



Aspergillus fijiensis n. sp. isolated from bronchial washings in a human case of bronchiectasis with invasive aspergillosis: the first report

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Abstract

Aspergillus species are frequently involved in human broncho-pulmonary diseases, especially in immunocompromised patients. Although *A. fumigatus* is the predominant species causing these diseases, *A. niger*, *A. flavus*, *A. nidulans*, *A. oryzae*, and *A. terreus* have occasionally been responsible. We report a case of invasive aspergillosis in a middle-aged male with a productive cough of 6 months' duration. In the bronchial washings, branched filaments compatible with septate, fungal mycelia were detected, in the absence of other pathogens. The pure culture on Sabouraud agar resembled that of *A. japonicus*, with a dark brown-black concentric rings on a whitish surface, yellow pigmentation on the reverse. The slide culture showed septate mycelia bearing globose vesicles, uniseriate, oval sterigmata and spherical echinulate spores. Multilocus sequence analysis of *benA*, *CaM* partial genes allowed us to identify it as *A. fijiensis*. This species was recently isolated from the soil of Fiji islands, identified as a new uniseriate species within *Aspergillus* Sect. *Nigri*. This report is the first on *A. fijiensis* in a pathogenic role.

Keywords: *Aspergillus fijiensis*, n. sp., molecular biological characterization, aspergilloma with bronchiectasis, first report itraconazole

Introduction

The black aspergilli (*Aspergillus* section *Nigri*) are an important group of species in food mycology, medical mycology and biotechnology. Many species cause food spoilage, but are also used in the fermentation industry. More recently they were shown to pose hazards to human and animal health as producers of ochratoxin A, and fumonisins, two important mycotoxins [1,2]. In addition, black aspergilli are receiving increasing attention as human fungal pathogens, although they occur in clinical samples less frequently than *A. fumigatus*, *A. terreus* or *A. flavus*. *Aspergillus* spp. which are widely documented as causative pathogens in invasive and non-invasive infections as well as in allergic reactions especially of Types III and IV. Richardson [3] stated that "Of the documented species of *Aspergillus*, *A. fumigatus* causes the large majority of cases of both invasive and non-invasive aspergillosis. Indeed, the allergic forms of the disease appear to be "almost exclusively caused by this organism". Over 95 % of all infections, are caused by three species: *Aspergillus fumigatus*, *A. flavus* and *A. niger*. However strains of black aspergilli are often misidentified as *A. niger* due to the difficulties of classifying and identifying of the species of this group [4].

Pulmonary *Aspergillus* infection is initiated by inhalation of airborne spores that are small enough (2–3 µm) to reach the alveoli, though inhalation of spores is not by itself sufficient

to cause disease. Host characteristics or predisposing factors are usually present to account for a heightened susceptibility to disease. *Aspergillus* infection can lead to multiple clinic-pathological syndromes, and progression from a less aggressive to a more aggressive form rarely occurs [5]. Host immunologic responses are central to the pathogenesis of allergic broncho-pulmonary aspergillosis (ABPA) and hypersensitivity pneumonia, whereas direct tissue injury by the fungi plays a more important role in invasive pneumonia. Immunocompromised patients such as those with congenital immunodeficiency syndromes of hyper-immunoglobulin (Ig) E syndrome, with chronic granulomatous disease, or with cystic fibrosis, may be at increased risk for ABPA [6]. This report describes a case of bronchiectasis, with invasive aspergillosis caused by a strain of black aspergilli which is the recently described species *A. fijiensis* [7], while it is the first report on its role as an opportunistic pathogen.

Materials and methods

Microbiology

Species identification was made because it is regarded as being therapeutically useful since strains may vary in drug-resistance (Richardson 1995).

Bronchial washings and brushings were studied on wet, unstained mounts by microscopy, and were cultured on Sabouraud agar plates and slide cultures incubated in air at

room temperature (ambient 28 - 30°C).

Molecular biology

Isolation and analysis of nucleic acids

The strain was deposited in the ITEM Collection (CNR-ISPA, Bari, Italy) with the number ITEM 15047. A suspension of spores from the fungal strain was grown in Wickerham medium, containing 40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and water up to 1L. The mycelium obtained was filtered and lyophilized for total DNA isolation. The fungal DNA was extracted with mechanical grinding using 5 mm iron bead in Mixer Mill MM 400 (Retsch), and "Wizard® Magnetic DNA Purification System for Food" kit (Promega), with some modifications, starting from 10 mg of lyophilized mycelium. The quality of genomic DNA was determined by electrophoresis and the quantification using a Spectrophotometer ND-1000 (Nano Drop). Beta-tubulin (*benA*, ca. 450 nt), calmodulin (*CaM*, ca. 650 nt), were amplified respectively using PCR conditions and primers described in literature: BT2a and BT2b primers of Glass and Donaldson [8], CL1 and CL2A primers of O'Donnell et al., [9]. After amplification, the products were purified with the enzymatic mixture EXO/SAP (Exonuclease I, *E. coli* / Shrimp Alkaline Phosphatase).

Bidirectional sequencing was performed for all loci and isolates. Sequence reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech) and analyzed on the "ABI PRISM 3730 Genetic Analyzer" (Applied Biosystems). The alignment of the two loci was performed using the software package BioNumerics 5.1 from Applied Maths.

Analysis of sequence data

Twenty-eight representative strains belonging to *Aspergillus* Sect. *Nigri*, together with *A. flavus* ITEM 7526^T as outgroup, were used to assess the species identity of the strain ITEM 15047. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [10]. Phylogenetic trees were prepared by the neighbor-joining method [11].

The evolutionary distances were computed using the Maximum Composite Likelihood method [12] and are in the units of the number of base substitutions per site. The analysis involved 30 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 781 positions in the final dataset. Maximum parsimony analysis was also performed for all data; branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The MP trees were obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The trees are drawn to scale, with branch lengths calculated using the average

pathway method and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). A tree possessing only branches that received MP bootstrap properties³ 70% was chosen to represent each of the two loci. This criterion prohibited poorly supported nonmonophyly at one locus from undermining well supported monophyly at another locus.

Results

Clinical features

Present history of the patient on admission

The patient, NGJ, was a male, 60 years-old, admitted to hospital in 2011, with a productive cough of 6 months' duration.

A clinical diagnosis of "Aspergilloma", was made. A chest X-ray in 2011, after the resection in 2007, showed no abnormality apart from bronchiectasis.

The presence of *diabetes mellitus* or a history of steroid use, have not been recorded.

Previous history

The patient had been treated 9 years previously in 2002 for 6 months, for pulmonary tuberculosis, followed by recovery. Haemoptysis occurred occasionally during the last 3 years. For the last 2 years, he had occasional fever, 'night sweating', and loss of weight.

In 2007, he was admitted to the General Hospital, Kandy, Sri Lanka with a non-productive cough of 6 months duration, haemoptysis and fever, shortness of breath, 'night sweats', loss of weight, and loss of appetite of 2 years duration. Bronchoscopy revealed - bleeding from the left upper-lobe bronchus, with bronchiectasis. Thoracotomy was done with a sub-total resection of right, superior lobe of the lung. The patient's written, informed consent was obtained for this publication.

Microbiological findings

Wet smear of the centrifuged deposit of bronchial washings. Branched filaments compatible with septate, fungal mycelia were seen (Figure 1).

Plate cultures on Sabouraud Agar, at room temperature for 7 days

Within one week, the *Aspergillus* with its characteristic features was fully grown. Zonation with concentric rings of black pigmentation on a white velvety colony was marked with the colony having sharply defined, thin edges. The entire colony was speckled with white spots (Figure 2). The agar culture had a yellow-pigmented reverse.

Slide cultures on Sabouraud Agar blocks stained with Lactophenol Cotton Blue showed non-septate conidiophores with conidia-bearing vesicles that were globose with uniseriate phialides (Figure 3) that terminated in globose, echinulate conidia (Figure 4,5). The hyphae in the mycelium were septate.

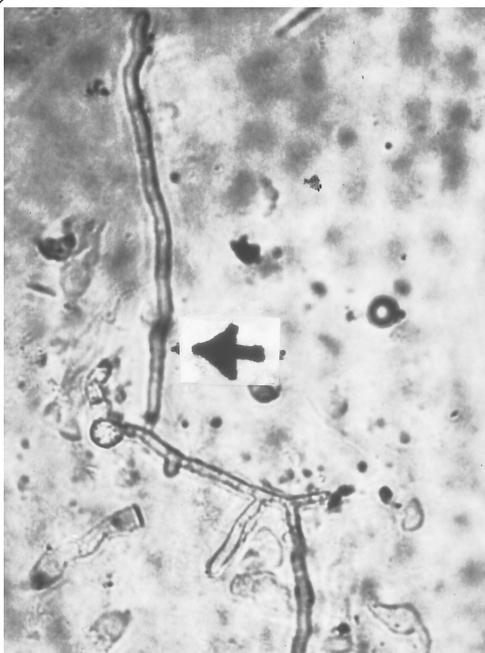


Figure 1. Septate mycelial fragment in bronchial washings. Unstained, wet preparation. Initial magnification x 1000.



Figure 2. Fungal isolate from bronchial washings, in pure culture on Sabouraud agar at 28 - 30°C after one week, showing black pigmentation in concentric zones on a white, velvety colony.

Pathological findings

The pathology report of the resected tissue with the cyst stated: "Lining epithelium composed of columnar ciliated cells with focal ulceration and focal squamous metaplasia. The submucosa shows intense infiltrate of lymphocytes and plasma cells. The blood vessels show endarteritis. The cyst

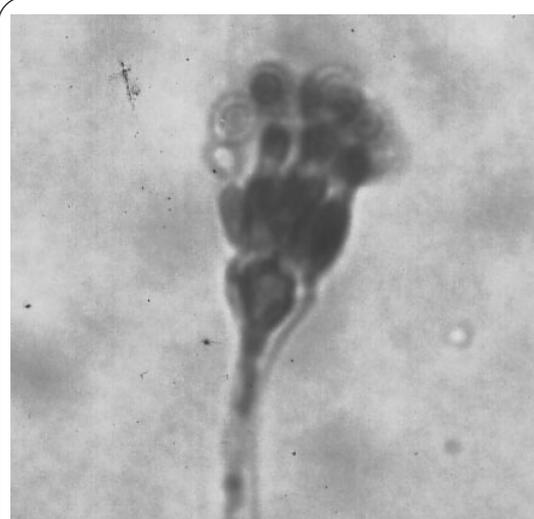


Figure 3. Fungal isolate on slide culture on Sabouraud Agar, 28 - 30°C after one week, stained with Lactophenol Cotton-Blue. Note globose vesicle with oval uniseriate phialides bearing spherical-oval conidia in chains. Initial magnification x 1000.

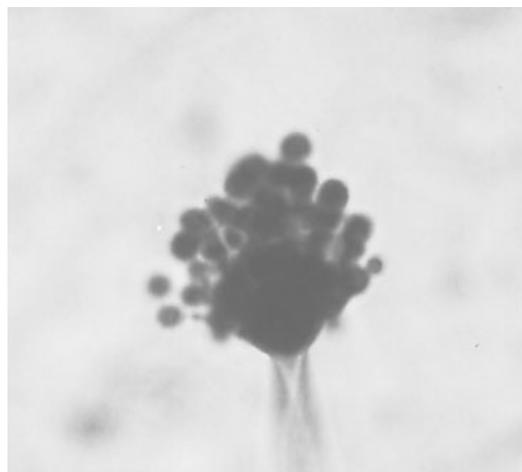


Figure 4. Fungal isolate on slide culture on Sabouraud Agar, 28 - 30°C after one week, stained with Lactophenol Cotton-Blue. Note globose vesicle with oval uniseriate phialides bearing spherical-oval conidia in chains. Initial magnification x 1000.

contains a mass of branching, filamentous fungi resembling *Aspergillus*. The surrounding tissue also shows similar cysts with bronchiectasis and fresh and old haemorrhages. Appearances are those of an aspergilloma within a cystically dilated bronchus with bronchiectasis".

Treatment

The patient responded to 3-months treatment with itraconazole 200 mg twice daily, which Richardson [3] found to be useful

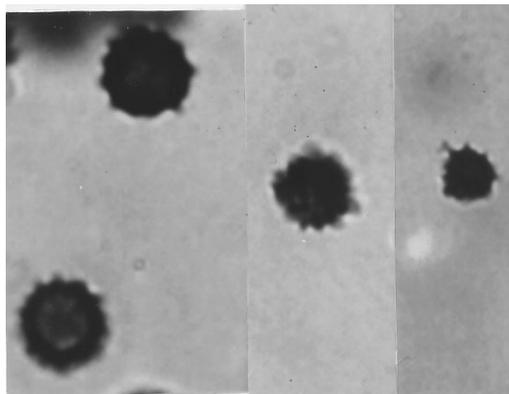


Figure 5. Conidia from the colony on Sabouraud agar showing echinulate surfaces. Initial magnification x 1000. Gram's stain.

in invasive, pulmonary aspergillosis.

Discussion

Molecular biological identification of the strain

The phylogenetic analysis was conducted firstly on the two single locus alignments and successively the combined alignment of the two loci was analyzed for inferring the organismal phylogeny. The neighbor-joining tree (**Figure 5**) obtained on the combined dataset of sequences has the optimal tree with the sum of branch length = 1.19543859. The percentage (> 70) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary history was also inferred using the Maximum Parsimony method. One of the 19 most parsimonious trees (length = 942) is used with the consistency index of (0,562137), the retention index is (0,842786), and the composite index is 0,505492 (0,473761) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Close-Neighbor-Interchange algorithm [14] with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). In both the analysis NJ and MP the strain ITEM 15047 clustered with a bootstrap value of 98% with the type strain of *A. fijiensis* CBS 119.49. These results indicated that the strain analyzed belongs to the *A. fijiensis* species and that this new recently described species could be found as a opportunistic human pathogen.

Clinical aspects

The patient's symptoms were typical of those described in invasive pulmonary aspergillosis. Although the clinical classification of pulmonary aspergillosis to include Chronic Necrotizing Pulmonary Aspergillosis, Chronic Invasive Pulmonary Aspergillosis, Subacute Invasive Pulmonary

Aspergillosis has been proposed, these entities amidst other states of aspergillosis have "not been rigorously defined, and an overlap in clinical and radiological features between these different entities probably exists" [3]. It is difficult to clearly assign our patient's pulmonary pathology to any of these states. It is possible that his productive cough was attributable to an exacerbation of his lung's bronchiectatic pathology that evidently had a history of 3 years, with haemoptysis of 2 year's duration, with an earlier history of pulmonary tuberculosis.

Aspergillosis has been frequently documented in patients with immunodeficiency but there was no data in this patient of a state that indicated immune-compromisation. However, the patient's age, 60 years, could reflect a waning immune-competence, though the white blood cells counts were regarded as normal. Extended tests of immune function, such as for humoral and cell-mediated immune competence were not done, while on the other hand, aspergillosis is known to supervene on already damaged lungs with a neutropaenia or immunosuppression [3], when the normally saprophytic *Aspergillus* becomes parasitic. Clinically, our patient had the selection criteria that Richardson [3] included in his chapter on Aspergillosis: fever, cough, haemoptysis, suggestive pulmonary radiology, most importantly the presence of mycelia in bronchial washings which produced a pure culture of the isolated described here. These findings were made possible by the examination of bronchial lavage specimens that Richardson [3] regarded "as often rewarding", as in our investigation of this case.

The colonisation by this strain of *Aspergillus* was probably a result of previous damage to the lung parenchyma following the bronchiectasis. Evidence for a pathogenic role of our isolate was (1) the appearance of mycelium on the direct smear from the bronchial washings, and (2) the isolation of the fungus in pure culture on fungal growth medium, and (3) the similarities (septate mycelium) of the organism on the direct smear and the organism in pure culture (septate mycelium).

Radiologically, three patterns of chronic pulmonary aspergillosis have been described [3]: "The first is characterized by the formation and expansion of multiple cavities, some containing fungus balls, which has been termed chronic cavity pulmonary aspergillosis (CCPA)", a condition analogous to that of our case. Another condition Chronic Necrotising Pulmonary Aspergillosis (CNPA) occurs in "...middle aged or older men with chronic or previously treated lung disease, such as tuberculosis,"; it is of interest that our patient was also an elderly man with a history of previous tuberculosis. An aspergilloma, which our patient had, is considered to be "the most familiar of the localized infections produced by *Aspergillus* spp" (Richardson 1995); the localization in the upper lobe of the lung in this patient was also typical.

Our patient's response to itraconazole parallel Richardson's comment [3] that: "More recent studies reaffirm the value of itraconazole in the treatment of invasive aspergillosis, especially where an early diagnosis is achieved".

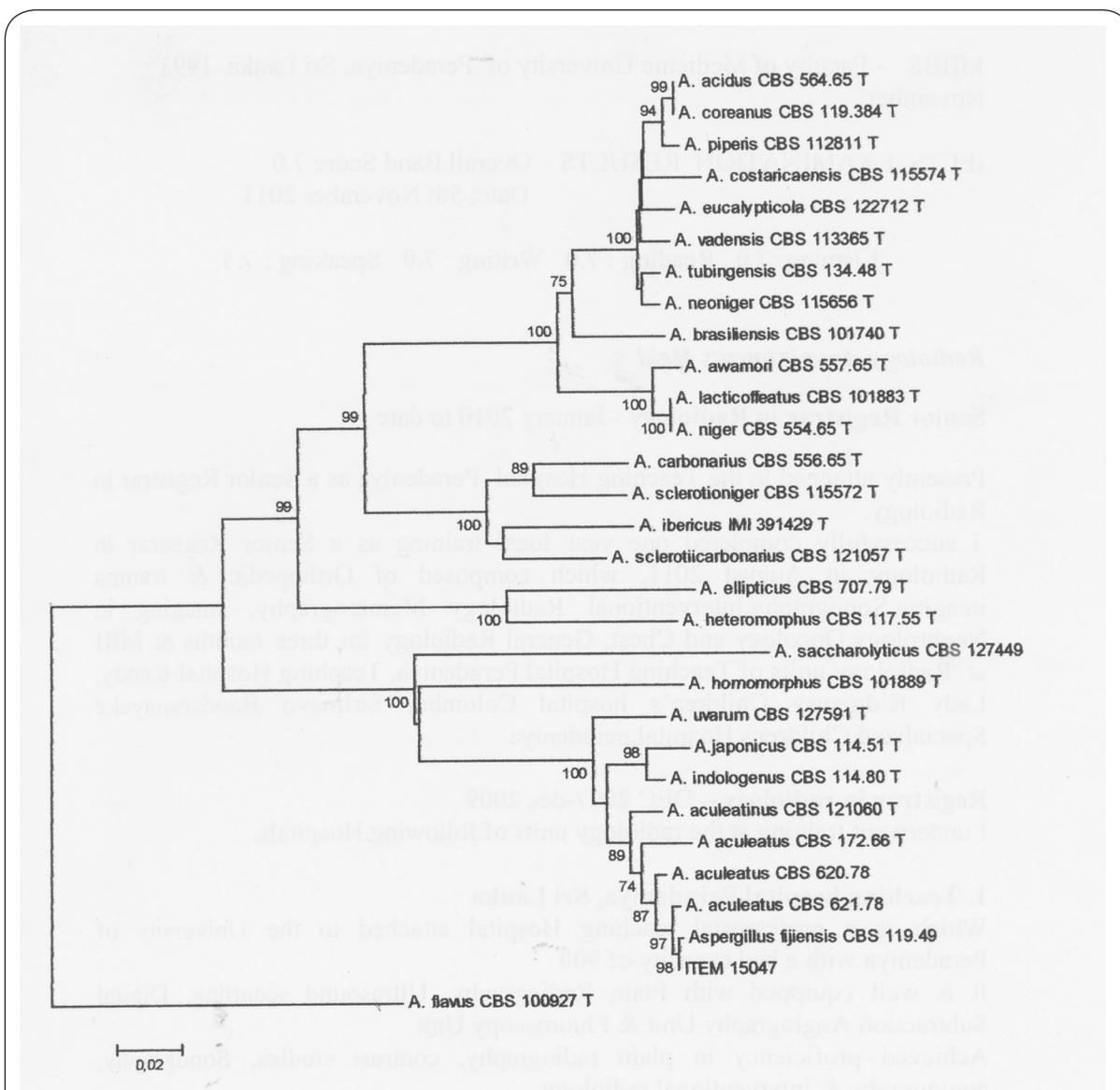


Figure 6. Phylogenetic tree produced from the combined sequence data of the two loci (CaM, benA) of 29 strains belonging to *Aspergillus* Sect. Nigri group. Numbers above branches are bootstrap values. Only values above 70% are indicated. The evolutionary history was inferred using the Neighbor-Joining method.

The literature does not include evidence of a pathogenic role of *Aspergillus fijiensis*, but documents that it is a uniseriate species (as found in our isolate), isolated from soil in Fiji and from *Lactuca sativa* in Indonesia, related to *Aspergillus aculeatinus* that we previously reported (Perrone et al., [15], Perrone et al., [16]) as the pathogen in human dacryocystitis. Varga et al., [7] described the colony of *A. fijiensis* as having a yellow-coloured reverse, and uniseriate conidiophores with globose vesicles and echinulate conidia, as in our isolate. In addition molecular analysis by sequencing the betatubulin and calmodulin partial genes strongly supported the assignment of this strain to the newly described species *A. fijiensis* (Figure 6), further supported by the findings of Varga et al., [7] in relation

to these two loci.

In conclusion, this report provides the first evidence of a human opportunistically-pathogenic role of the newly described species, *Aspergillus fijiensis*, based on direct microscopy of bronchial lavage specimens, the isolation in pure culture of an *Aspergillus* with identical morphological similarities to those seen on direct microscopy, the microscopic features that correspond to those of *Aspergillus fijiensis*, while the clinical characteristics of the patient compatible with the opportunistic nature of this *Aspergillus*.

Competing interests

The authors declare that they have no competing interests.

Publication history

Editor: Arianna Tavanti, Affiliation: University of Pisa, Italy.
Received: 10-Jul-2013 Revised: 29-Jul-2013
Accepted: 09-Aug-2013 Published: 16-Aug-2013

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Citation:

Perrone G, Stea G, Kulathunga CN, Wijedasa H and Arseculeratne SN. ***Aspergillus fijiensis* n. sp. isolated from bronchial washings in a human case of bronchiectasis with invasive aspergillosis: the first report.** *Microbiol Discov.* 2013; **1**:9.
<http://dx.doi.org/10.7243/2052-6180-1-9>