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Development of selective media for the isolation of yeasts and filamentous fungi from the sputum of adult patients with cystic fibrosis (CF)

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Abstract

Yeasts and filamentous fungi are beginning to emerge as significant microbial pathogens in patients with cystic fibrosis (CF), particularly in relation to allergic-type responses, as seen in patients with allergic bronchopulmonary aspergillosis (ABPA), *Aspergillus* bronchitis and in invasive fungal disease in lung transplant patients. Four fungal media were compared in this study, including Sabouraud Dextrose Agar (SDA) and Medium B, with and without the addition of selective antibiotics, where antibiotic-supplemented media were designated with $^{+}$. These media were compared for their ability to suppress contaminating, mainly Gram−ve pathogens, in CF sputa (*Pseudomonas aeruginosa*, *Burkholderia cepacia* complex [BCC] organisms) and to enhance the growth of fungi present in CF sputum. Medium B consisted of glucose (16.7 g/l), agar (20 g/l), yeast extract (30 g/l) and peptone (6.8 g/l) at pH 6.3 and both SDA^{+} and Medium B^{+} were supplemented with cotrimethoxazole, 128 mg/l; chloramphenicol, 50 mg/l; ceftazidime, 32 mg/l; colistin, 24 mg/l). Employment of SDA^{+} or Medium B^{+} allowed an increase in specificity in the detection of yeasts and moulds, by 42.8% and 39.3%, respectively, over SDA when used solely. SDA^{+} had a greater ability than Medium B^{+} to suppress bacterial growth from predominantly Gram−ve co-colonisers. This is a significant benefit when attempting to detect and isolate fungi from the sputum of CF patients, as it largely suppressed any bacterial growth, with the exception of the BCC organisms, thus allowing for an increased opportunity to detect target fungal organisms in sputum and represented a significant improvement over the commercial medium (SDA), which is currently used. Overall, both novel selective media were superior in their ability to suppress bacteria in comparison with the commercially available SDA medium, which is routinely employed in most clinical microbiology diagnostic laboratories presently. Alternatively, Medium B^{+} had a great ability to grow fungi than SDA^{+} and when employed together, the specificity of combined use was 82%, with a sensitivity for yeasts, filamentous fungi, and combined overall fungi of 96.0%, 92.3% and 96.0%, respectively. Overall, when employing one fungal selective medium for the routine detection of yeasts and filamentous fungi in the sputum of CF patients, we would recommend employment of Medium B^{+}. However, we would recommend the combined employment of SDA^{+} and Medium B^{+}, in order to synergistically isolate and detect the greatest number of fungi present in CF sputa.

Keywords: Fungi; Yeasts; *Aspergillus*; *Exophilia*; *Scedosporium*; ABPA; Lung transplantation; *Aspergillus* bronchitis; Medium; Culture

1. Introduction

Patients with CF may suffer increased morbidity and mortality from colonisation, allergic reaction (allergic bronchopulmonary aspergillosis; ABPA) and invasive infection, from yeasts and filamentous fungi. The presence of fungi in the

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airways is particularly important in the context of CF lung transplantation. The frequent recovery of *Aspergillus* spp. from respiratory tract secretions is well recognised. However, there have been very few reports of other fungi being of clinical significance in CF patients and the isolation rates of fungi from CF patients vary widely in the reported literature. One possible reason for the under-reporting of such mycological agents in CF may be due to inadequacies in the microbiological detection of fungi in sputum from CF patients, which is relatively insensitive and requires significant improvement, due to difficulties in cultural separation of the yeasts and filamentous fungi from the patients' sputum from a mainly Gram−ve bacterial flora, approximating to 10^8–10^9 colony forming units of bacteria (cfu)/ml sputum [1].

Laboratory culture of sputum continues to be the diagnostic cornerstone in the detection of medically important fungi, colonising/infecting patients with CF. However, microbiological culture specific for fungi is not performed as a routine examination in most clinical laboratories. However, it is estimated that many fungi may be missed using this conventional culture method, due to (i), the inhibition of fungal growth by bacteria, particularly *Pseudomonas aeruginosa* and *Burkholderia cepacia* and (ii) overgrowth by rapidly growing bacterial organisms.

Therefore, the aim of this study was to define a mycological culture medium that could be employed in routine microbiological laboratories supporting CF units, specifically for the examination of CF sputum with a high burden of bacterial organisms, in order to promote selectivity and sensitivity in the recovery of yeasts and filamentous fungi from sputum of adult patients with CF, whilst inhibiting the bacterial co-flora in the sputum of these patients.

2. Materials and methods

2.1. Basal medium

Two novel fungal basal media, i.e. "Medium B" and "Medium C" were formulated and compared against a commonly employed and commercially available fungal medium, i.e. Sabouraud Dextrose Agar (SDA), for the ability to proliferate yeasts and filamentous fungi. Medium B was formulated based on calculating mean constituent components from 28 commercially described fungal culture media (http://www.oxoid.com) and contained glucose (16.7 g/l), agar (20 g/l), yeast extract (30 g/l) and peptone (6.8 g/l) at pH 6.3. Medium C was formulated by employing glucose (20 g/l), agar (20 g/l) and pooled adult CF sputum (100 g/l). The mean pH of freshly expectorated sputum from adult CF patients was determined to be 7.3 and the pH of Medium C was corrected to this value. SDA (Oxoid CM0041, Oxoid Ltd., Basingstoke, UK) was also included in this study and consisted of mycological peptone (10.0 g/l), glucose (40.0 g/l), agar (15.0 g/l) with a pH 5.6±0.2. The specificity of SDA, Medium B and Medium C was examined semi-quantitatively with eight fungi, selected from an archive of highly characterised fungal taxa previously isolated from CF sputum, including: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Mucor* sp. and *Penicillium* sp. SDA, Medium B and Medium C were streaked individually with 20 μl inocula of each of the above fungi and incubated aerobically at 37 °C for 1 week, prior to examination of the resulting plates. Growth was scored in a semi-quantitative basis, using the range no growth, + (few), ++ and +++ (confluent) growth (see Table 1).

2.2. Addition of selective antibiotic agents to suppress bacterial co-flora

Six antibiotic agents were included in the basal fungal media (SDA, Medium B and Medium C) to inhibit the growth of bacterial co-flora in the sputum, in order to avoid interference and potential inhibition of fungal growth by bacteria and overgrowth by rapidly growing bacterial organisms. All antibiotic agents were obtained from Sigma Corp. (St. Louis, USA) and included vancomycin, gentamicin, cotrimoxazole, chloramphenicol, ceftazidime and colistin. Antibiotic susceptibility of 13 species wildtype bacteria, which had been isolated previously from CF sputum, including. *P. aeruginosa* ($n=5$), *Pseudomonas fluorescens* ($n=1$), *B. cenocepacia* ($n=21$), *Aeromonas salmonicida* ($n=1$), *Alcaligenes* sp.($n=1$), *Acinetobacter baumannii* ($n=1$), *Klebsiella oxytoca* ($n=1$), *Morganella morganii* ($n=1$), methicillin-resistant *Staphylococcus aureus* (MRSA) ($n=1$), *Stenotrophomonas maltophilia* ($n=1$), *Proteus mirabilis* ($n=1$) and *Methylobacterium mesophilicum* ($n=1$), were examined with reference to their resistances against the six selected agents combined in a matrix of 10 combinations of the six agents. Eight of these combinations employed a combination of three antibiotic agents, with the remaining two combinations, having the inclusion of six and four agents, respectively, in order to determine the ability of the fungal basal media+antibiotic combination, to suppress the growth of the bacterial isolates examined. All 36 isolates were prepared by culture of pure inoculum onto Columbia Blood Agar (Oxoid CM0331) supplemented with 5% (v/v) defribinated horse blood and incubated at 37 °C for 24 h. Following this, serial dilutions of a stock bacterial suspension (approximately 10^8 cfu/ml in phosphate buffered saline (PBS)) were prepared and each bacterial isolates was streaked onto Medium+antibiotic combination, which was incubated at 22 °C for 72 h and the

Table 1
Comparison of growth of eight fungal species on unsupplemented SDA, Medium B and Medium C

Fungi	SDA	Medium B	Medium C
Aspergillus niger	+++	+++	+
Penicillium sp.	++	+++	++
Mucor sp.	−	+	+
Aspergillus fumigatus	+++	+++	+++
Aspergillus flavus	++	++	++
Candida albicans	+	+++	+
Candida dubliniensis	+	+++	+
Candida parapsilosis	+	+++	++

+++, confluent growth; ++, semi-confluent growth; +, few; −, no growth.