

## Case Reports

**Phaeohyphomycosis due to *Alternaria infectoria*: a single-center experience with utility of PCR for diagnosis and species identification**

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The term phaeohyphomycosis refers to a rare group of fungal infections characterized by the presence of dark-walled hyphae or yeast-like cells in affected tissues. Herein, we report on the clinical and epidemiological characteristics of six cases of phaeohyphomycosis due to *Alternaria* spp. that occurred in our hospital over a 30-month period (from January 2008 to June 2010). Interestingly, whereas histopathological examinations were positive and fungal cultures yielded molds in all cases, mycological identification using conventional phenotypic methods was never possible despite prolonged incubation of the isolates. Identification of *Alternaria infectoria* species complex was obtained for each isolate by amplification and sequencing of the internal transcribed spacer of the ribosomal DNA (ITS rDNA). All patients had favourable outcomes following the introduction of azole-based antifungal therapy. This case series describes the clinical course of these six patients and highlights the utility of molecular identification to help in the identification of the etiologic agent when classical mycological methods have failed.

**Keywords** phaeohyphomycosis, *Alternaria infectoria* species complex, molecular identification, ITS rDNA sequencing, azole antifungals

**Introduction**

Incidence of invasive fungal infections has increased during the past two decades as a result of the ever-growing number of immunocompromised patients, such as solid-organ transplant recipients or patients with haematological

malignancies. Whereas invasive infections due to hyalohyphomycetes such as *Aspergillus* or *Fusarium* have been extensively studied, those caused by less common fungi such as those responsible for phaeohyphomycosis have been poorly investigated. Phaeohyphomycosis refers to the presence of dark-pigmented fungal elements found through histopathological examination of infected tissues. Melanized fungi are widely found in our environment, being frequently recovered as laboratory contaminants. However, they are increasingly reported as opportunistic pathogens in immunocompromised patients. At present, 130 species belonging to 70 genera including *Alternaria* have been involved in human infections [1]. *Alternaria* spp. are responsible for a wide spectrum of diseases, ranging

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from allergic disorders such as sinusitis or asthma to opportunistic, mostly focal superficial infections in immunocompromised hosts [2]. Almost 200 cases of human alternariosis have been reported in the literature from 1933 to the present [2,3]. Nevertheless, the real prevalence of phaeohyphomycosis due to *Alternaria* species is probably underestimated because of diagnostic issues, such as the polymorphic aspect of the lesions or lack of conidial production by the fungus makes mycological identification impossible.

We report our experience with six cases of culture and biopsy-proven phaeohyphomycosis due to *Alternaria infectoria* that occurred in our hospital between January 2008 and June 2010. We emphasize the difficulties encountered with mycological identification, as well as the utility of ITS rDNA sequencing for the identification of the causative agent.

## Case reports

Clinical and mycological characteristics of the six cases are reported in Table 1.

### Patient no. 1

A 73-year-old female patient was admitted in June 2008 to the infectious diseases department due to cutaneous nodules on her right leg. Her past medical history included high blood pressure, diabetes mellitus and hepatitis C infection. At physical examination, the nodules, located on the external and posterior sides of her right leg appeared indurate and violaceous, oozing, painless and non-pruritic (Fig. 1A). Biological investigations revealed hypogammaglobulinemia (6.7 G/l) and lymphopenia (1.1 G/l). Diabetes mellitus was well-balanced (hemoglobin A1c = 6.8%). A skin biopsy was performed for further investigations. Histopathological examination of Periodic Acid Schiff (PAS) and Gomori-Grocott stained material revealed numerous septate hyphae and fungal structures suggestive of dictyospores (spore divided into segments by both transverse and longitudinal walls; Fig. 1B). Fungal cultures on Sabouraud's dextrose agar were positive for a mold that remained sterile despite prolonged incubation for several weeks. The diagnosis of alternariosis due to *A. infectoria* was finally obtained by amplification and sequencing of the ITS rDNA region (isolate no. 17333882). Antifungal therapy with itraconazole (200 mg/day) was started at the end of June 2008. Two months later, the lesions had completely regressed, leading to withdrawal of itraconazole. One year later, five new budding lesions were noticed, suggesting a relapse of the disease. Again, histopathology, fungal cultures and ITS rDNA analysis confirmed the

identification of the etiologic agent as *A. infectoria* (isolate no. 70022013). Itraconazole was therefore reintroduced (400 mg/day as a loading dose followed by 200 mg/day). At the end of November 2009, after 6 months of antifungal therapy, cutaneous lesions had completely regressed, leading to discontinuation of itraconazole. No relapse was noted at the last follow-up in July 2011.

### Patient no. 2

A 54-year-old male patient was admitted in January 2008 to the dermatology department due to a single crusted and inflammatory cutaneous lesion located on his left calf. His past medical history included chronic renal insufficiency leading to kidney transplantation in June 2007. At the time of admission, his immunosuppressive regimen consisted of tacrolimus (1.5 mg/day) and mycophenolate mofetil (1440 mg/day). His creatinine clearance estimated by the MDRD formula was 48 ml/min. The patient had neither fever nor systemic symptoms. Skin biopsies of the cutaneous lesion were performed. Histopathological examination after hematoxylin-eosin safran (HES) staining revealed an epidermal hyperplasia and a dermal polymorphic inflammatory infiltrate consisting of microabscesses, multinuclear giant and epithelioid cells, as well as granulomatous foci. Examination of Gomori-Grocott and PAS stained material revealed the presence of septate hyphae. A mold was recovered in culture but did not form conidia despite prolonged incubation. The identification of *A. infectoria* was obtained by ITS rDNA sequencing (isolate no. 17320399). At this time, because of the spontaneous regression of the lesion, no antifungal treatment was started. Six months later, a new budding lesion was noted on his left knee, close to the former one, leading to the initiation of fluconazole therapy for 4 weeks (50 mg/day orally). Two months later, without any change of immunosuppressive therapy dosage, significant regression of the lesions was noted. The patient remained free of symptoms at his last follow-up more than three years later.

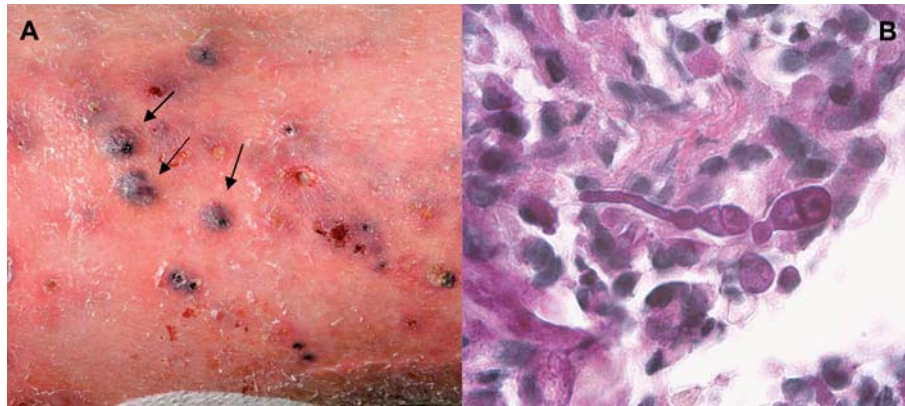
### Patient no. 3

A 75-year-old female patient was admitted in January 2009 in our hospital because of the recent discovery of a pulmonary mass with mediastinal adenopathies associated with hemoptysis and deterioration of her general condition. Serum galactomannan level was positive (index 13, cut-off value 0.5, Platelia *Aspergillus*, Biorad, Marnes la Coquette, France). Her past medical history included kidney transplantation for IgA glomerulonephritis five years before her present hospitalization, high blood pressure, venous thrombosis, and chronic obstructive pulmonary disease. At the

**Table 1** Epidemiological, clinical, histological and mycological features of the six cases of phaeohyphomycosis due to *Alternaria infectoria* species-group.

Patient no.	Age/sex	Profession/hobbies/area of living	Underlying conditions	Anatomical sites involved	Clinical features	Histopathology/direct examination	Fungal culture (time to positive culture)	Molecular identification	Antifungal therapy	Outcome
1	73 / F	Lives in the countryside	Diabetes mellitus	Right leg (multiple lesions)	Firm purplish and crusted nodules with a budding aspect at relapse	Positive (numerous fungal hyphae)	Sterile hyphae (two days)	<i>Alternaria infectoria</i> species group	ITR as initial therapy being reintroduced one year later after relapse of the disease	Favourable outcome
2	54 / M	Lives in the countryside	Kidney transplantation	Left calf (single lesion) followed by left knee (relapse)	Infiltrated papule (initial lesion), budding lesion (relapse)	Positive (numerous hyphae along with granuloma)	Sterile hyphae (three days)	<i>Alternaria infectoria</i> species group	No antifungal treatment at the initial diagnosis followed by FLC at relapse	Favourable outcome
3	75 / F	Retired farmer	Kidney transplantation	Right hand 4th and 5th fingers (multiple lesions)	Purplish cutaneous nodules	Positive (numerous fungal hyphae along with dermal inflammatory infiltrate)	Sterile hyphae (four days)	<i>Alternaria infectoria</i> species group	VRC	Favourable (no relapse). Death 1 year after (unrelated cause)
4	56 / M	Carpenter	Heart and lung transplantation	Right knee (single lesion)	NA	Positive (Fungal hyphae with necrotic areas and abscesses)	Sterile hyphae (three days)	<i>Alternaria infectoria</i> species group	Surgery in combination with VRC followed by POS	Favourable outcome (no relapse)
5	77 / M	NA	CMML, dysimmunity and long-term corticotherapy	Left hand and right leg	Budding lesions and nodules	Positive (numerous fungal hyphae along with granuloma and inflammation)	Sterile hyphae (two days)	<i>Alternaria infectoria</i> species group <sup>(1)</sup>	ITR <sup>(2)</sup>	Favourable. Death 6 months later (unrelated cause)
6	41 / F	Occasional gardening	No known underlying disease	Sinus and palate	Superinfected and crusted lesions, hypervascularity and palate ulcerations at the initial diagnosis. Relapse with palate osteolysis	Positive (numerous fungal hyphae with granuloma infiltrate and microabscesses)	Sterile hyphae (four days)	<i>Alternaria infectoria</i> species group <sup>(1)</sup>	ITR followed by POS after relapse	Favourable outcome (ongoing)

M, male; F, female; NA, not available; FLC, fluconazole; VRC, voriconazole; CAS, caspofungin; ITR, itraconazole; AMB, amphotericin B; POS, posaconazole; CMML, chronic myelomonocytic leukaemia. <sup>(1)</sup>Diagnostic of alternariosis due to *A. infectoria* species group was also performed directly on the specimen biopsy. <sup>(2)</sup>The patient was previously given liposomal amphotericin B for systemic cryptococcosis.



**Fig. 1** (A) Violaceous oozing and crusted nodules (arrows) on the right leg. (B) Fungal structure suggestive of a dictyospore after PAS staining (magnification  $\times 1000$ ). Note that dictyospores are rarely seen in clinical samples.

time of admission, her immunosuppressive regimen consisted of tacrolimus (3 mg/day orally) and prednisone (7.5 mg/day orally). The diagnosis of probable invasive aspergillosis was made and treatment with voriconazole was started (600 mg/day). Simultaneously, several violaceous cutaneous nodules which had been developing for two to three months and located on the 4th and 5th fingers of her right hand were investigated. Histopathological examination of a skin biopsy revealed a dermal perivascular polymorphic infiltrate with numerous septate hyphae and ovoid-shaped structures. Cultures inoculated with a portion of the biopsy yield a mold which remained sterile. Identification of *A. infectoria* was again only obtained by ITS rDNA sequencing (isolate no. 70012838). Antifungal therapy with voriconazole (600 mg/day) was continued for four additional months. No relapse was observed on her last follow-up in 2009. Unfortunately, the patient died of an unrelated cause one year later.

#### Patient no. 4

A 56-year-old male patient, whose medical history included idiopathic fibrosis and pulmonary hypertension, was admitted in June 2009 to the thoracic and cardiovascular surgery department for heart and lung transplantation. His immunosuppressive regimen consisted of cyclosporine (target blood level: 280–320 ng/ml), mycophenolate mofetil (2 g/day) and prednisolone (20 mg/day). During the post-operative course, he experienced acute pulmonary edema, bacterial pneumonia, and *Candida albicans* colonization treated with fluconazole (100 mg/day). Two months after transplantation, a diagnosis of *Abiotrophia defectiva* endocarditis led to mitral valve replacement. Despite antibiotic therapy, the patient's condition worsened, and mediastinitis was suspected. Three months after transplantation, a surgical drainage was performed. During surgery, a 5-mm diameter single lesion,

located on his right knee of several weeks duration was excised, and fluconazole was replaced with voriconazole. Histopathological examination revealed dermal necrosis and several abscessed areas with septate hyphae. In light of this invasive fungal infection occurring in a highly immunocompromised patient, caspofungin was added (70 mg as a loading dose followed by 50 mg/day). In the following days, the patient experienced hallucinations and confusion, and voriconazole was switched for posaconazole (600 mg/day). Fungal cultures of the skin biopsy were positive for a mold that remained sterile (isolate no. 70029785). *A. infectoria* was identified by ITS rDNA sequencing, leading to withdrawal of caspofungin and continuation of posaconazole for three additional weeks. Two years after the initial diagnosis, no relapse was noted.

#### Patient no. 5

A 77-year-old male patient was admitted in June 2010 to the infectious diseases department for the investigation of several budding and violaceous subcutaneous lesions located on his left hand and right leg. His past medical history included several autoimmune diseases including Gougerot-Sjögren syndrome for which he was receiving long-term corticotherapy (prednisone, 5 mg/day). Moreover, he had been given azacitidine since July 2008 for chronic myelomonocytic leukemia. The day after admission, the patient developed fever and a culture of a blood sample yielded *Cryptococcus neoformans*. Antifungal therapy with liposomal amphotericin B was started (3 mg/kg/day). Biopsies of the skin lesions revealed several septate fungal elements after histopathological examination. A mold was recovered in cultures inoculated with a biopsy sample but as in other cases, identification of the fungus could only be obtained by ITS rDNA sequencing (*A. infectoria*) (isolate no. 70052399). The diagnosis of *A. infectoria* infection

was also confirmed by a pan-fungal PCR performed directly on the biopsy specimen. Three weeks later, liposomal amphotericin B was replaced by itraconazole (400 mg/day orally), and the patient was discharged from hospital. Clinical evolution of both cryptococcosis and phaeohyphomycosis seemed to be favourable, with regression of the cutaneous lesions. Unfortunately, the patient died in November 2010 from *Rhodococcus equi* sepsis despite broad-spectrum antibiotherapy.

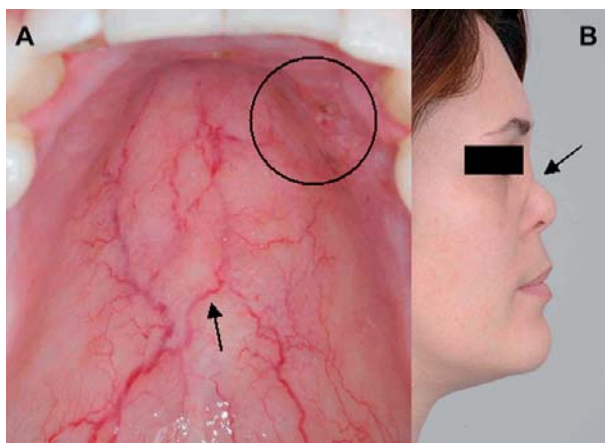
#### Patient no. 6

A 40-year-old female patient was admitted to the otorhinolaryngology department for chronic rhinitis which had been developing since 2004. In 2005, the chronic rhinitis deteriorated, with palatin impairment and saddle nose deformity (Fig. 2A). In 2006, small and painful palatin ulcerations developed in association with superinfected crusts and hypervascularity (Fig. 2B). Examination of a nasal biopsy performed at this time only showed a granulomatous and inflammatory reaction without necrosis. The patient had no systemic symptoms and was in good general status. A chronic inflammatory disease such as Wegener's granulomatosis was suspected, but without any biological evidence. In January 2008, because of palatin ulcerations evolving for 15 days, biopsies were performed. Histological examination of HES stained sections showed ulceration and acute inflammatory lesions with microabscesses and granulomas. PAS and Gomori-Grocott stained material revealed the presence of numerous septate hyphae. A mold grew on Sabouraud's agar plates but remained sterile, as described in previous cases. *Alternaria infectoria* was once again identified by ITS rDNA sequencing (isolate no. 17320171). The diagnosis of *A. infectoria* infection was also confirmed by a pan-fungal PCR performed directly on

the biopsy specimen. Interestingly, the patient was HIV-negative, had neither low CD4 cell count ( $942/\text{mm}^3$ ) nor hypogammaglobulinemia (including IgG subclasses) and no acquired or congenital immune dysfunction. Itraconazole (400 mg/day) was started in May 2008. Antifungal therapy was discontinued after one year, as the patient's conditions had gradually improved with a regression of symptoms and a palate reossification on tomodensitometry. Nevertheless, physical examination revealed that the nasal septum healed with persistent hypotrophic after effects. At the next clinical follow-up in October 2010, CT-scan revealed palate osteolysis, and positron emission tomography showed a nasal metabolic-active area, suggesting a relapse. Once again, histopathological examination confirmed the presence of numerous septate hyphae and *A. infectoria* was identified from cultures by ITS rDNA sequencing (isolate no. 70068244). Posaconazole was initiated in January 2011 (400 mg twice a day). Clinical and radiological follow-up in July 2011 highlighted a significant clinical improvement. At present, the patient is still under posaconazole therapy.

#### Mycological methods and findings

All biopsy specimens were cultivated on Sabouraud and Sabouraud-chloramphenicol agar slants without cycloheximide (bioMérieux, Marcy l'Etoile, France) and incubated at both 22°C and 37°C. *A. infectoria*, recovered from all patients, grew as woolly whitish to pinkish colonies, with colonies of up to 5 cm in diameter after 7 days of incubation at 25°C. As discussed above, none of the isolates formed conidia, even after prolonged incubation of several weeks. Notably, the use of several other media (2% agar, potato-dextrose agar, potato-carrot agar and malt extract agar) did stimulate spore formation. Finally, mycological identification of each isolates was only obtained by sequencing analysis of the ITS1, 5.8S and ITS2 ribosomal DNA regions. Briefly, DNA was extracted from fungi growing in culture using the Macherey-Nagel Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) and amplification of the ITS rDNA was achieved using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers on an Applied Biosystems 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Reaction mixtures contained 0.5 µmol/l of each primer, 10 µl of 5 × buffer, 2 mmol/l of MgCl<sub>2</sub>, 0.2 mmol/l of each deoxyribonucleoside triphosphate, 0.03 U of GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA) and sterile water up to a final volume of 50 µl. Amplification parameters were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and elongation at 72°C for 1 min. A final extension step at



**Fig. 2** (A) Hypervascularity (arrow) and palate ulcerations (circle). (B) Nasal saddle.

72°C for 7 min was included at the end of the amplification. PCR products were purified and sequencing was performed using a BigDye terminator sequencing kit on an ABI PrismR 3130 genetic analyzer (Applied Biosystems). Nucleotide sequences were analyzed using the Seqscape software (Applied Biosystems). The same protocol was used with fresh biopsy samples from patients 5 and 6, but similar samples were not available from other patients.

As discussed above, comparison of the nucleotide sequences from each isolate with Genbank database revealed a close homology with *Lewia infectoria* (teleomorph of *A. infectoria*). More precisely, *A. infectoria* isolates from patients 2 to 5, as well as the first isolate from patient 1 had 100% homology with *L. infectoria* ATCC 12054 (GenBank accession number AF229480). Notably, the second isolate from patient 1 differed from the former by a single base substitution, suggesting either a reinfection by an almost identical strain or a genetic microevolution of the same isolate. Since sequencing was performed twice it is unlikely that this difference could be attributed to polymerase error. Finally, both isolates from patient 6 were identical and yield 99.6% homology with *L. infectoria* ATCC 12054 (GenBank accession number AF229480), but differed from the other strains. Nucleotide sequences of the isolates (ITS rDNA) have been deposited in GenBank under accession numbers JQ082440 to JQ082447. The isolates 70012838 (patient 3) and 70052399 (patient 5) have been deposited at the University of Alberta Microfungus Collection and Herbarium under the accession numbers UAMH 11629 and 11630, respectively.

Antifungal susceptibility testing according to the EUCAST methodology could not be performed due to the absence of conidia. Fontana-Masson staining was performed retrospectively on the first biopsy sample from each patient that was sent to the laboratory for histopathological examination and permitted the diagnostic of fungal infection. Fontana-Masson was positive for samples from four of the patients (patient 1 to 3 and 6).

## Discussion

Clinical manifestations of *Alternaria* infections are usually cutaneous or subcutaneous lesions, with ocular, sinusal, or ungueal injuries being uncommon [2]. Defined as an opportunistic infection, phaeohyphomycosis caused by *Alternaria* is often associated with severe immunodepression. Hence, patients at high risk are mostly those receiving solid organ transplantation, hematopoietic stem cell transplantation, long-term corticotherapy or having haematological malignancies [1,2,4]. To a lesser extent, patients with unbalanced diabetes mellitus and rarely, immunocompetent subjects, could also be at risk, as shown for two patients (patients 1 and 6) in our study [5–10]. Notably, the clinical presentation

of phaeohyphomycosis in the 6th patient is very unusual, and was, to the best of our knowledge, reported only once in a single patient in the literature [6].

Regarding the portal of entry of *Alternaria*, infection is often initiated after cutaneous injury that follows contact with plants and/or soil (gardening, farmer). Indeed, country people, and more generally those living in rural areas, are at a higher risk [2]. Notably, most of our patients lived in such areas, some practicing gardening, but none of the patients remembered having had a traumatic incident (Table 1). Giving advice about gardening (i.e., wearing gloves) to high-risk patients such as solid organ transplant recipients could probably reduce the fungal burden of these infections.

Polymorphic characteristics of lesions render clinical diagnosis of phaeohyphomycosis caused by *Alternaria* difficult, leading to a delayed in establishing its etiology [3]. Histopathological examination is of major importance because it enables an early diagnosis of fungal infection, allowing the start of antifungal therapy while awaiting mycological identification. Granuloma could be the typical presentation of the disease, especially in lesions evolving for several months [11–14]. Importantly, fungal hyphae usually appeared hyaline after HES staining, especially since the hyphae of *A. infectoria* are often poorly melanized. As discussed previously, the absence of melanin, a well-known virulence factor in several fungal pathogens, could explain the low virulence of *A. infectoria* [15]. Altogether, histopathological diagnosis of cutaneous phaeohyphomycosis caused by *Alternaria* is particularly tricky in routine practice [12]. Hence, the use of the specific melanin stains, e.g., Fontana-Masson, may be useful even if inconsistent results have been reported [4,12,16]. Here, the retrospective analysis of the use of this stain in studies of the biopsy of each patient revealed that it was positive for four of them.

Mycological culture is the cornerstone for the identification of the fungal agent. Unfortunately, even if *Alternaria* grows easily on most media such as Sabouraud's, some species, especially clinical isolates of *A. infectoria*, often lose their ability to sporulate [1,14,15]. Hence, pan-fungal PCR as done here can be useful, being either applied to sterile mycelium in culture or directly on the biopsy specimen [17,18]. The latter approach that was successful for two of our patients provided both a rapid diagnosis, avoiding delays of mycological cultures, and identification to the species level.

Clinical management of phaeohyphomycosis due to *Alternaria* spp. is still not standardized despite the large number of cases reported worldwide. Reduction of immunosuppressive therapy could promote improvement of the disease [19,20]. In the case of well-delimited lesions, excision alone can lead to a total resolution of the disease,

but antifungal therapy is frequently prescribed in order to avoid relapse [5,18]. However, no consensus regarding the best antifungal drug and duration of therapy has emerged [2,3]. Azoles, especially triazoles, seem to be the best choice considering the susceptibility of most dematiaceous molds to these drugs [1]. Among them, good experiences have been reported with itraconazole but there are limited data for posaconazole [2,3,21]. As shown in Table 1, two patients in our study have been given posaconazole with relative success.

In light of our study, diagnosis of phaeohyphomycosis due to *Alternaria* spp. is challenging when only histopathological and mycological routine methods are used. Fontana-Masson staining, as well as ITS rDNA sequencing, for the identification of non-sporulating isolates are therefore of major interest. Finally, this case series underlines the importance of discussions between physicians, mycologists and histopathologists for efficient diagnosis and therapeutic management of these infections.

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