n = 188)		Patients positive in:			
			pure cultures		mixed cultures	
	N	%	N	%	N	%
S. aureus	148	78.7	37	25	111	75.0
P. aeruginosa	109	58.0	16	14.7	93	85.3
H. influenzae	25	13.3	2	8.7	23	90.3
H. parainfluenzae	15	8.0	-	-	15	100
B. cepacia	13	6.9	1	7.7	12	92.3
S. maltophilia	12	6.4	1	8.3	11	91.6
A. xylosoxidans	5	2.7	-	-	5	100
P. mirabilis	4	2.1	-	-	4	100
S. marcescens	4	2.1	-	-	4	100
E. cloacae	3	1.6	1	33.3	2	66.7
E. coli	3	1.6	-	-	3	100
S. pneumoniae	2	1.0	-	-	2	100
A. baumannii	1	0.5	-	-	1	100
K. pneumoniae	1	0.5	-	-	1	100

Frequencies are calculated according to the number of CF patients (n = 188) who had at least one sputum sample or tracheal aspiration microbiologically positive during the study period.

(78.7% and 58%, respectively), followed by *Haemophilus influenzae* (13.3%), *Haemophilus parainfluenzae* (8%), *Burkholderia cepacia* complex (6.9%), and *Stenotrophomonas maltophilia* (6.4%). Other bacterial species, such as *Achromobacter xylosoxidans*, *Proteus mirabilis, Serratia marcescens, Enterobacter cloacae*, *Escherichia coli*, *Streptococcus pneumoniae, Acinetobacter baumannii* and *Klebsiella pneumoniae* were occasionally isolated, often occurring in mixed cultures. Overall, the mucoid phenotype of *P. aeruginosa* was identified in 81% of cases.

Regarding bacteria known as the most relevant in the respiratory tract of CF patients, *S. aureus* and *P. aeruginosa* were isolated mostly in combination with other bacteria and/or fungi, while *B. cepacia* complex was detected in combination only with other bacteria or in a pure culture (Tab. III).

CF patients positive for *S. aureus* and *P. aeruginosa* at their first microbiological control were grouped by age. Figure 1 shows that *S. aureus* was the most common species isolated during the first decade of life. Its frequency progressively decreased until the fourth decade, together with a concomitant increase in the prevalence of *P. aeruginosa.* Very close isolation percentage were seen for these two species during the third decade of life; whereas the latter became the predominant species in older patients (31% vs 79.3% for patients aged 31-40). Among the enrolled patients, 15 (11 females and 4 males, aged between 7 and 42 years) were continuously positive for *S. aureus* and/or *P. aeruginosa* in all examined clinical samples. The remaining patients resulted positive for other species.

Mycological investigations were positive in 52 out of 188 patients (27.6%) (Tab. IV).

Candida albicans was the most frequent fungus (19.1%), followed by *Aspergillus fumigatus* (9.6%), *Aspergillus flavus* (2.1%), *Candida glabrata* and *Scedosporium apiospermum* (0.5%), mainly in combination with bacteria (usually *S. aureus* and *P. aeruginosa*, and to a lesser extent *S. maltophilia*).

Tab. III. Distribution of the most relevant bacterial pathogens observed in respiratory secretions from 188 CF patients admitted to CF Reference Centre (2002-2010).

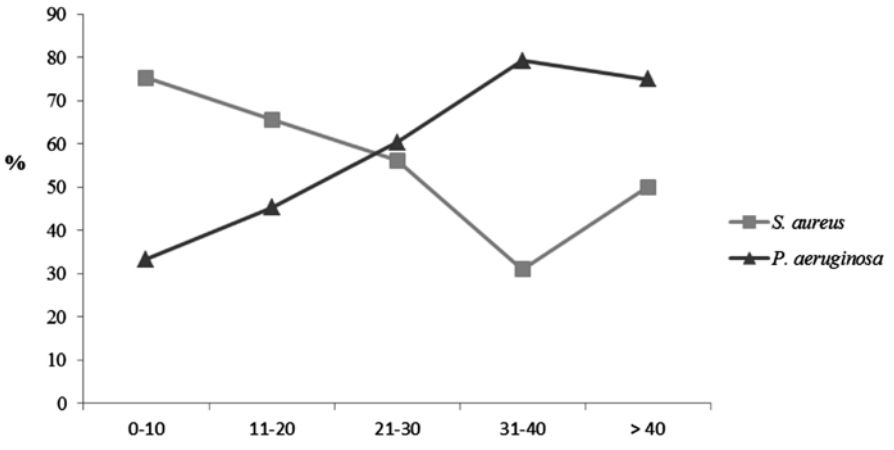
Microorganisms	Positive samples (%)	Positive patients (%)
S. aureus	719 (59.1)	148 (78.7)
Alone	221 (30.7)	37 (25.0)
With other bacteria	332 (46.2)	68 (45.9)
With fungi	55 (7.6)	6 (4.1)
With fungi and bacteria	111 (15.4)	37 (25.0)
P. aeruginosa	725 (59.6)	109 (58.0)
Alone	250 (34.5)	16 (14.7)
With other bacteria	312 (43.0)	58 (53.2)
With fungi	72 (10.0)	3 (2.7)
With fungi and bacteria	91 (12.5)	32 (29.4)
B. cepacia	107 (8.8)	13 (6.9)
Alone	44 (41.1)	1 (7.7)
With other bacteria	63 (58.9)	12 (92.3)
With fungi	0	0
With fungi and bacteria	0	0

Data correspond to the number of positive samples and of positive patients among the 1217 clinical samples and the 188 CF patients examined. In parentheses are indicated frequency percentages.

Sequential clinical samples were obtained from 129 patients during a period longer than 3 years. From the analysis of these samples, a high persistence (81.3%) was observed for *B. cepacia*, lower for *P. aeruginosa* (50.9%) and *S. aureus* (38.6%). Regarding to mycological investigations, a high persistence was observed for *A. fumigatus* and *A. flavus* (54.1% and 50%, respectively).

Clinical outcomes were unfavourable for 16/188 examined patients (8.5%). Of the remaining patients, 12 underwent transplantation (lung, liver or renal transplantation in 6, 4 and 2 patients, respectively). Three women completed a pregnancy, one with twins.

Fig. 1. Isolation percentage of *S. aureus* and *P. aeruginosa* according to the age of the patients at first microbiological control.



Discussion

It is widely known that a lung disease and recurrent lung infections are the major cause of morbidity and mortality in CF patients [16]. Many patients follow a characteristic pattern of infection that begins in the early stages, usually in childhood, with *S. aureus* and *H. influenzae* infection. These microorganisms seem to prepare the bronchial tree for more aggressive microorganisms, such as *P. aeruginosa*, typical in adolescent patients, often associated or replaced by *B. cepacia* complex in young adults [17]. Fortunately, today the ability of these bacteria to invade the respiratory tract is countered by new therapeutic protocols aiming at the eradication of the infection, which has significantly changed the prognosis in these patients [18].

In Apulia, a neonatal screening program is not yet operational. In addition, the presence of many *mild* mutations determines blurred clinical signs and infections caused by different etiological agents. Infact in our study, a wide variety of microorganisms was frequently recovered such as *S. aureus, P. aeruginosa, A. fumigatus*, *B. cepacia*, etc, while others were seldom isolated (i.e. *E. coli, C. glabrata, A. baumannii, S. apiospermum*). *Staphylococcus aureus* was the main detected bacterial species, particularly in the first decade of life and among the younger patients, than to *P. aeruginosa* (mean age 15.7 ± 11.1 and 22.3 ± 13.3, respectively; $p < 0.001$). Furthermore, *S. aureus* infection tended to become chronic in some patients, as demonstrated by repeated isolations in the years, in pure cultures for some patients or in combination with *P. aeruginosa* for others.

Pseudomonas aeruginosa was recovered from 58% of examined patients. According to other authors [19], it appeared in the first two decades of life with low frequency, and increased up to 79% in adulthood. In these patients, chronic colonization of the respiratory tract was noted, especially by the mucoid phenotype and drug-resistant strains of *P. aeruginosa,* often related to a decline in lung function and a poor prognosis (unpublished data).

Burkholderia cepacia complex is responsible for unfavorable prognosis [20]. Although it was isolated with low frequency (6.9%), it showed a high persistence rate (> 80%) in our patients.

Tab. IV. Fungal communities in respiratory secretions from CF patients with positive mycological cultures.

Microorganisms	Positive samples n. (%)	Positive patients n. (%)
C. albicans	129 (10.6)	36 (19.1)
Alone	8 (6.2)	4 (11.1)
With bacteria	120 (93.0)	31 (86.1)
With other fungi	1 (0.8)	1 (2.8)
With other fungi and bacteria	-	-
C. glabrata	1 (0.08)	1 (0.5)
Alone	-	-
With bacteria	-	-
With other fungi	1 (100)	1 (100)
With other fungi and bacteria	-	-
A. fumigatus	100 (8.2)	18 (9.6)
Alone	-	-
With bacteria	81 (81.0)	10 (55.5)
With other fungi	8 (8.0)	2 (11.1)
With other fungi and bacteria	11 (11.0)	6 (33.3)
A. flavus	24 (2.0)	4 (2.1)
Alone	-	-
With bacteria	14 (58.3)	3 (75.0)
With other fungi	-	-
With other fungi and bacteria	10 (41.7)	1 (25.0)
S. apiospermum	20 (1.6)	1 (0.5)
Alone	-	-
With bacteria	20 (100)	1 (100)
With other fungi	-	-
With other fungi and bacteria	-	-

Data correspond to the number of positive samples and of positive patients among the 1217 clinical samples and the 188 CF patients examined.

In a retrospective study, Millar et al. [21] observed an important emergency of *Stenotrophomonas maltophilia* in CF patients, largely favouring the progression of lung disease. Valdezate et al. [22] reported that the same strain

of *S. maltophilia* was isolated 11 times in seven years from one patient, and other subjects showed chronic *S. maltophilia* infection especially in addition with *P. aeruginosa*. Also in our study *S. maltophilia* was frequently isolated, representing the sixth bacterial species from polymicrobial cultures and the eighth species when fungi were included. It appeared in association with *S. aureus* in 91.2% of cases, and to a lesser extent with *P. aeruginosa* and *A. fumigatus.*

Regarding to results of mycological surveillance, the overall frequency of fungal isolation did not appear high in our patients (27.6%), although there was no prophylactic antifungal treatment, in line with the protocols of our CF Reference Centre. Our data, especially the low occurrence of filamentous fungi (i.e. *A. fumigatus* 9.6%), are conflicting with those obtained in similar studies performed in other European countries [23, 24], suggesting a particular local epidemiology.

In Apulia, there are many mutations (about 60) from different classes and with different phenotypic outcomes; some of these are specific to Southern Italy and others are private mutations [15, 25]. Recently, Green et al. [26] asserted that the mutations, which determine a mild genotype by retaining some residual CFTR function, may result in a delay in the colonization of the respiratory tract by some microorganisms. In our study, 30.3% of patients had mild genotype, and only 21% of them had mycological cultures positive. Recently, some authors emphasized that the transition from colonization to fungal infection, as well as the course of CF disease, are not only based on the genetic structure but are also determined by other genetic factors, so-called "modulator genes" and by environmental factors [15, 27, 28]. The modulator genes produce proteins that can affect the clinical manifestations of CF disease. The interactions between CFTR mutations and allelic modulators genes result in different phenotypic expressions and in part explain why individuals with the same genotype can have different level in severity of the disease. Moreover, the fungal genotype analyses conducted in recent years demonstrated that a dominant genotype tended to settle with the ageing colonization [29, 30]. In our study, a persistent colonization with *Aspergillus* species was observed for some patients. Besides, a patient, after colonization by *A. fumigatus*, was infected by *S. apiospermum* of which an unique genotype was identified. Twelve months later, the patient died after a cerebral involvement, despite antifungal treatment [31].

This study suggests the need for an increased microbiological monitoring in CF patients in order to determine whether the microbial flora is the same in a clinically stable phase and during an exacerbation, aiming at correlating microbiological data to mutations and/or to clinical outcomes of these patients, defining possible therapeutic strategies that can prevent, or at least delay, new infections. Additionally, a better knowledge of local fungal epidemiology may be helpful, particularly when a decision regarding transplantation is made, which consequently may contribute to improving life expectancy in CF patient population.

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