



Isolation of *Alternaria alternata* from an emollient cream: implications for public health

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Received 5 April 2002; accepted in final form 10 December 2002

Abstract

A specimen of emollient cream, which was observed to be contaminated peripherally with a filamentous fungus was examined for the presence of fungi and the resulting fungal colonies were examined phenotypically and genotypically. Subsequent DNA extraction and PCR amplification of the large internal transcribed spacer region [ITS1-5.8S-ITS2] yielded an amplicon of 512 bp. Sequence analysis identified this as *Alternaria alternata* at the 100% homology level with all 512/512 bases called. This organism has been previously reported as a cause of opportunistic infections involving skin and immunocompromised patients. This is the first report of an emollient cream as a source of this organism. It highlights the need for proper management of such preparations in order to minimize the potential spread of fungi to susceptible patient populations.

Key words: *Alternaria alternata*, emollient cream, ITS, PCR, rRNA

Introduction

To date, there has been very limited data on the mycobiota found in emollient creams employed as base preparations in a wide variety of dermatological applications. Although these emollient products are prepared under stringent aseptic packing conditions at pharmaceutical manufacture, they generally have several risk factors for fungal colonisation associated with their subsequent handling by the patient, which may predispose such preparations to fungal colonisation and overgrowth, including: (i) an extended shelf-life of several years, (ii) storage at ambient temperature, (iii) packaged in wide-mouth containers, (iv) intermittent use of a product by patients over a prolonged time period and (v) lack of effective antifungal preservatives.

It was therefore the aim of this study to identify a fungal contaminant in an emollient cream by conventional and molecular based approaches. An additional objective was to review the current literature on *Al-*

ternaria spp. associated skin infections and propose simple control measures to minimize potential overgrowth of *Alternaria* spp. in emollient preparations, thus minimising the risk of potential acquisition by susceptible patients.

Materials and methods

Source and microbiological examination of emollient cream

An emollient cream (500 g) dispenser was examined microbiologically for the presence of bacterial and fungal contaminants. Cream (1 g) was aseptically removed from the core of the reservoir and serially diluted 10-fold in 0.1% peptone saline (Oxoid Ltd, Dorset, England) diluent. The dilution was plated onto (a). plate count agar (PCA) (CM325, Oxoid Ltd) for the enumeration and identification of endogenous bacteria. The medium was incubated at 30 °C for 48h be-

fore enumeration. (b). Sabouraud dextrose agar (SAB) (CM41, Oxoid Ltd., England), which was incubated at 22 °C for 5 days, prior to fungal enumeration.

In addition, filamentous fungi from an area of gross fungal overgrowth situated at the interface of the cream and wall of the plastic dispenser unit were subcultured onto SAB agar (as above).

Phenotypic identification

Phenotypic identification was made by subculturing the isolate onto malt extract agar plates (Oxoid CM0059, Oxoid Ltd., England) at 26 °C and 37 °C for three to five days. Both macroscopic and microscopic examination of the isolate was made, where the latter assay was performed using lactophenol cotton blue mount and by slide block culture, as previously described [1]. Identification was made in accordance with the phenotypic description [2].

PCR identification of fungi

DNA extraction

All DNA isolation procedures were carried out in a Class II Biological Safety Cabinet in a room physically separate from that used to set up reaction mixes and also from the "post-PCR" room in order to minimise the production of false positive results. In all methods tested and where applicable, molecular grade water was employed (Biowhittaker Inc, Maryland, USA, LAL Grade cat no: W50-100) to reduce contamination. Fungal culture material was extracted as per the recommended instructions issued with the Roche High Purity PCR Template Kit (Roche Diagnostics Ltd., England). The fungal extraction procedure was as per the commercial kit above except for the following modification, i.e. after washing with Tris-HCl, the sample was incubated with Tris-EDTA (0.98 ml; 10 mM Tris HCl pH 8.0 containing 1mM EDTA) and lyticase (20 µl, 0.5 mg/ml, Roche, UK) for 1 h at 30 °C, prior to heating at 100 °C, after which the sample was freeze/thawed twice and subsequently centrifuged at 13,000 g for 15 min. Extracted DNA was transferred to a clean tube and stored at -20 °C prior to PCR.

PCR amplification

All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and the amplification and post-PCR room in order to minimize contamination. Reaction mixes (50 µl) were set up as follows: 10 mM Tris-HCl, pH 8.3, 50 mM KCl,

2.5mM MgCl₂, 200 µM (each) dATP, dCTP, dGTP and dTTP; 1.25U of *Taq* DNA polymerase (Amplitaq; Perkin Elmer), 0.2 µM (each) of the appropriate primers targeting both the smaller 5.8S-ITS1-28S rRNA internal transcribed spacer region (ITS3/ITS4), ITS3 (forward) 5'-GCA TCG ATG AAG AAC GCA GC-3' and ITS4 (reverse) 5'-TCC TCC GCT TAT TGA TAT GC 3', as well as the larger 18S-ITS1-5.8S-ITS2-28S (ITS1/ITS4), ITS1 (forward) 5' TCC GTA GGT GAA CCT GCG G-3' and ITS4 (reverse) 5'-TCC TCC GCT TAT TGA TAT GC 3', as previously described [3], and 4 µl of DNA template. The reaction mixtures following a "hot start" were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96 °C for 3 min followed by 40 cycles of 96 °C for 1 min, 55 °C for 1min, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. During each run molecular grade water was included randomly as a negative control and appropriate DNA templates from *Candida albicans* were included as a positive control as appropriate.

Detection of amplicons

Following amplification, aliquots (15 µl) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, UK) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3), stained with ethidium bromide (5 µg/100 ml). Gels were visualised under UV illumination using a gel image analysis system (UVP Products, England) and all images archived as digital graphic bitmap files (*.bmp).

Sequencing of amplicons and analysis of sequence data

All amplicons were purified using a QIAquick PCR purification kit (Qiagen Ltd., UK) eluted in Tris-HCl (10 mM, pH 8.5) prior to sequencing, particularly to remove dNTPS, polymerases, salts and primers. For the short ITS2 region, the sequencing primer ITS3 was employed in the forward direction and for the large ITS1-5.8S-ITS2 region, the ITS1 primer was employed in the forward direction. In both cases, the sequencing primer was labelled with Cy-5' and was used in conjunction with the ALF Express II (Amersham-Pharmacia Ltd., Buckinghamshire, England) employing the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, UK; catalogue

Table 1. A review of *Alternaria alternata* skin-associated infections

Country	Year	Clinical description	Reference
Canada	2001	Onychomycosis of the toe caused by <i>Alternaria alternata</i> . Treated unsuccessfully with itraconazole with neither a mycological nor clinical cure recorded	[9]
Italy	2001	Cutaneous phaeohyphomycosis in a 95 year old male manifested by verrucoid lesions of the hand and wrist caused by <i>Alternaria alternata</i> . The patient was concurrently on methylprednisolone for eczema. Treatment of <i>Alternaria alternata</i> infection was with itraconazole (100 mg daily for 1 month) followed by 200 mg daily for a further month, followed by 100 mg daily for a further 60 days, with complete mycological and clinical recovery.	[10]
Italy	1985–1999	Eight cases of cutaneous phaeohyphomycosis caused by <i>Alternaria alternata</i> manifested by dystrophy and distal subungual hyperkeratosis of one or two nails of the feet or hands. Successful treatment in six cases with itraconazole.	[11]
UK	2001	A 55 year old male cardiac transplant patient with cutaneous nodules on limbs caused by <i>Alternaria alternata</i> . Unsuccessful oral fluconazole (200 mg daily/3 weeks) treatment. Successfully treated with curettage, cautery and double-freeze/thaw cryotherapy.	[12]
Greece	1998	A 78 year old farmer with a history of idiopathic pulmonary fibrosis and skin lesions caused by <i>Alternaria alternata</i> . Successfully treated with itraconazole (200 mg daily) for 3 months.	[13]
UK	1998	An immunocompromised patient with unusual leg ulceration caused by <i>Alternaria Alternata</i>	[14]
Italy	1997	Ungual phaeohyphomycosis due to <i>Alternaria alternata</i> treated with itraconazole	[15]
Italy	1997	Two cases cutaneous phaeohyphomycosis due to <i>Alternaria alternata</i> and <i>A. tenuissima</i> in immunocompromised patients treated by surgical excision of nodule and itraconazole therapy	[16]
Spain	1996	A 47 year old male with CREST syndrome (calcinosis, Raynaud's phenomenon, oesophageal dysfunction, sclerodactyly and telangiectasia) being treated with prednisolone developed cutaneous phaeohyphomycosis spreading from his knees to his ankles was successfully treated for <i>Alternaria alternata</i> with high dose oral itraconazole	[17]
USA	1994	A 6 year old male with aplastic anaemia presented with skin lesions caused by <i>Alternaria alternata</i> which was successfully treated with antifungal therapy	[18]
Germany	1994	A 5 year old female with agranulocytosis of unknown origin developed cutaneous nodules containing <i>Alternaria alternata</i> and <i>Aspergillus terreus</i> .	[19]
New Zealand	1993	An 81 year old male with nephrotic syndrome for which he was receiving prednisone developed a spreading eczematous-appearing rash on the dorsum of his right hand and forearm, originating from three small post-traumatic ulcers over his knuckles. The phaeohyphomycosis cleared with 100 mg itraconazole daily accompanied by a reduction in his prednisone dosage.	[20]
Spain	1990	A 60 year old male receiving therapy for Non-Hodgkin's lymphoma developed multiple papuloerythematous cutaneous lesions from which <i>Alternaria alternata</i> was cultured and was successfully treated with amphotericin B	[21]
Spain	1988	Cutaneous phaeohyphomycosis in a patient with nephrotic syndrome being treated with corticosteroids. Good results were noted for the treatment of the disease with ketoconazole and topical ciclopirox.	[22]
USA	1976	A 69 year old farmer with acute lymphocytic lymphoma developed multiple ulcerated haemorrhagic cutaneous lesions	[23]

no: RPN 2438) (96 °C 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 5 s, followed by a 4 °C hold). The resulting sequences obtained were compared with those stored in the GenBank Data system using BLAST alignment software (<http://www.blast.genome.ad.jp/>). and sequence homology identity determined.

Results and discussion

A filamentous fungus observed overgrowing the edge of the cream was phenotypically identified as *A. alternata*, which was confirmed genotypically by PCR and direct sequencing. Subsequent DNA extraction and PCR amplification yielded an amplicon of 512bp with the large ITS region, i.e. ITS1-5.8S-ITS2. Sequence analysis identified this to be *A. alternata*, and this sequence has been deposited in GenBank with Accession Number AF492461. Neither bacteria nor fungi were detected (<100 colony forming units/g) throughout the cream from the dispensing unit.

Alternaria alternata is a common plant pathogen, which causes a variety of diseases of fruits and plants, including black spot [4] and late blight fungal disease [5]. Taxonomically, the genus, *Alternaria*, belongs to the mitosporic family *Pleosporaceae* where 42 species, including *A. alternata*, are currently described. Although a relatively uncommon fungal pathogen in human clinical infections, *A. alternata* is the most common species to cause a variety of infections, including sinusitis [6, 7] and hypersensitivity pneumonitis [8], in addition to its more frequent occurrence in skin and nail infections (see Table 1). From the reports outlined in Table 1, a major risk factor for the acquisition of *A. alternata* associated cutaneous infections has been prior and continuous treatment with immunosuppressive therapy, particularly corticosteroids. In addition, the main portal of entry to the skin of this organism appears to be through traumatic inoculation of soil contaminated material [10]. Emollient creams are useful in a wide variety of dermatoses and in conditions such as eczema, where they are the principal form of treatment. Some studies suggest a partial deficiency in T-cell immunity in patients with eczema would render these patients particularly susceptible to fungal infections [24].

Our data suggest that the emollient cream was not contaminated throughout the container with *A. alternata*, but that contamination was limited to a specific region, probably due to sporadic inoculation from

environmental spores when left exposed. Such contamination may thus increase the risk of exposure of susceptible patients, as detailed in Table 1, which should be avoided. Therefore, several simple controls may be put in place to help minimize this risk of acquisition, including: (i) improved education of immunosuppressed patients to the potential risks of fungal infections, (ii) avoidance of large volume dispenser units (500 g), in preference to small sealed tubes (15 g), (iii) improved employment of antifungal preservative in pharmaceutical packaging material and emollient creams, to prevent spore outgrowth from sporadic environmental contamination.

In conclusion, this is the first report of the isolation of *A. alternata* from an emollient cream and highlights the need for proper management of such preparations once opened in order to minimize the potential spread of this fungus to susceptible patient populations.

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