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Isolation of Fungi, Especially *Exophiala dermatitidis*, in Patients Suffering from Cystic Fibrosis

A Prospective Study

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Key Words
Cystic fibrosis · Black yeasts · *Exophiala dermatitidis* · Sputum

Abstract
Background: Patients with cystic fibrosis (CF) are at an increased risk of pulmonary colonisation by opportunistic micro-organisms. Using specialised methods, the black yeast *Exophiala dermatitidis* could consistently be cultured from CF patients. Isolation rates from sputum samples ranged between 1.8 and 15.7%. Occasionally, infection could be recognised. ***Objectives:*** This study aimed at investigating the isolation rates of *E. dermatitidis* in samples taken from CF patients at the University of Bonn, Germany. ***Methods:*** Altogether, 439 respiratory specimens taken from 81 CF patients were screened for the occurrence of *E. dermatitidis* over a period of 18 months. For the selective isolation of this fungus erythritol-chloramphenicol agar (ECA) produced in house was applied. ***Results:*** The isolation rate of *E. dermatitidis* was 1.1% from all specimens, 1.6% from all sputum samples and 6.2% in all patients examined. ***Conclusions:*** Prior to the introduction of ECA, *E. dermatitidis* had never been isolated in our laboratory, either from CF, or from any other patient. During this study, *E. dermatitidis* was found to colonise the respiratory tract of some CF patients. The use of additional selective culture media is necessary for the recognition of uncommon fungi, e.g. *E. dermatitidis*, in CF patients.

Introduction

The viscous sputum of patients suffering from cystic fibrosis (CF) is frequently contaminated by certain facultative pathogens (e.g. *Pseudomonas aeruginosa*, *Aspergillus fumigatus* and *Candida albicans*) due to colonisation of the respiratory tract. In 1990 and 1992, pulmonary infections due to the black yeast *Exophiala dermatitidis* were described for the first time [1, 2]. A new culture medium was developed for the efficient recovery of this species, the erythritol-chloramphenicol agar (ECA) [3]. Since then, the fungus was found frequently in sputum samples of CF patients. This paper reports on the isolation rate of *E. dermatitidis* in CF patients in Bonn, Germany, during a period of 18 months.

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Table 1. Culture media used in this study

Medium	Columbia-Sheep blood agar	Columbia-Sheep blood-nalidixic acid agar	Bacitracin-chocolate agar	MacConkey's agar
Manufacturer	Oxoid	Oxoid	self production	Oxoid
Time of incubation	2–3 days	2–3 days	2–3 days	2–3 days
Culture conditions	35–37 °C, 10% CO_2	35–37 °C, 10% CO_2	35–37 °C, 10% CO_2	35–37 °C
Selective properties	no selection	gram-positive bacteria	*Haemophilus* spp.	gram-negative bacteria
Medium	**Cetrimid agar**	**Candi-select agar**	**Sabouraud's glucose agar**	**ECA**
Manufacturer	Merck	BioRad	Oxoid	Becton Dickinson, Sigma; self production
Time of incubation	3–5 days	2–3 days	10 days	28–30 days
Culture conditions	35–37 °C	35–37 °C	28–30 °C	35–37 °C
Selective properties	*P. aeruginosa*	yeasts	hyphomycetes	*E. dermatitidis*

Materials and Methods

During a period of 18 months, 439 specimens (315 sputum samples, 122 oral swabs, 1 nasal swab, 1 tracheal secretion) from patients suffering from CF were cultured for the presence of *E. dermatitidis*. The samples were taken from 81 CF patients, among whom there were 6 sister-brother pairs. The male-to-female ratio was 1.1/1 (42 male/39 female patients). Patients' ages ranged between 3 months and 42 years (mean age: 18 years). A minimum of 1 specimen and a maximum of 12 specimens were analysed from each patient. Specimens from an individual patient were collected at intervals ranging from a few weeks up to a few months.

The specimens were delivered to the Institute by a courier from the Children's Hospital of the University of Bonn. They were plated within 2–4 h on to the culture media shown in table 1.

For the selective isolation of *E. dermatitidis*, ECA produced in house [3] was applied. Strain CBS 148.90 (CBS Culture Collection) was used for quality assessment of every batch of ECA. The medium was stored at 8 °C until use, for a maximum of 14 days. After inoculation, the plates were incubated at 36 ± 1 °C for 28–30 days and examined for fungal growth every 2–3 days. Black fungi were identified down to species level using morphological criteria, growth at 40 °C and nitrate assimilation [4, 5].

Results

Neither fungi nor bacteria were cultured from single tracheal secretion; *Haemophilus influenzae* and other members of the mucosal microflora of the respiratory tract were isolated from nasal swabs. The results of oral swabs and sputum samples are summarised in table 2. There was no apparent correlation between age or sex of the patients and detection of specific pathogens. From most of the patients examined, *P. aeruginosa* and *C. albicans* were cultured, which is in accordance with the data from the literature [6].

The number of different bacterial and fungal species cultured from sputum samples was higher than that from oral swabs (table 2, fig. 1, 2). Figure 2 shows a more detailed analysis of the fungal isolates from sputum samples (SS) and oral swabs (OS) and compares them. Non-*albicans Candida* species were the only fungi, which were isolated at a higher frequency from oral swabs than from sputum samples. *E. dermatitidis* could not be cultured from any of the oral swabs. The fungi listed as 'other hyphomycetes' in table 2 and figure 2 did not develop any conidia that would have allowed microscopic identification. Because there were no clinical signs of infection in the patients from whom the samples were taken, no further attempts were made to identify these isolates.

In figure 3, moulds detected using SGA are compared with those grown on ECA. Using ECA, mainly several isolates of *Aspergillus* and *Penicillium* spp. were obtained in addition to two strains of *Pseudallescheria boydii. E. dermatitidis* was cultured on SGA only once, but 5 times from 5 sputum samples when ECA was used. Colonies were recognised after 5–16 days of incubation, with an average time of about 10 days. In contrast to cultures incubated with SGA and CSA, no strains of *P. aeruginosa* grew on ECA, which may be a reason for the higher isolation rate using this selective medium. In general, during this study, *E. dermatitidis* was isolated from 1.1% of all specimens, from 1.6% of all sputum samples (table 2, fig. 2) and 6.2% from all patients examined (table 4).

All 5 ECA samples positive for *E. dermatitidis* were from a single sample, although the number of samples analysed from the *E. dermatitidis*-positive patients ranged between 1 and 11. In 2 cases, family members also suffered from CF, but in these patients no black yeasts were observed. The data of the 5 *E. dermatitidis*-positive patients are compiled in table 3, including all isolates from each patient. The results of the samples, from which *E. dermatitidis* was cultured exhibit a greyish background. These patients' ages ranged between 9 and 35 years (mean age, 21.8 years); male to female ratio was 1:4. Except patient 3, all patients had suffered from CF for more than 1 year and received glucocorticoids as well as antibiotics during this study. No special factor (e.g. antimicrobial treatment or time of CF diagnosis) was found to predispose to colonisation by *E. dermatitidis*. None of the 5 patients showed any signs of malnutrition. *E. dermatitidis* was invariably accompanied by *P. aeruginosa*, which grew

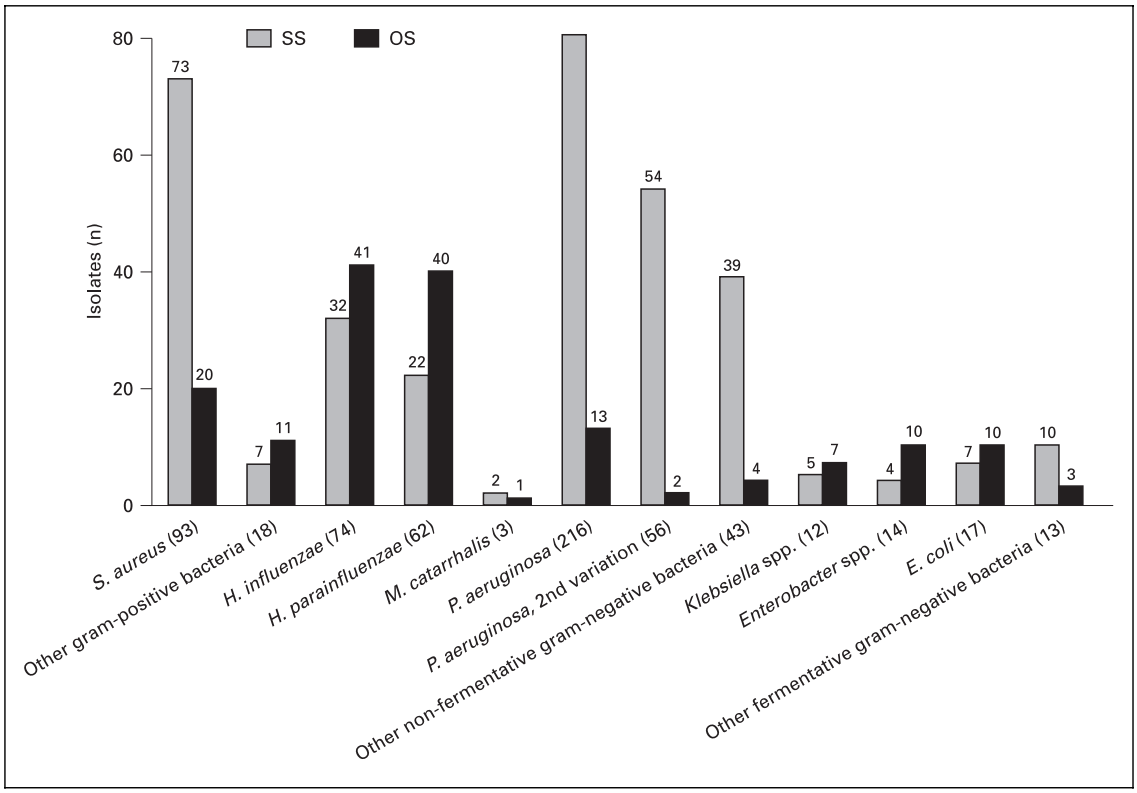

Fig. 1. Comparison of bacterial groups and species isolates from oral swabs (OS) and sputum samples (SS). Numbers of complete isolates are given in brackets behind the name of the isolated micro-organism.

Table 2. Bacteria and fungi isolated from 81 CF patients

Material	Total	Oral bacterial flora	*S. aureus*	Other gram-positive bacteria	*H. influenzae*	*H. parainfluenzae*	*M. catarrhalis*	*P. aeruginosa*	*P. aeruginosa*, 2 biovars	Other non-fermentative gram-negative bacteria	*Klebsiella* spp.	*Enterobacter* spp.
Total	439	424	93	18	74	62	3	216	56	43	12	14
Total, %	100	97.0	31.3	4.2	16.9	14.1	0.7	49.2	12.5	9.8	2.8	3.2
OS	122	119	20	11	41	40	1	13	2	4	7	10
OS, %	100	97.5	16.4	9.0	33.6	32.8	0.8	10.7	1.6	3.3	5.7	8.2
SS	315	304	73	7	32	22	2	203	54	39	5	4
SS, %	100	96.5	23.2	2.1	10.2	7.0	0.6	64.4	12.4	12.4	1.6	1.3

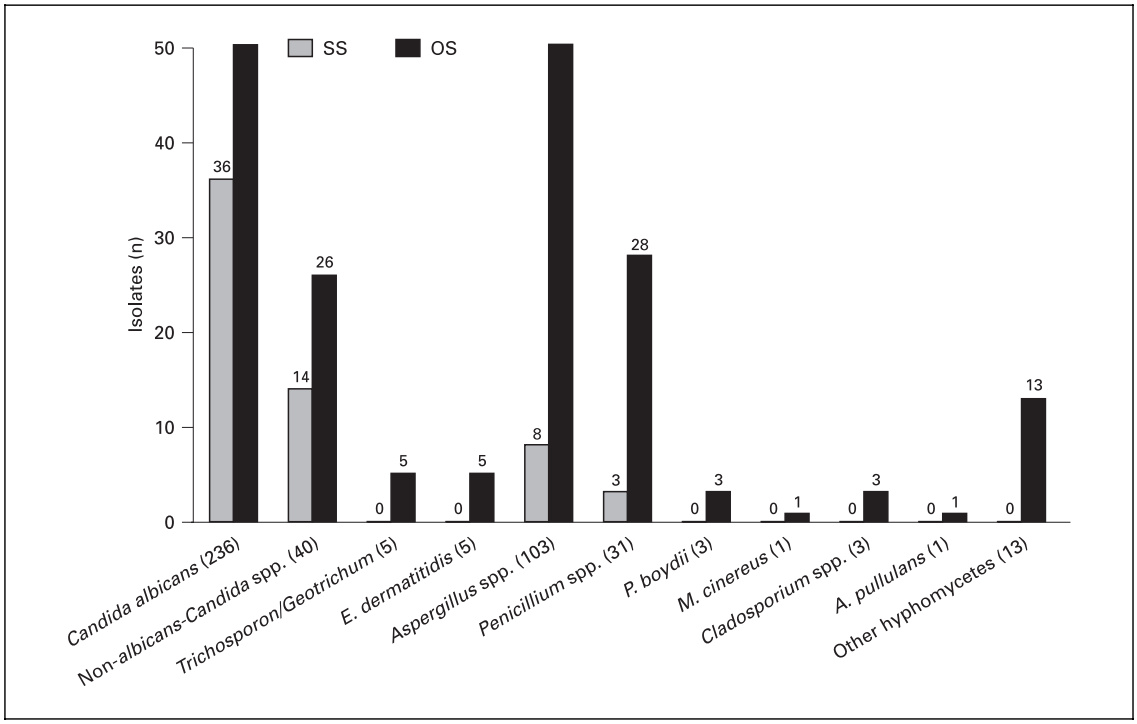

Fig. 2. Comparison of fungal groups and species isolates from oral swabs (OS) and sputum samples (SS). Number of complete isolates are given in brackets behind the name of the isolated fungus.

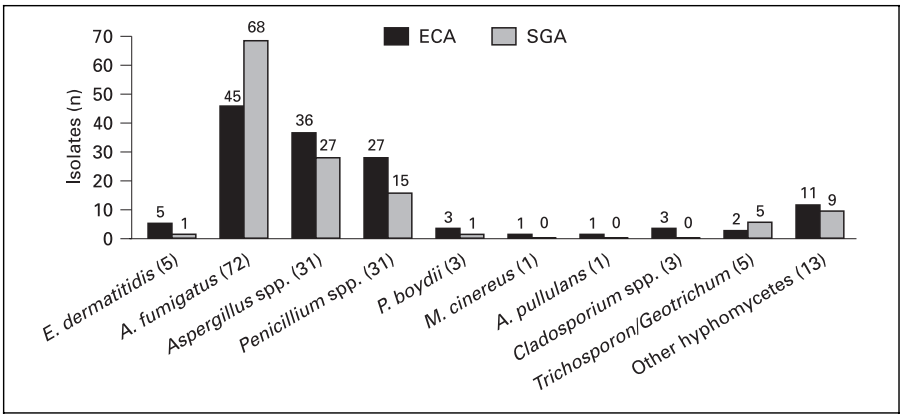

Fig. 3. Comparison of results obtained using SGA and ECA for mould detection. Number of complete isolates are given in brackets behind the name of the isolated fungus.

E. coli	Other fermentative gram-negative bacteria	*C. albicans*	Non-*albicans Candida* spp.	*Trichosporon/ Geotrichum* spp.	*E. dermatitidis*	*Aspergillus* spp.	*Penicillium* spp.	*P. boydii*	*M. cinereus*	*Cladosporium* spp.	*A. pullulans*	Other hyphomycetes
17	13	236	40	5	5	103	31	3	1	3	1	13
3.9	3.0	53.8	9.1	1.1	1.1	23.5	7.1	0.7	0.2	0.7	0.2	3.0
10	3	36	14	0	0	8	3	0	0	0	0	0
8.2	2.5	29.5	11.5	0.0	0.0	6.6	2.5	0.0	0.0	0.0	0.0	0.0
7	10	200	26	5	5	95	28	3	1	3	1	13
2.2	3.2	63.5	8.3	1.6	1.6	30.2	8.9	1.0	0.3	1.0	0.3	4.1

Table 3. Data of the patients from whom *E. dermatitidis* was isolated

Patient No.	Age	Sex	Samples taken during this study	Samples/ patient	Samples OS/SS	*S. aureus*	Other gram-positive	*P. aeruginosa*	*E. coli*	Other gram-negative	*C. albicans*	*A. fumigatus*	*Exophiala dermatitidis*	Other fungi
1	18	f	5 in 16 months	1	SS			1	1	2	1			
				2	OS				1					
				3	SS	1			2		1			
				4	SS	1				1			1	
				5	SS				2	1	1			
2	9	f	9 in 13 months	1	SS	1		1			1	1	1	1
				2	SS			1						1
				3	SS			2			1			
				4	SS					2	1	1		
				5	SS	1		1		1	1			1
				6	SS	1		2						
				7	SS	1		2			1			1
				8	SS	1		2		2				1
				9	SS			1			1	1		1
3	31	f	1 during 18 months	1	SS	1	1	1			1	1	1	1
4	32	m	5 in 11 months	1	SS			1			1			1
				2	SS			2				1		
				3	SS			1		1	1			1
				4	SS			2						
				5	SS	1		1					1	2
5	19	f	11 in 13 months	1	SS	1				1	1			
				2	SS	1				2	1			
				3	OS	1		1		1	1			
				4	SS	1				1	1			
				5	SS	1				1				
				6	SS	1		2			1			
				7	SS	1				1	1			
				8	SS	1		1		1			1	
				9	SS	1				1	1			
				10	SS					1	1			1
				11	SS			2			1			1

SS = Sputum sample; OS = oral swab.

Table 4. Summary of the isolation rate of *E. dermatitidis* from sputum samples from CF patients described in the literature

Reference	Region	Patients	Positive	Clinical significance
7	Germany, Dresden	51	8 (15.7%)	several isolates from each patient
8	Germany, Dresden	96	15 (15.6%)	7 with clinical symptoms
5	Germany, Frankfurt and Aachen	121	11 (9.1%)	several isolates from 9 patients, 8 with specific positive antibodies
9	Germany, Bonn	81	5 (6.2%)	none reported
10	The Netherlands, region unknown	331	6 (1.8%)	none reported
11	Germany, Essen	no data given	3	several isolates from 2 patients
12	Germany, region unknown	no data given	8	several isolates from each patient
13	North America, region unknown	11	11	pulmonary infiltrations
1, 2	Germany, Aachen	1	11	pneumonia

[1] Single case report.

on most media used, except for ECA. The presence of *E. dermatitidis* could not be related to any adverse effect on the health of any of the patients. During this study, none of our patients was hospitalised because of a fungal respiratory infection. However, 21 of the CF patients (25.9%) received in-hospital treatment due to other reasons. All of them showed more known risk factors for colonisation or infection due to *E. dermatitidis*, e.g. malnutrition or long-term antibiotic and/or antifungal treatment, than the 5 *E. dermatitidis*-positive patients, but *E. dermatitidis* was cultured from none of them.

In table 4, the isolation rate of *E. dermatitidis* is compared with the results of other studies published.

Discussion

Facultative opportunistic pathogenic fungi (e.g. *C. albicans* and *A. fumigatus*) are often present in the respiratory tract and, in general, do not predispose to fungal infection. Those patients are at risk for fungal infection nearly exclusively when their immunity is reduced and/or the flora of the respiratory tract is disturbed. Infections due to *C. albicans* are mainly endogenous due to the patients' own colonizing strains, while infections due to *A. fumigatus* are often exogenous as a result of inhaling the conidia, which are pervasive in the environment. In both cases, humans are permanently in contact with fungi. In infections due to 'uncommon fungi' such as *E. dermatitidis*, the situation is clearly different, because these fungi are not as widely present as *C. albicans* or *A. fumigatus*, either in the patient or in the environment. Besides the patient's immunological status, the risk of infection may also depend on the kind of contact and the fungal mass. Until now, the risk of infection due to *E. dermatitidis,* especially in CF patients, is still a moot point. A total of 2,021 nosocomial infections by *E. dermatitidis* have been reported following intravascular [14] or intra-articular injection [15] of contaminated drugs. Some of these cases resulted in lethal outcomes [16].

Prior to the introduction of ECA, *E. dermatitidis* had never been isolated in our laboratory, either from CF, or from other patients. Although the detection of this fungus did not correlate with the clinical status of any of our patients, a medical significance of the organism cannot be completely excluded. Blaschke-Hellmessen et al. [8] observed clinical and radiological signs of pulmonary infections in a significant number of their patients. In another study, *E. dermatitidis*-specific antibodies were found in the sera of CF patients from whom this fungus had been isolated [17]. These data show that colonisation due to *E. dermatitidis* may result in pulmonary infections in humans, especially those suffering from CF. As other human opportunistic pathogens, e.g. *Burgholderia cepacia*, fungi such as *E. dermatitidis* are known to colonise the respiratory tract of CF patients and may lead to infection if additional predisposing factors are present [18]. Routine isolation techniques are insufficient to detect fungi in specimens from CF patients [18], especially, when *P. aeruginosa* is present in the sample, because pyocyanin and 1-hydroxyphenazine produced by these bacteria may inhibit fungal growth [19]. Therefore, additional diagnostic methods for the detection of fungi are necessary in CF patients. Bakare et al. [18] demonstrated that the amount of fungi isolated using antibiotic-containing culture media was much lower than that detected using fungal staining methods such as calcofluor white. However, in this study, the fungal culture media used were incubated and inspected only on 3 consecutive days [18], while prolonged incubation up to 4 weeks is more effective. With the ECA medium used in the study presented here, growth of *E. dermatitidis* and other fungi (table 2, fig. 2, 3) needed 5 to nearly 20 days for detection.

E. dermatitidis can survive for many months in the environment, preferentially under a relative humidity between 49 and 70% [20]. It is known to occur in the direct vicinity of humans, e.g. in bathrooms [20] or in steam baths [21]. In contrast to *Penicillium* or *Aspergillus* spp., *E. dermatitidis* occurs nearly exclusively in a humid environment [20]. It is not a common airborne fungus. Therefore, airborne contamination of the ECA medium can be excluded. In the literature data compiled in table 4, the isolation rates of *E. dermatitidis* from sputum samples of CF patients ranged between 1.8 and 15.7%. Glucocorticoid treatment, antibiotic therapy and malnutrition are established risk factors for the colonisation of the respiratory tract by *E. dermatitidis* in CF patients [5]. Genetic factors may also play a role, as proposed for the colonisation by *P. aeruginosa* [22]. This would be in line with the observation that *E. dermatitidis* is a well-known causative agent of cerebral infections, preferentially in Asians [23], despite its environmental distribution being worldwide.

The opportunist *P. boydii* was encountered 3 times in our study, in all cases without associated clinical symptoms of disease. However, colonisation by this therapy-refractory species may imply a health risk for CF patients [24].

Surveillance of CF patients, from whom opportunistic pathogenic fungi have been isolated, will be necessary in order to start adequate therapy in case of respiratory

infection as early as possible. In contrast, the situation is clearly different when the mainly saprophytic or plant pathogenic fungi *Aureobasidium*, *Cladosporium* and *Microascus* are isolated. Their pathogenicity is very low or questionable and human infections are mostly due to traumatic inoculation. In our routine practice, those isolates are kept frozen for up to about 6 months and discarded when no signs of infection can be recognised during this time and isolation cannot be repeated culturing another further specimen.

In case of the development of pulmonary fungal infections, the recognition of the occurrence of fungi, e.g. *E. dermatitidis*, by repeated isolation may predict infection by those fungi. During the last few years, bacterial detection in sputum samples from CF patients became increasingly cost-effective. Lots of selective isolation media are commercially available for the detection of 'new' pathogenic bacteria, e.g. *Pseudomonas* spp., *B. cepacia*, methicillin-resistant *Staphylococcus aureus* (MRSA). In contrast, less attention has been paid to the detection of fungi. Today, nearly exclusively fast-growing fungi like *Candida, Aspergillus* and *Penicillium* spp. will be detected with standard protocols [18].

Although lots of other human opportunistic pathogenic fungi have been described during the last decade [25, 26], their role in CF patients is controversial. More data are necessary on the occurrence and the pathogenicity of those 'uncommon new pathogenic fungi' in CF patients, and therefore, specialised selective isolation methods should be used and incubation of the media should be prolonged for up to 4 weeks.

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