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Case Study
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First case of human chromoblastomycosis caused by *Alternaria alternata* in Brazil: a case report

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Abstract

Chromoblastomycosis (CBM) is a neglected, chronic subcutaneous disease with progressive development, caused by a variety of melanized fungal species. The main etiological agent of CBM in Brazil and throughout the world is *Fonsecaea pedrosoi*. However, there is a significant knowledge gap regarding the relationship between other CBM-causing species and the host, which needs to be further investigated. We report a case of CBM in which *Alternaria alternata* was identified as the etiological agent in a patient with no history of immunodeficiency or other medical conditions that impair the immune response. In particular, the patient's skin exhibited a predominant T-regulatory immune response, marked by IL-10 production, which may have contributed to the persistence of the fungus in the tissue. This is the first report identifying *Alternaria alternata* as the etiological agent of CBM in Brazil.

Keywords: *neglected tropical disease, chromoblastomycosis, Alternaria alternata, T cell response, cytokines*

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1. Introduction

Chromoblastomycosis (CBM) is a chronic subcutaneous disease caused by at least 41 species of melanized saprophytic fungi, with the genera *Fonsecaea* and *Cladophialophora* having greater medical and epidemiological relevance due to the number of infected patients [1]. The disease is mainly reported in tropical and subtropical regions of the world, but the exact number of affected patients is unknown due to lack of monitoring [2]. CBM is considered an occupational disease, since the etiological agents are inoculated traumatically in the skin of humans during field work [3]. Socioeconomic condition is associated with this disease, as access to protective equipment and medical services remains a challenge in countries [3]. Recently, CBM was included in the list of neglected tropical diseases by the World Health Organization [2].

The onset of human infection starts after a traumatic inoculation of the filamentous forms of fungus in the skin, which rapidly differentiate into the parasitic form of the fungi, called muriform cells, which are spherical and highly pigmented [4, 5]. The muriform cell has been considered an adaptation to extreme environments such as those provided by the immune response of the host [6]. Such forms are easily observed in histopathological sections stained with hematoxylin and eosin, and their presence in tissue has been considered the gold standard for the diagnosis of CBM [7–9]. At the site of a traumatic lesion, where the fungi accessed the dermis, an initial CBM lesion develops slowly as an asymptomatic papule [10]; the epidermis progressively thickens and can develop into one of the five different clinical forms: nodular, tumoral, verrucous, plaque, and cicatricial [11]. The thickening of the epidermis occurs through pseudo-epitheliomatous hyperplasia and hyperkeratosis, which correspond to the thickening of the spinosum and corneum layers, respectively; these histopathological features are findings frequently observed in CBM lesions [12–14].

Brazil is the country with the highest number of reported cases of CBM in Latin America, and, although different species of etiological agents can cause this infection, most of them are caused by *Fonsecaea pedrosoi,* which can cause all clinical forms of the disease [6, 15–17]. Due to the medical and epidemiological relevance of this species, *F. pedrosoi* has been the focus of numerous published studies related to the relationship between fungi and the host [18–21]. During the first steps of infection, macrophages act as the first line of defense against *F. pedrosoi*, developing a granulomatous response and activating T cells. These cells, along with neutrophils and dendritic cells, are responsible for recognizing hyphae and conidia implanted in the skin [18, 19, 21]. However, *in vitro* experiments have shown that, although macrophages are capable of phagocytosing *F. pedrosoi* conidia, they are unable to eliminate the intracellular form of fungi [20, 22]. Immunological evasion of the fungus has been associated with the presence of melanin in the cell wall; this component can neutralize the action of hydrolytic enzymes and reactive oxygen and nitrogen species produced by phagocytic cells [22, 23].

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In the context of acquired immune response, participation of $CD4^+$ and $CD8^+$ T lymphocytes was observed in all clinical forms of CBM; however, disease severity was associated with high levels of IL-10 production, while milder forms showed higher production of IFN-γ [24]. On the other hand, d'Ávila et al. (2002) [25] demonstrated that the skin CBM patients (undetermined species) exhibited a higher density of $CD8^+$ compared to $CD4^+$ T cells, suggesting that the pattern of immunity can be associated with the infecting species [25].

It is important to emphasize that the treatment of CBM is challenging and prolonged. Its effectiveness is influenced by the causative fungal species, the extent of the lesions, and the side effects of therapeutic agents [10]. In cases of localized and well-defined lesions, surgical excision may be a valid therapeutic option. However, chemotherapeutic approaches that use antifungal or immunomodulatory agents are used in all severities of CBM [26]. The antifungal agents currently used belong to the azole class [27, 28], which inhibit the demethylation of lanosterol, a precursor of ergosterol, an essential component of the fungal cell membrane [29]. However, the efficacy of azoles depends on the progression stage of the lesions and the specific fungal species involved. Therefore, combination therapies with antifungal drugs, such as 5-fluorocytosine and terbinafine, are often recommended [30–32]. These agents act by interfering with DNA synthesis and ergosterol production, respectively [29]. A significant obstacle related to the efficacy of treatment is the presence of fungal melanin, which can hinder the efficacy of antifungal agents by binding directly to these compounds or acting as a physical barrier that limits drug penetration [33].

Despite the slow expansion of knowledge about the species that cause CBM, there is a lack of understanding about the host–fungi relationship, such as the case of *Alternaria alternata*, which has been implicated as an etiologic agent of CBM in only one published report [34]. Another species of the same genus, *Alternaria infectoria*, was recently reported as an agent of CBM [35]. This genus has been described as a pathogenic genus of plants and some animals [36]. Although *Alternaria* species have been associated with alternariosis and CBM, to date, there are no reports associating *A. alternata* infection with CBM in Brazil. Therefore, this work aimed to present a case of CBM caused by *A. alternata* and characterize the inflammatory infiltrate histologically and immunologically to uncover the features of CBM caused by *A. alternata*. The lack of identification of the etiological agents of CBM results in limited recognition of uncommon species causing this disease, such as *Alternaria*, limiting the understanding of its histopathology and treatment, considering that the success of treatment is also influenced by the causative species [26].

2. Case presentation

We present a report of a patient with CBM admitted to the outpatient Dermatology service of the 'Hospital das Clínicas da Universidade de São Paulo'. The skin biopsy from the lesion was collected in the "Ambulatório de Micologia do Departamento de Dermatologia do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo", São Paulo, SP, Brazil, during 2014. In 2024, the etiological agent from this biopsy was identified, enabling the description of the present case report. At the time of sample collection, no pictures were taken of the patient's lesion or culture plate. The patient was a 74-year-old man, retired carpenter, resident of São Paulo city, São Paulo State, Brazil, for 30 years. Previously he was a resident of the Rio Real city, Bahia State, Brazil. He had systemic arterial hypertension and had no history of diabetes or autoimmune disease. Although CBM is a slow and progressive subcutaneous mycosis, the patient only sought medical attention at an advanced stage of the disease, so it was not possible to record the onset of the skin disease. His lesion was classified as a verrucous plaque in the proximal interphalangeal space of the left fourth finger. The treatment used was a combination of itraconazole (200 mg/day) and terbinafine (250 mg/day).

Histopathological analysis of the skin histological sections showed pseudoepitheliomatous hyperplasia and hyperkeratosis (**Figure 1a**). The inflammatory infiltrate was composed of macrophages, polymorphonuclear cells, lymphocytes, and plasma cells (**Figure 1b**). Muriform cells (**Figure 1b**, black arrow) were detected primarily in the dermis of the patient, as well as macrophages fused into giant cells (GC), as shown in **Figure 1b**.

To identify the etiological agent of CBM, DNA was isolated from paraffin-embedded skin, and PCR reactions were performed with the internal transcribed spacer (ITS) ITS1–ITS4 primer pairs. As a positive control, DNA of *F. pedrosoi* was submitted to a PCR reaction employing ITS1 (5′-CCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and, as an internal control, the β-actin gene was amplified using the following primer pairs: forward (5′-GACAGGATG-CAGAAGGAGAT-3′) and reverse (5′-TTGCTGATCCACATC-TGCTG-3′); DNA from human blood cells was used as a positive control for β-actin reaction. The PCR products were analyzed by electrophoresis on 1% agarose gel containing 0.1% GelRed (Uniscience). Visualization of amplified genes was performed in a Gel Logic 212 PRO transilluminator (Carestream). The PCR products were purified using the *QIAquick® PCR Purification* kit (QIAgen), and sequencing of the amplified ITS1–ITS4 regions was performed using an AB 3500 Genetic Analyzer automatic sequencer. It was possible to observe that the PCR reaction for ITS1–ITS4 produced an amplified product of ~600 bp (**Figure 1c**), and the reaction for β-actin resulted in a fragment of ~250 bp (**Figure 1d**). Partial sequencing of ITS1–ITS4 revealed a sequence containing 336 nucleotides, which matched with *A. alternata* sequence, deposited in GenBank (access number: ON411173.1). The homology of amplified sequences with those present in GenBank was 94.6% (**Figure 2a**). An alignment between the patient-derived sequence and the positive control of *F. pedrosoi* was also performed (**Figure 2b**). A phylogenetic tree analysis was performed using the Neighbor-Joining method with the MEGA12 software, exhibiting that the patient sequence is more related to *A. alternaria* than *F. pedrosoi* ITS1–ITS4 sequence (**Figure 2c**).

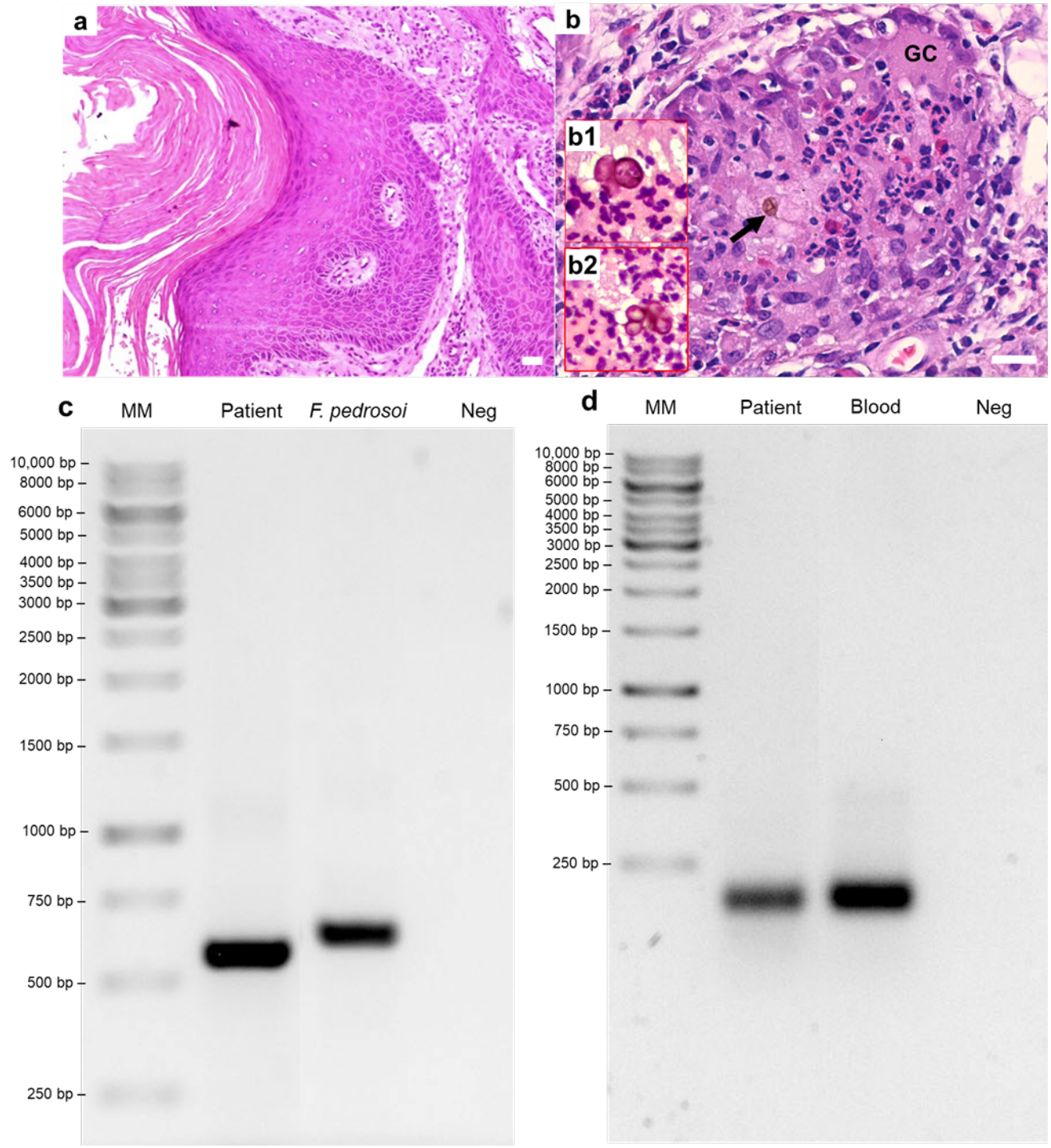

Figure 1 • The skin histological sections stained with hematoxylin and eosin exhibit pseudoepitheliomatous hyperplasia and hyperkeratosis (**a**). Muriform cells (black arrow; insights (**b1**) and (**b2**)) and giant cells (GC) were observed in the inflammatory infiltrate (**b**). Electrophoretic analysis of ITS1–ITS4 transcripts from the patient and positive control (**c**) and of β-actin transcripts from the patient and positive control (**d**). Photomicrographs are recorded at magnifications of 10× (**a**) and 40× (**b**). MM—molecular marker (bp); *F. pedrosoi*—DNA extracted from *F. pedrosoi* culture; Blood—DNA extracted from human blood cells; and Neg—negative control.

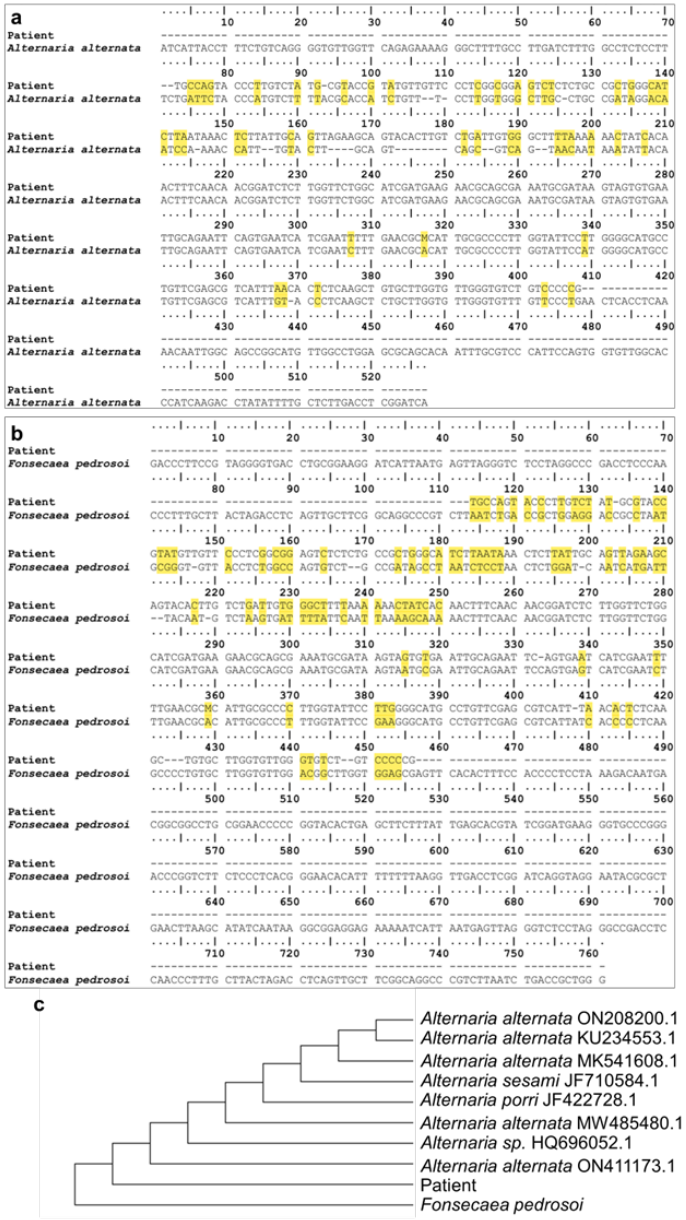

Figure 2 • Alignment of the ITS1–ITS4 sequence of the patient with the *Alternaria alternata* sequence exhibits a similarity of 94.6% (**a**). Alignment of ITS1–ITS4 sequence of the patient with the positive control *Fonsecaea pedrosoi* (**b**). Phylogenetic tree analysis (**c**). Highlights indicate areas of discrepancy between the two sequences (**a**,**b**).

For immunological evaluation, silanized slides were prepared from paraffin-embedded biopsy. Histological sections were dewaxed and hydrated in xylene and a decreasing series of alcohol, respectively. Subsequently, endogenous peroxidase was blocked. Three different recovery buffers were used according to the primary antibody: (1) citrate buffer was used for IFN-γ, IL-4, IL-10, and CD8 (clone C8/144B) antibodies; (2) EDTA buffer was used for the reaction performed with IL-17A antibody; and (3) Tris-EDTA buffer for the reaction with the CD4 antibody (clone OKT-4) antibody. Antigen retrieval was performed in a water bath (96–99 °C), and nonspecific binding was avoided using a blocking solution produced with 6% nonfat milk in PBS (*w/w*). The primary antibodies were diluted in the following dilutions: anti-CD4 (1:10), anti-CD8 (1:25), anti-IFN-γ (1:125), anti-IL-4 (1:150), anti-IL-10 (1:700), and anti-IL-17A (1:500) in PBS containing 1% BSA—PBS-BSA (*w/w*). A negative control for the reaction was established by omitting the primary antibody from the PBS-BSA solution during incubation. After an overnight incubation, the histological sections were washed three times with a solution containing PBS plus 0.05% Tween 20 (PBS-T). Subsequently, incubation with post-primary antibody (Novolink, Newcastle Upon Tyne, UK) was performed, followed by five washes with PBS-T. Finally, the slides were incubated with the polymer-HRP (Novolink, UK) for 30 min and washed five times with PBS-T. The reaction was revealed by the addition of 3,3-diaminobenzidine (DAB) (DakoCytomation, Carpinteria, CA, USA) for 5 min. The reaction was stopped by immersing the histological sections in distilled water. The sections were counterstained with hematoxylin and washed in distilled water. Dehydration and clearing of the sections were then performed using an increasing series of alcohol and xylene, respectively. To analyze the densities of immunolabeled cells, 10 random fields of the inflammatory infiltrate from the immunolabeled slide were captured using an optical microscope connected to a microcomputer. The immunostained cells were quantified considering differential staining in the histological sections, and cell density (d) was calculated using the ratio d = N/A, where N—number of immunostained cells and A—area of each recorded histological image.

Analysis of immunostained skin histological sections revealed the presence of $CD4^+$ (**Figure 3a,c**) and $CD8^+$ (**Figure 3a,d**) T lymphocytes in the inflammatory infiltrate, as well as IL-10- (**Figure 3b,e**), IFN-γ- (**Figure 3b,f**), IL-17- (**Figure 3b,g**), and IL-4 producing cells (**Figure 3b,h**). Immunohistochemistry reactions using the same antibodies were performed in the skin of healthy individuals who were submitted to plastic surgeries; in this specific case, immunolabeled cells were absent or present at very low densities.

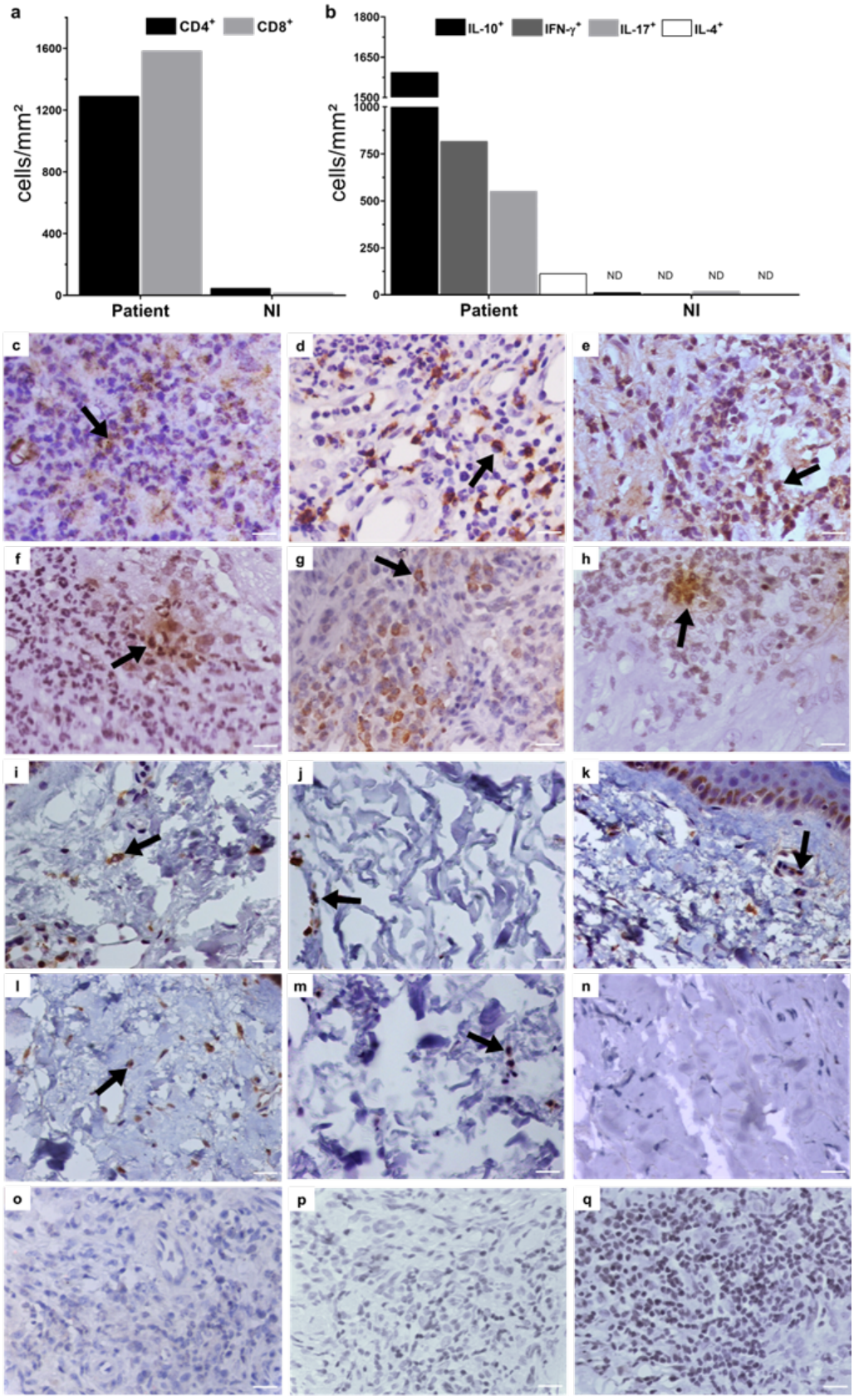

Figure 3 • Quantification of $CD4^+$ and $CD8^+$ T cell densities (**a**) and $IL\text{-}10^+$, $IFN\text{-}\gamma^+$, $IL\text{-}17^+$, $IL\text{-}4^+$ and cell densities (**b**). Data displayed as NI refer to healthy (non-infected) skin. Photomicrographs of immunostained skin histological sections from patient showing $CD4^+$ cells (**c**), $CD8^+$ cells (**d**), $IL\text{-}10^+$ cells (**e**), $IFN\text{-}\gamma^+$ cells (**f**), $IL\text{-}17^+$ cells (**g**), $IL\text{-}4^+$ cells (**h**); photomicrographs of immunostained healthy skin histological sections showing $CD4^+$ cells (**i**), $CD8^+$ cells (**j**), $IL\text{-}10^+$ cells (**k**), $IFN\text{-}\gamma^+$ cells (**l**), $IL\text{-}17^+$ cells (**m**), $IL\text{-}4^+$ cells (**n**); photomicrographs of negative control of immunostaining of different recovery buffers: Tris-EDTA (**o**), citrate (**p**), EDTA (**q**). Labeled cells are shown with black arrows. Photomicrographs taken with a 40× objective lens; scale bar = 20 μm.

3. Discussion

The patient reported here was diagnosed with CBM through clinical and histopathological study. The patient's cutaneous lesion presented as a verrucous lesion that has been considered one of the most prevalent clinical forms of CBM [10]. Additionally, all histopathological features recorded in the skin of this patient were related to the changes observed in the CBM with the presence of pseudoepitheliomatous hyperplasia and hyperkeratosis. Pseudoepitheliomatous hyperplasia is a typical feature of CBM lesions, also observed in skin histological sections of patients infected with *F. pedrosoi* [12], *C. carionii* [37], *P. verrucosa* [38], *R. aquaspersa* [39], and with the following uncommon species related to CBM: *A. alternata* [34], *Bipolaris cynodontis* [40], and *Rhytidhysteron rufulum* [41]. Hyperkeratosis, although common, is not seen in all CBM cases; it is a striking feature of verrucous lesions and can be found less frequently in other types of lesions [10]. It has been observed in CBM caused by *F. pedrosoi* and *F. monophora* [42], *C. carionii* [43], *Cladosporium tenuissimum* [44], *Phialophora chinensis* [45], and species less reported such as *A. infectoria* [35] and *Chrysosporium keratinophilum* [46]. Importantly, muriform cells were observed sparsely distributed within the patient's inflammatory infiltrate, a significant feature to diagnose CBM [6]; these cells can divide to form aggregates divided by longitudinal or transverse septa [6].

Using molecular biology techniques, based on the amplification and sequencing of the genetic region ITS1–ITS4, the etiological agent of this patient was identified. The ITS1–ITS4 genetic fragment is a non-coding region of ribosomal DNA that has been used as a universal barcode to identify fungal species as it contains certain characteristics, such as the following: (1) it is a multicopy region, which facilitates its amplification and sequencing, even with low amounts of genetic material, which is the case showed herein, since the fungal genetic material originates from muriform cells in the histological sections of the patient's biopsy; (2) it possesses well-conserved specific initiation sites and highly variable adjacent regions that, combined with (3) high availability of sequences for comparison, it allows the identification of unknown samples; additionally, (4) the ITS region had greater amplification success and resolving efficiency for species discrimination within the Dikarya group than other ribosomal regions such as LSU, SSU, and the *RPB1* protein-coding region [47]. Thus, considering the superiority of ITS1–ITS4 over other genetic regions, the study presented herein used this region to identify the etiological agent of CBM in the skin of the patient studied herein. It was possible to identify a partial sequence of ITS1–ITS4, allowing the identification with 94% of certainty that *A. alternata* is the etiological agent of CBM in the Brazilian patient, suggesting that *A. alternata* may have a broader occurrence as an etiological agent of CBM, especially considering that this mycosis is not a notifiable disease and PCR analysis is not always used for species identification [48].

It is important to note that, in this report, the fungal species *A. alternata* was identified for the first time as the etiological agent of CBM in Brazil. This species has been described as a pathogenic species of plants and some animals [36, 49], whose airborne spores are associated with allergic reactions in humans [50, 51] and, when its hyphae are inoculated in the skin, cause mycoses such as keratitis, phaeohyphomycosis, and alternariosis [52, 53]. *A. alternata* has been implicated as an etiological agent of CBM in only one case in 2014, involving a 50-year-old immunocompetent farmer living in Ningbo, China, who presented two clinical forms of the disease, an erythematous plaque surrounded by verrucous hyperplasia on the dorsum of the left hand [34]. The lesion appeared after trauma and gradually increased over six months. Histopathological studies showed pseudoepitheliomatous hyperplasia and granulomatous inflammation in the dermis with the presence of muriform and giant cells, features also found in CBM caused by other species. The initial treatment was oral itraconazole (400 mg/day for 15 weeks), and then the use of 5-aminolevulinic acid associated with photodynamic therapy (ALA-PDT) was started. At the end of the treatment, the lesion healed completely [34]. In Brazil, this is the first report suggesting that *A. alternata* may be incriminate as a causative agent of CBM in Brazil; furthermore, it would be important to note that the presence of this etiological agent can be more common than reported in the literature, as CBM is not a notifiable disease, and the etiological agent is not identified in all patients with CBM [48]. In Brazil, *A. alternata* has been strictly associated with plants such as coffee and potatoes [54, 55]; therefore, injuries caused by handling these vegetables can mediate the inoculation of this fungus in the skin. This finding is relevant and possibly underscores the importance of the species for public health in Brazil as a CBM agent. Therefore, identification of the etiological agent can facilitate the search for treatments, as the action of antifungals varies according to the species that causes the disease [26].

Regarding the composition of the inflammatory infiltrate, the cells commonly found in CBM lesions include polymorphonuclear cells, macrophages, and other mononuclear cells composed of lymphocytes and plasma cells [11]. In the case presented herein, the main component of the inflammatory infiltrate was macrophages that appeared as a single cell or fused cells, characterizing the giant cells, which are a common immunological occurrence in CBM, and, regardless of the fungi species, the main aims are to restrain and eliminate the parasitic forms of the fungus [56–59]. Polymorphonuclear cells were also present in the skin; this cell type contributes to the granulomatous characteristic observed in the histopathology of the lesions [11]. Furthermore, macrophages and neutrophils represent the first line of defense in fungal infections due to their phagocytic and microbicidal properties [60].

Lymphocytes are commonly found in the skin inflammatory infiltrate of patients with CBM [56]. $CD4^+$ T cells can determine the immune response in CBM through cytokine production [61]. Due to the quantification of IFN-γ, IL-17, IL-4, and IL-10-producing cells, it was possible to determine that the patient reported in this study exhibited a mixed immune response, as documented in previous studies [24, 56, 62]. The Th1 immune response is predominant in patients with milder forms of CBM, characterized by single lesions in plaque and nodular forms [24], since IFN-γ activates and polarizes macrophages to a microbicidal profile, which become able to eliminate the intracellular fungi [24, 56]. Another important cytokine found in patients with the verrucous form caused by *F. pedrosoi* is IL-17 [63–65], which is associated with neutrophil influx to the site of infection [66]. High densities of IFN and IL-17-producing cells in the inflammatory infiltrate tend to eliminate intracellular fungi due to macrophage activation and induction of antimicrobial compounds in neutrophils [24, 66].

On the other hand, a high density of IL-10-producing cells was recorded in the skin of the patient, suggesting an active immune regulatory response in the inflammatory environment. IL-10 is frequently observed in CBM with one or more extensive lesions [24, 25, 62], and elevated levels of this cytokine have been associated with IFN-γ inhibition in patients with CBM prior to treatment [62]. Furthermore, IL-10 can inhibit antigen presentation and downregulate the proinflammatory activity of IL-17, leading to the persistence of etiological agents of CBM [62]; consequently, the infection progresses. Despite the low number of IL-4$^+$ cells, these cytokine-producing cells may reduce Th1 and Th17 immune responses, as this cytokine polarizes macrophages to the anti-inflammatory M2 profile [67], favoring fungus survival.

CD8$^+$ T cells were also evidenced in the inflammatory infiltrate of the patient reported here. This subset of lymphocytes induces apoptosis in target cells, controlling intracellular infections, as described in patients with CBM [25]. This subpopulation of T lymphocytes can be activated when hosts exhibit a compromised or insufficient CD4$^+$ T cell response [25], as is the case with this patient who presented a mixed immune response that activates and deactivates the microbicidal immune response. The influx of CD8$^+$ T lymphocytes into the inflammatory infiltrate observed here may be related to the partial control of fungal infection, considering the clinical form of CBM observed in this patient [56].

4. Conclusions

The partial sequence of IT1-ITS4 recorded allowed the identification of *A. alternata* as a possible etiological agent of CBM, reinforcing the participation of this species in the cycle of this infectious disease, as previously reported. Furthermore, this is the first description of *A. alternata* as a probable causative agent of CBM in Brazil. The data presented here suggest that *A. alternata* causes a verrucous clinical form of CBM with histopathological features similar to those of other species. In addition, this infection establishes a mixed immune response, based on Th1, Th17, and Treg responses.

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Author contributions

Conceptualization, L.F.D.P.; methodology, I.N.C., M.D.L., W.B.J., C.H.C.d.C. and L.F.D.P.; validation, I.N.C. and L.F.D.P.; formal analysis, I.N.C., M.D.L., W.B.J., C.H.C.d.C. and L.F.D.P.; investigation, I.N.C., W.B.J. and M.D.L.; resources, M.D.L. and L.F.D.P.; data curation, I.N.C. and L.F.D.P.; writing—original draft preparation, I.N.C. and L.F.D.P.; writing—review and editing, I.N.C., M.D.L., W.B.J., C.H.C.d.C. and L.F.D.P.; visualization, I.N.C., M.D.L., W.B.J., C.H.C.d.C. and L.F.D.P.; supervision, L.F.D.P.; project administration, L.F.D.P.; funding acquisition, M.D.L. and L.F.D.P. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

Data availability statement

All data supporting the findings of this publication are available within this article.

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethics committees of the São Paulo State University and the Hospital das Clínicas of the University of São Paulo (protocol codes #55998421.2.0000.5411 and #55998421.2.3001.0068, and date of approval 2023 Apr 27).

Informed consent statement

Informed consent was waved by the ethics committees of the São Paulo State University and the Hospital das Clínicas of the University of São Paulo, due to the patient is no longer in follow-up.

Additional information

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