Paracoccidioidomycosis: eco-epidemiology, taxonomy and clinical and therapeutic issues

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Review
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Acquired by inhalation of the thermal dimorphic fungi *Paracoccidioides* spp. conidia, paracoccidioidomycosis ranges from symptomatic to severe and potentially fatal disseminated disease. The main focus of this review is to highlight clinical aspects of paracoccidioidomycosis and, its pathogens' diversity ecology and particularities. In addition, we present strategies for therapy, including DNA vaccines and nanostructured drugs. Molecular and morphological data supported the split of the *Paracoccidioides* genus into two species, *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. An acute form of the disease affects approximately 5% of cases and involves the phagocytic mononuclear system, resulting in progressive lymphadenopathy. The chronic form affects adult men and frequently involves lungs, skin and mucous membranes, lymph nodes, and adrenal glands. The clinical manifestations depend on the ability of the host to control the fungal multiplication and dissemination. The long survival time of the fungus in the host tissues allows it to evade immune responses; therefore, successful treatment often requires long-time therapy. The consensus for treatment must consider the severity of the disease and includes sulfone derivatives, amphotericin B and azoles. Novel strategies for therapy, based on DNA vaccines and nanostructured drugs are also presented and discussed in this review.

Epidemiology & ecology

Paracoccidioidomycosis (PCM), caused by the human fungal pathogens from the *Paracoccidioides* genus, is the highest cause of mortality among systemic mycoses in Brazil and the eighth most important cause of mortality from chronic infectious diseases, causing 1.65 deaths per 10^6 inhabitants [1]. PCM is endemic to populations that live in rural areas. This mainly affects individuals related to agricultural activities who, by manipulating the soil, generate aerosols containing fungal spores, which are inhaled [2]. The annual incidences of new cases vary within endemic areas ranging from 1 to 3 new cases per 10^5 inhabitants [3]. The disease is geographically restricted to central and South America (from Mexico to Argentina) showing high prevalence in Brazil, Colombia, Venezuela and Argentina [4]. Imported cases have been recorded in the USA, Europe and Asia [5]. In some regions of Brazil, such as the state of Rondonia, more than 1170 cases have been reported between 1998 and 2005 [Durlacher R, Lima S, Pers. Comm.]. The large predominance observed in male adult patients has not been observed in child or young adult patients (1–2 men to each woman) [3]. The ability of estrogens to inhibit the transformation of mycelium or conidia to yeast or the higher exposure of men than women to the soil in rural areas may explain these differences [6]. Transmission of the mycosis from one person to another has not been reported. Tobacco and alcohol intake increased the risk of PCM as shown in a case–control study in Brazil [7].

According to geospatial technologies, association between climatic factors and clinical diagnosis of PCM, *Paracoccidioides* should occur preferentially at sites of soil with a high rainfall index and optimum permeability, which is associated with a high relative humidity and abundance of vegetation and

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watercourses. The water volume during the rainy season should be satisfactory, and a variable temperature between 18 and 28°C could be favorable for sporulation and aerial dispersion of the fungus [8,9]. Influences of soil water storage, absolute air humidity higher than normal and the climatic anomaly caused by the 1982/1983 El Niño Southern Oscillation were associated with a cluster of acute/subacute cases 1–2 years later in a southern region of Brazil [8].

Although the route of infection has been established (respiratory tract via fungal propagules), the saprophytic habitat of the fungus continues to be investigated [10]. *Paracoccidioides* occurs as saprobic mycelium in soil, with decaying organic material acting as a source of nutrients, as confirmed by direct isolation [11] or molecular tests [12,13]. When infectious propagules (i.e., hyphal fragments or conidia) are inhaled, they are deposited in the human lungs and the morphology of the fungus differentiates to yeast form by increasing the temperature to 36–37°C, thus establishing the disease [14]. Despite the absence of a teleomorphic status, molecular and morphological data revealed the possibility of a sexual cycle in the genus *Paracoccidioides* [15]. Alternatively, the ecological niche of *Paracoccidioides* is found to be living in association with warm-blooded animals and the fungus is frequently isolated from armadillos (*Dasypus novemcinctus* and *Cabassous centralis*) from endemic regions of PCM [16,17]. Armadillos are usually in close contact with soil, as they have a habit of digging tunnels and living in underground burrows, which could contribute to the spread of fungal spores. Armadillos are attractive hosts for *Paracoccidioides* as they have an ideal body temperature and relatively low cellular immunity, which may favor the development of infections and may have played a role in the evolution of *Paracoccidioides* to the zoophilic condition despite the adaptation to animal tissue [12,18]. In addition, *Paracoccidioides* has also been isolated from dogs [19], two-toed sloths [20] and penguins as well as bat droppings [21,22]. Serological, intradermic tests and/or molecular analysis of the fungus indirectly suggest that it is present in domestic animals [23–25], primates [26,27] and in road-killed wild animals [28]. Recently, molecular analyses using nested PCR allowed identification of *Paracoccidioides* spp. in aerosol at the entry of armadillo burrows, which proved to be a valuable tool in the identification of pathogens [29].

The *Paracoccidioides* genus & species recognition

The genus *Paracoccidioides* is placed in the thermodimorphic fungal pathogens from the family Ajellomycetaceae, order Onygenales, which includes the anamorphs *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Emmonsia parva, Emmonsia crescens* and *Lacazia loboi*. Molecular evolutionary analysis clustered *Paracoccidioides* and *L. loboi* as sister groups, considering the *Coccidoides* genus (Onygenaceae) as an outgroup [30,31]. The family Ajellomycetaceae is adapted to vertebrate hosts, a characteristic shared by all members of this family [31]. PCM can be acquired by inhalation of infectious propagules from two species: *Paracoccidioides brasiliensis* [32] and *Paracoccidioides lutzii* [30,33]. *P. brasiliensis* has been considered a single species since its discovery and several studies including molecular and morphological data supported the split of *Paracoccidioides brasiliensis* into two species: phylogenetic species recognition based on genealogical concordance (GCPSR) criteria [34,35]; a long period of genetic isolation 24–32 mya [30,36]; lack of gene flow between *P. brasiliensis* and *P. lutzii* as evidenced by recombination analysis [30]; random amplification of polymorphic DNA (RAPD) data [34,37]; unique whole-genomic features, such as genome size and gene content, protein families expansions/contraction, and differential transposable elements distribution [38,39]; and differential morphology in conidial cells (Figure 1) [30,33,36]. *P. lutzii* is composed of a single monophyletic and recombining population found in central, south-western and north-western regions of Brazil and Ecuador [29,30,33,35,36]. *P. brasiliensis* harbors a complex of at least four different cryptic species (S1, PS2, PS3 and PS4; Figure 2) that are characterized by different evolutionary traits and geographic distributions [40]. *P. brasiliensis* and *P. lutzii* display particular morphological characteristics. Besides the typical bicorn cocked hat- and barrel-shaped conidia, produced by both species, *P. lutzii* frequently produces elongated, rod-shaped conidia, which could be used for species diagnosis [30,33,36]. Yeast cells from both species produce no significant variation in size and shape, with the exception of *P. lutzii* Pb01 isolate, which exhibits larger yeast cells and *P. brasiliensis* PS2, which commonly presents elongated yeast cells, similar to pseudohyphae [30,36]. *P. brasiliensis* S1 represents a monophyletic and recombining population widely distributed in South America and has been associated with the majority of cases of PCM detected

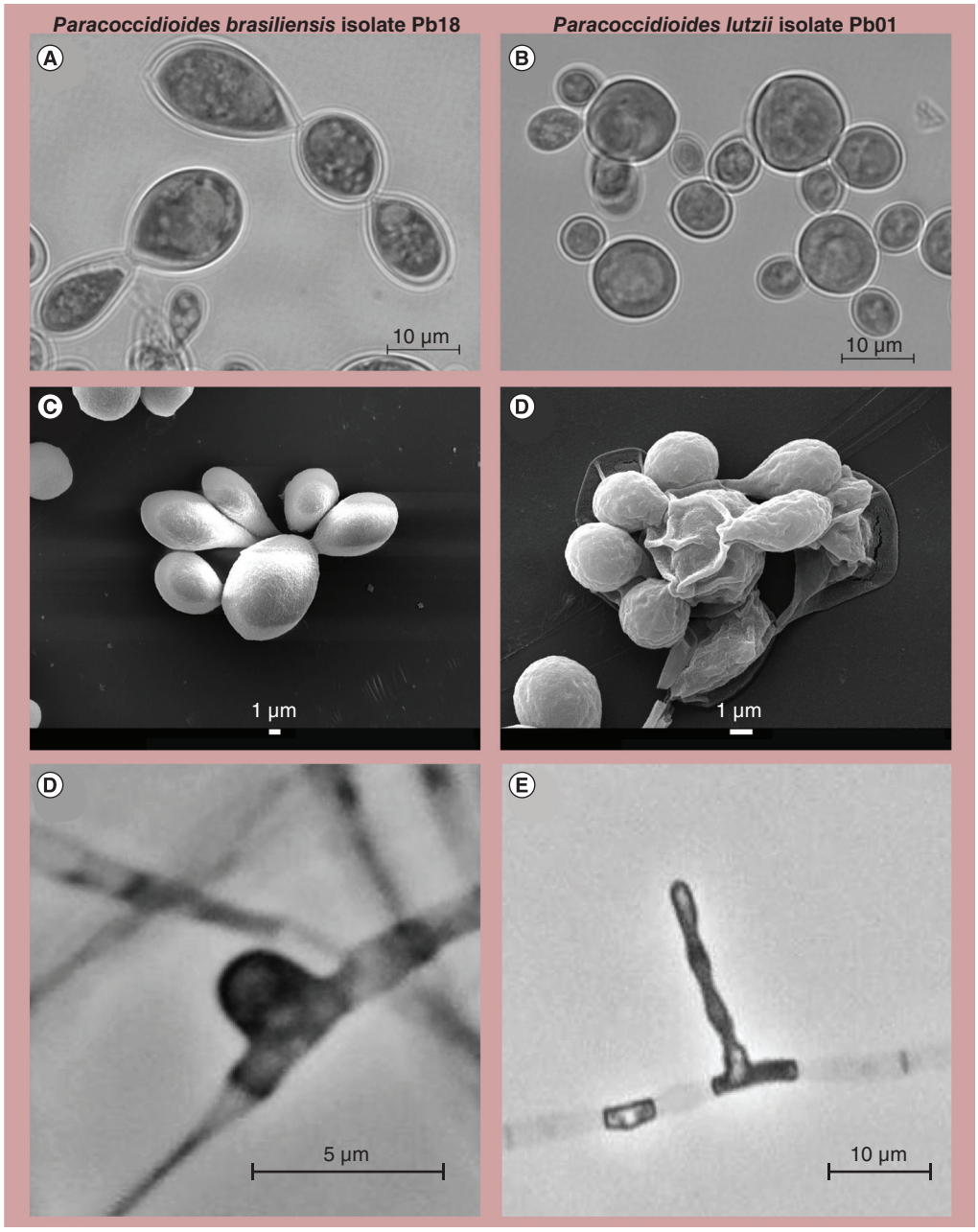

Figure 1. Morphological differences between *Paracoccidioides brasiliensis* isolate Pb18 and *Paracoccidioides lutzii* isolate Pb01 yeast cells and conidia. The budding yeast cell size differences are shown by **(A & B)** light micrograph and **(C & D)** electronic sweep microscopy; and **(E & F)** conidial shape differences between *P. brasiliensis* and *P. lutzii* isolates are shown by light micrograph.

until now. Isolates belonging to *P. brasiliensis* S1 were already recovered from Armadillos, soil and penguin feces [40]. *P. brasiliensis* PS2 is a paraphyletic and recombining population and has only been identified in Brazil and Venezuela so far [30,40]. *P. brasiliensis* PS3 is made up of a monophyletic and clonal population and is exclusively found in Colombia. Beyond clinical samples, *P. brasiliensis* PS3 has also been isolated from armadillos [34]. *P. brasiliensis* PS4 was recently suggested and appears to be a monophyletic population of clinical isolates recovered from Venezuela [36,41]. The radiation of *Paracoccidioides* started in the north-western region of South America, around 11–32 mya, according to biogeographic inferences. Vicariant events were probably responsible for the divergence of S1/PS2 and *P. lutzii* and a recent dispersal raised the PS3 species, which is geographically restricted to Colombia [36]. Owing to the difficulties of producing conidia in the laboratory and the morphological exclusivities among

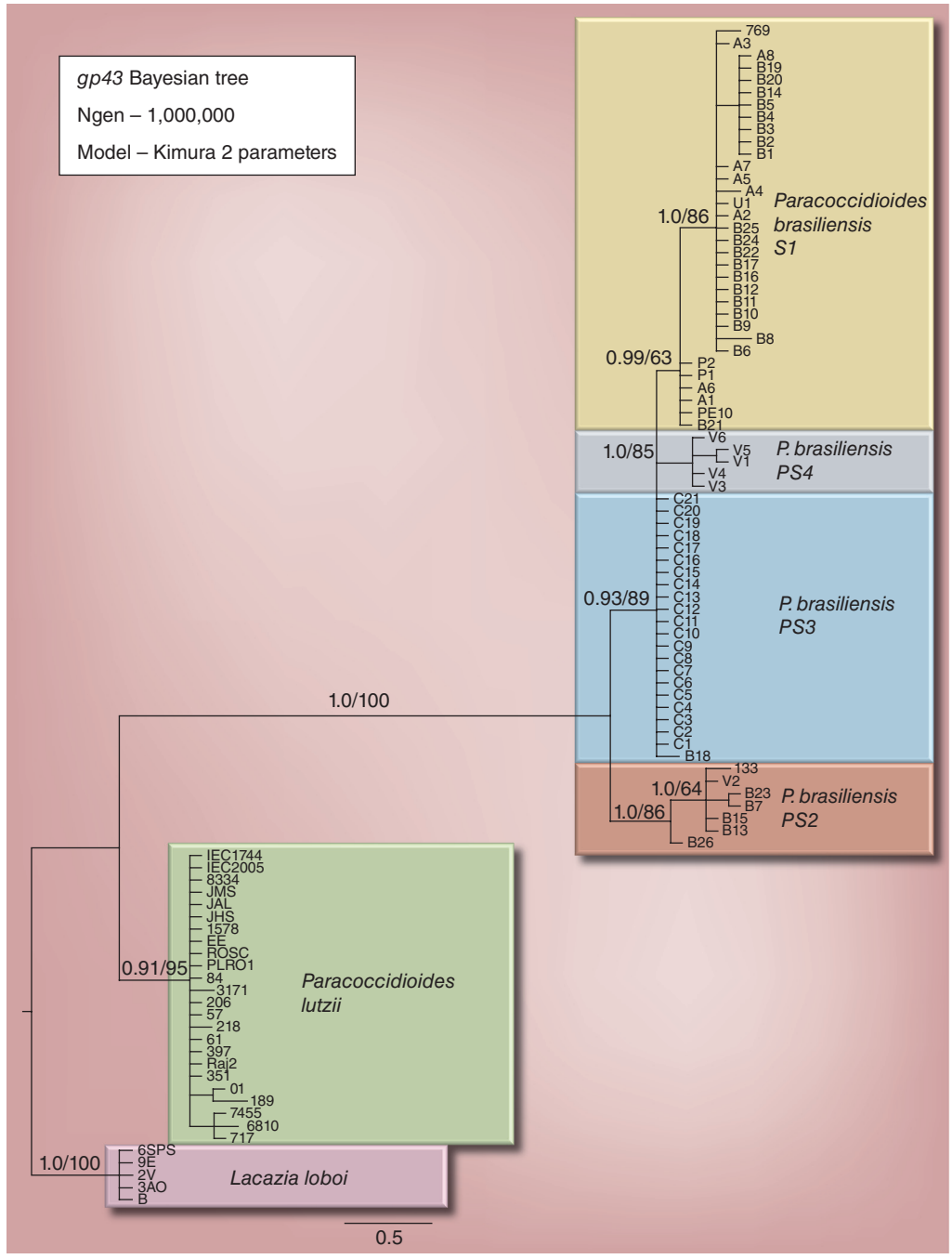

Figure 2. Phylogenetic representation of the genus *Paracoccidioides* based on partial sequences of the *gp43* gene. Sequences were retrieved based on previous reports evaluating the evolution of the *Paracoccidioides* genus [29,30,33,34,39]. Bayesian and maximum likelihood analyses were performed using MrBayes and MEGA 5.0, respectively, and the nucleotide substitution model selected was the Kimura 2-parameters model. *Lacazia loboi* was added as an outgroup, and confident bootstrap and posterior probability values representing the branch supports were inferred and added next to the branches. Scale bars of each tree refer to the number of substitutions per site analyzed.

species, molecular diagnoses of *Paracoccidioides* species have become a common tool of choice among mycologists. Several molecular markers were already applied in population studies in the genus *Paracoccidioides* and gp43 and hsp70 loci are the best choice for species delineation, owing to the high frequency of polymorphic sites shared among species [30,33,36,40]. The evolutionary history of *Paracoccidioides* is not a simple matter owing to the constant migration of human hosts, the long latency of PCM, the lack of information about the clinical history of

patients and the fact that environmental isolates are poorly sampled, thus hindering the exact location of the fungi [40].

Host–fungal interactions

The immunological responses to PCM have been investigated in experimental models and in patients to better understand the host–fungal interaction. In resistant and susceptible strains of isogenic mice, anti-*P. brasiliensis* protection is directly related to high levels of IFN-γ and IL-2, and to the production of IgG2a antibodies (Th1 response), while susceptibility is characterized by secretion of IL-4, IL-5, IL-10 and TGF-β, as well as eosinophilia with preferential production of IgG2b and IgA (Th2 response) [42]. In pulmonary murine PCM, a dual role of IL-4 was observed in IL-42-depleted mice with different genetic backgrounds suggesting a role for IL-4 in the modulation of the immune response [43]. Da Silva studied the formation of granulomas in Pb18-infected Swiss mice, and observed that granulomas began predominantly with macrophage infiltration in the lungs [44]. Fibrosis was observed within 2–4 weeks of infection and organized granulomas were present at 8 weeks postinfection. After the eighth week, several organized granulomas – formed by macrophages, epithelioid cells, giant cells and fungal cells – were observed inside the lungs.

Recently, other aspects of adaptive immunity have been investigated. The costimulatory molecule CD28 was shown to participate in initial protection via T cells, but also by inducing the response of regulatory T cells with fungal dissemination and early murine mortality [45]. Additional information about the innate immune response on PCM has been obtained from Toll-like receptor-defective and knockout murine models. Toll-like receptor 2-defective mice had preferential Th17 immunity associated with impaired expansion of regulatory T cells [46].

The lymphocytes from patients with the chronic form of PCM stimulated with nonpurified fungal antigen or the 43 kDa glycoprotein of *P. brasiliensis* (gp43) produced low levels of IFN-γ when compared with patients who received the clinical cure [43], corroborating the antigen-specific immunosupression observed during development of the disease, as well as the protective role of high levels of IFN-γ during the infection [47].

The acute form of this mycosis is characterized by secretion of IL-4, IL-5 and IL-10, and decreased levels of lymphocyte proliferation and IFN-γ, which results in depression of the cellular immune response and consequent severe manifestation of the disease. On the opposite side are the healthy infected individuals with high levels of lymphocyte proliferation and IFN-γ and low levels of IL-10. The chronic form shows an intermediate pattern of immune response with mixed production of these cytokines [48].

The direct relation between high levels of IFN-γ and protection in human PCM was also reported when investigating intracellular cytokines. Healthy or subclinical *P. brasiliensis*-infected individuals showed higher numbers of IFN-γ-positive lymphocytes when compared with patients with both chronic and acute disease [49]. In an immunohistochemical study, cells expressing IL-17 or Foxp3 were found to be distributed on both inflammatory infiltrates of skin and mucosal lesions. Foxp3-positive cells, however, were increased in compact granulomas from mucosal lesions, further corroborating the hypothesis of an important modulating role of regulatory T cells in the lesions [50]. In addition to these phenotypical aspects, the role of genetic determinants and inherited immunodeficiencies has been posited as a factor affecting susceptibility to PCM [51].

A higher frequency of HLA classes I A2, A9, B7, B21, B13, B40 and Cw1 was observed in infected patients when compared with control groups [51]. Regarding the frequency of alleles *DRB1* and *DQB1* from HLA class II genes, the allele *DRB1*11* was statistically more frequent in patients with the less severe form of PCM (the chronic unifocal PCM) [51]. In addition to HLA allelic frequencies, polymorphisms in cytokine genes have also been reported; associations between functional genetic variants in the IL-4 promoter as well as upregulation in production of this cytokine have also been observed in patients [52].

Clinical aspects

The disease spectrum varies from oligosymptomatic course to severe and potentially fatal disseminated disease. The incubation period is unknown. The disease has been reported in children 3 years of age or older who had lived for some years in the endemic area [Shikanai-Yasuda MA *et al.* Unpublished Data].

The clinical forms of PCM were classified in the International Colloquium on PCM, held in 1986 in Medellín, Colombia, where the relationship between clinical aspects and natural history of the disease and definitions were established [53]. Some definitions are important,

such as: infection; acute/subacute form (juvenile type) that can be classified as moderate or severe; chronic form (adult type) that can be classified based on unifocal and multifocal lesions; and sequels [53].

This classification of the disease considers its incidence soon after the primary infection (acute/subacute type) or after a long period of latency (chronic type), followed by the localization in different organs and the degree of severity on the basis of general and nutritional status and organ dysfunction. Therefore, unifocal lesions may be associated with high disease severity, in some cases with CNS or other vital function commitment [53].

Infection

Evidence from surveys suggests that first contact between host and fungi typically occurs two decades prior to the presentation of symptoms. Usually, the only expression of the infection is the cellular immune response to fungal antigens. Primary pulmonary complex had been registered in PCM and histopathological studies have confirmed the prevalence of this disease in endemic areas in individuals without a previous diagnosis [54].

Disease

Acute form (juvenile type)

Approximately 5% of cases are acute (juvenile type), which is characterized by involvement of the phagocytic mononuclear system. It usually affects children, adolescents and young adults (under 35 years of age), and affects men and women equally. Symptoms include fever and weight loss and mild/moderate anemia for 2–3 months or longer. Cervical, axillary and inguinal nodes are the most commonly enlarged. Symptoms and signs of obstructive jaundice may arise when hepatic perihilar lymph nodes are affected. Lymph nodes are initially hard but evolve with inflammatory signs and sometimes become fluctuant. The liver and spleen are often moderately enlarged and bone marrow may be involved. Multiple skin and mucosal lesions may occur. In approximately 50% of cases, the digestive tract is affected, as determined by radiological analyses [55]. High transient blood eosinophilia (up to 30,000/mm^3) has been described [56]. In the blood, some alterations are observed in the first months, such as a high rate of erythrocyte sedimentation and inflammatory markers such as CRP, α2 proteins and mucoprotein. After treatment, these parameters decreased [3].

Chronic form

The chronic form of the disease accounts for more than 90% of cases and usually occurs in 30–60-year-old individuals who have worked in agricultural areas. Males are more commonly affected in a ratio of 10–25 males to one female. Characteristically, it presents insidious and chronic evolution, usually with mild or moderate severity but resulting in sequels in about one-third of cases. Unifocal forms involve only one localization and the multifocal form represents canalicular and/or hematogenous dissemination of the disease. The lungs are the most commonly affected organ (80%), followed by, in chronic form, the skin (lesions in 45.7%) and mucous membranes (oral, larynx, trachea and digestive tract; ulceration of the oral or nasal mucosa in 50.9%) [57]. Lymph nodes, adrenal glands and other organs or tissues could also be involved. Less frequently, the intestine, CNS (brain, cerebellum and meninges), bones, spleen, eyes, genitourinary system or cardiovascular system could be involved. The course of the disease is insidious, fever is uncommon and, when present, secondary pulmonary infection or association with TB should be suspected. Patients may complain of weakness, weight loss, dyspnea, cough, sometimes purulent sputum, and, rarely, hemoptysis. As the course is chronic, when patients present with dyspnea, a marked contrast is observed between the paucity of clinical symptoms and signs and the multiple findings of the chest radiography: bilateral, asymmetrical, reticulonodular interstitial infiltrates in the middle and lower parts of the lungs with alveolar infiltrates (**Figure 3A & 3C**). In rare cases, apical cavities and pleural effusions can be found [50]. Mucosal lesions are predominant in the mouth and/or oropharynx. Infiltrative moriform stomatitis is characteristic of this mycosis, appearing as shallow ulcers with a granular surface showing multiple hemorrhagic points with papules. In the respiratory tract, the larynx and trachea could be involved; hoarseness and dysphonia occur in association with laryngeal lesions and may evolve with laryngeal obstruction. Cutaneous lesions could be single or multiple and are observed as papules, pustules, ulcers, crusted ulcers, vegetative, sarcoid-like, verrucoids or acneiform cutaneous lesions mainly on the face. Few lymph nodes may be involved and the involvement of the adrenal glands has been described in 50% of autopsies [3]. Partial adrenal insufficiency was found in approximately 33–40% of cases, but only 3–10% were symptomatic [58,59].

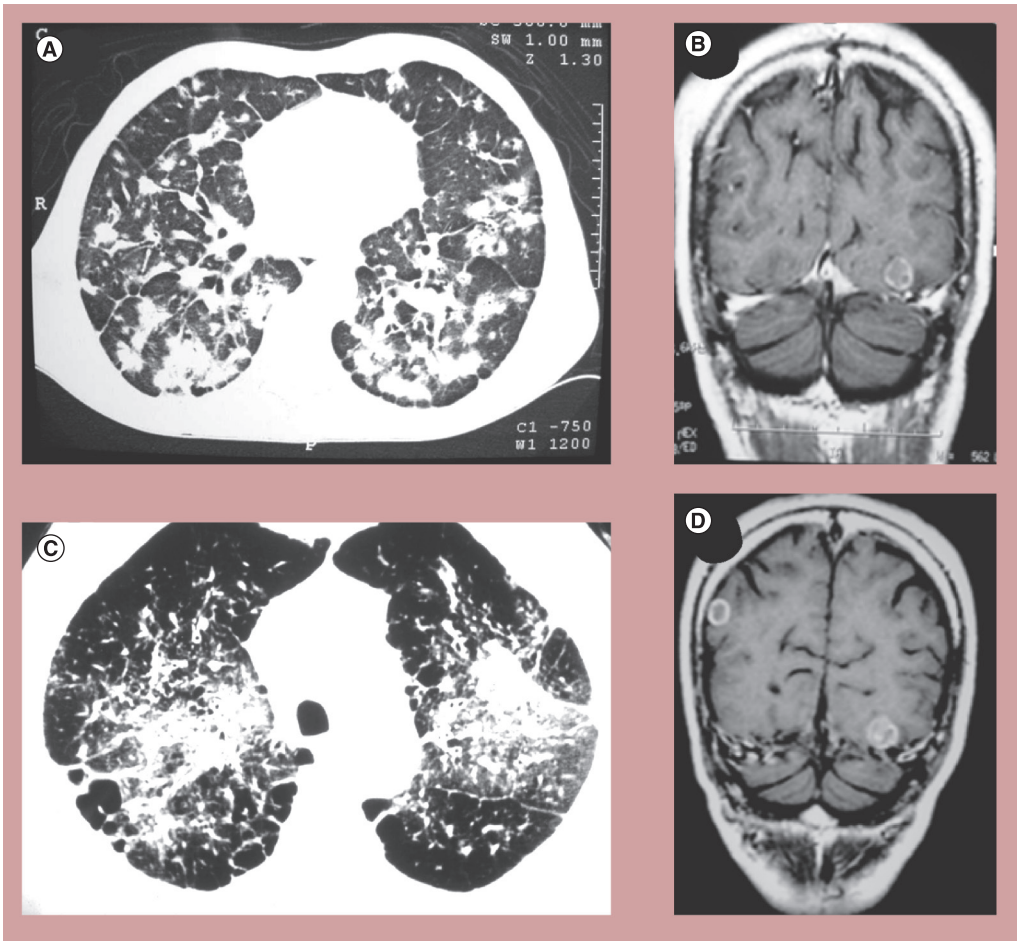

Figure 3. Radiological aspects of paracoccidioidomycosis patients. (A) Pulmonary paracoccidioidomycosis, asymmetric alveolar and interstitial macronodular reticulonodular infiltrates and alveolar infiltrates. **(C)** Fibrosis in the lower fields of both lungs. **(B & D)** Paracoccidioidomycosis of the CNS showing ring-enhancing lesions in the cerebral cortex **(B)** prior to the treatment and **(D)** 4.5 years later.

CNS involvement is described in 6–25% of patients, affecting the cortex, cerebellum, brain or all regions (Figure 3B & 3D), leading to epilepsies, expansive lesions and cerebellar signs and symptoms, usually present in 20–30% of these cases. CT scans show the affected area and, if possible, magnetic resonance is more sensitive than CT scan for detection of cerebellar and brain regions. Recently Reis *et al.* showed that an MRI of patients with neuro-PCM could help to differente the PCM lesion from other inflammatory lesions [60].

Sequels

Although fungal infection can be controlled using conventional chemotherapy, impairment of vital functions can prove to be fatal. The natural evolution of the lesions, without early administration of treatment, is to heal by fibrosis, so sequels such as microstomia, laryngeal or tracheal stenosis and intestinal obstruction are normally observed in patients. Pulmonary fibrosis leading to cor pulmonale and respiratory insufficiency is usually associated with a previous smoking habit. Sequels have been described in 30.3% and deaths in 7.6% of all PCM cases [61]. In the juvenile/acute form, sequels represent 6.3% and deaths 9.5% of PCM cases. Obstructive and restrictive patterns of ventilatory defect have been found in about 36 and 16% of patients, respectively; approximately 30% of these patients may die as a result of respiratory or cardiorespiratory failure [62]. Adrenal insufficiency was observed in 15–50% of patients [63]. Concerning the lymph node fibrosis, lymphatic blockage at the level of the mesenterium leads to ascytis associated with enteric loss of protein and severe cellular and humoral immunodeficiencies leading to death caused by intra- and extracellular infections [64]. Chemotherapy is able to control fungal multiplication but impairment of vital functions might lead to death [65].

Comorbidities

Concomitant TB is observed in approximately 10–15% of pulmonary PCM cases. No association has been found between PCM and neoplasias. Carcinoma has been described in the same location or close to previous fungal lesions in the lungs, mouth and upper respiratory tract [66], although controlled studies related to the high incidence of smokers in both diseases have not been performed. Immunosupression could be associated with reactivation in patients with the chronic form with pulmonary involvement followed by hematogenic dissemination seen in the acute form, which is expressed by generalized skin lesions, and involvement of the liver, spleen and lymph nodes. In kidney transplant patients, a few reported cases presented with characteristics of chronic PCM, with predominant lung involvement. These lesions were similar to those observed in immunocompetent patients but the exsudative patterns were similar to pneumonia [67–69]. In a controlled study involving HIV-infected and non-HIV-infected patients with PCM, fever, lymphadenomegaly, hepatomegaly, splenomegaly and skin lesions were more frequent in HIV-infected patients [57]. The response to therapy was 73.5 and 87% in HIV-infected and non-HIV-infected patients, respectively, and deaths owing to mycosis at 6 months of therapy were 12 and 6%, respectively.

Diagnosis

The main differential diagnosis is considering lung involvement in TB. Chest x-ray of mycosis shows asymmetrical involvement of both sides mainly in the two-thirds of the lower fields. Cavitation in the upper field similar to that observed in TB is rarely found in paracoccidioidomycosis. The diagnosis is confirmed by the presence of the fungus *P. brasiliensis* or Koch's bacillus (TB). Histoplasmosis, pneumoconiosis, cryptococcosis and coccidioidomycosis need to be considered in the differential diagnosis of lung PCM. Considering the difficultly of making an accurate clinical diagnostic to distinguish PCM and TB, western blot has been experimentally evaluated and shown to be important to confirm PCM diagnosis, even when negative results are found by other serological tests, such as double immunodiffusion [70,71]. In the acute form, involvement of regional or disseminated lymph nodes have been observed, as described for other diseases, such as histoplasmosis TB, leukemia and lymphoma. The definitive diagnosis is made by finding blast cells in the leukogram or in biopsies from bone marrow or lymph nodes. For differential diagnosis of oropharyngeal lesions, histoplasmosis, leishmaniasis and malignant tumors need to be considered; isolation of *Histoplasma* or *Leishmania* in culture or histopathology accompanied by immunohistochemistry of the lesions allow the identification of the parasite.

The gold standard for diagnosis is the identification of the fungus by direct microscopy as isolated cells, histopathology as proliferative and/or exudative reactions with granulomas containing intra- or extra-cellular *Paracoccidioides* ssp, and serological testing.

Immunological tests

Immunodiffusion (Ouchterlony) and counter immunoelectrophoresis are useful for detecting anti-*Paracoccidioides* antibodies for diagnosis when the lesions are not easily accessible and for therapeutic control. Cross reactions are mainly with other systemic mycoses, such as histoplasmosis, aspergillosis, cryptococcosis and candidiasis. Enzyme immunoassays employing nonpurified PbAgs are highly sensitive but less specific, and the use of gp43 as an antigen results in high sensitivity and specificity by ELISA. Studies evaluating polymorphisms of gp43 revealed a high level of amino acid residue substitutions between *P. brasiliensis* and *P. lutzii* [30]. In addition, the *gp43* gene is under positive selection in the *Paracoccidioides* population, which increases the genetic diversity within species, thus increasing the risks of false-negative serological results [72].

The specificity and affinity between antibodies from patients infected by *P. lutzii* and exoantigens and/or cell extracts from *P. brasiliensis* are low and the serological tests show a false-negative result. The use of exoantigens produced by the B339 reference strain showed a low level of positivity in the Rondonia state of Brazil (prevalence of *P. lutzii*) in which only 7% (in 2007) and 1.8% (in 2008) of PCM patients were positive [Durlacher R, Lima S, Unpublished Data]. Moreover, when the serological assays were carried out with exoantigens extracted from isolate 510-B (*P. lutzii* isolate) the positivity was 92.3 and 41.3% for Mato-Grosso and São Paulo patients, respectively. By contrast, when B339 exoantigens were used in serological tests, the positive recognitions were 26.2 and 100% of the sera of patients from Mato-Grosso and São Paulo, respectively, indicating geographic limits in the use of the standard exoantigen [73]. Immunoblot is used as a confirmatory test and shows more sensitivity and specificity than the serological tests. However, the cost limits its use as a diagnostic assay [74]. Also, one alternative to overcome the high levels

of negative results using gp43 could be the use of gp70 [75]. Antibody titers tend to decrease approximately 6–9 months after starting specific therapy, becoming stable after 10 months and disappearing after 1.5–5 or more years of treatment. Circulating gp43 and gp70 antigens were detected in 100% of cerebrospinal fluid and almost all serum samples of patients with neuro-PCM [76].

Prognosis

The lesions from patients with moderate or mild disease involute soon after the introduction of the treatment in the majority of cases. Normalization of cell-specific responses, particularly of the skin test (paracoccidioidin), indicates a good prognosis. By contrast, severe sequels and severe acute or chronic cases may lead to death.

PCM treatment: conventional & new approaches

Conventional & experimental therapies

The clinical aspects and treatment approaches for patients infected with *P. brasiliensis* have been reviewed by several groups [77–80]. The consensus is that there are many therapeutic options available, which include sulfone derivatives (sulfadiazine, sulfadoxine, sulfamethoxypyridazine, cotrimazine and trimethoprim–sulfamethoxazole), amphotericin B, azoles (ketoconazole, itraconazole, fluconazole, voriconazole and posaconazole) and terbinafine. The treatment option must consider the severity of disease.

For mild-to-moderate clinical forms, the standard treatment is itraconazole. A randomized trial comparing itraconazole, ketoconazole and sulfadiazine showed that all drugs are efficient at promoting the clinical cure of severity PCM. Besides, none of these drugs proved to be a better regimen comapred with the others [77]. PCM patients treated with ketoconazole showed significant failure and relapse ratios. Instead when ketoconazole was associated with other antifungal drugs it showed an increased efficacy [79]. Voriconazole is also effective for treating patients with mild and moderate PCM, and demonstrated the same effect as itraconazole in a pilot randomized clinical trial [81]. Similarly, voriconazole can be used in patients with neuro-PCM, since it penetrates better into the CNS than other drugs [82]. Beyond the severity of disease, different genotypes must be considered for treatment of PCM. Patients infected with *P. lutzii* isolates demonstrated better responses to trimethoprim–sulfamethoxazole than patients infected with *P. brasiliensis* isolates [73].

Patients with severe and disseminated forms of PCM should use amphotericin B in conventional or lipid formulations. The amphotericin B deoxycholate preparation is still used in Latin American countries despite its known adverse effects. One important aspect during the severe form of treatment is the intense inflammatory reaction. *P. brasiliensis* infection induces severe tissue damage and impairment of organ function and, eventually, fibrosis and retraction. According to Benard *et al.*, during antifungal therapies these tissue reactions are intensified and the concomitant use of corticosteroids has suggested clinical improvement and/or complication prevention. Despite the small number of patients in the study, the corticosteroids should be considered for use in PCM patients with severe and disseminated forms [83].

The PCM treatments occur over long periods of time. The main concerns during the treatment period are noncompliance, high frequency of relapses and sequels, and fibrosis. The fibrotic scars and sequels impair the patient's quality of life. Recently, it was proposed that pentoxifylline could be used as complementary treatment in the pulmonary PCM. The combined therapy with itraconazole and pentoxifylline resulted in a significantly more rapid reduction of granulomatous inflammation and pulmonary fibrosis [84].

Another strategy used to decrease fibrosis is increasing the host cellular immune response. Vaccine DNA of HSPs, such as hsp65 from *Mycobacterium leprae*, has shown prophylactic and therapeutic effects in experimental models of diseases, including TB, leishmaniasis and PCM [85,86]. The DNA vaccine used as a treatment in experimental PCM decreased the fungal burden and changed the granulomatous inflammatory reaction, with smaller and more mature granulomas and a reduction of fibrosis formation (**Figure 4**) [85,86]. Another candidate to modulate the immune response is the gp43 and its 15-amino acid inner peptide P10. gp43, the major 43 kDa antigenic glycoprotein of *P. brasiliensis*, is able to induce a protective immune response. Animals immunized with gp43 DNA showed a specific and long-lasting humoral and Th1/Th2 cellular immune response [87]. The P10 plasmid immunization induced a strong protective response and the treatment eradicated the fungus in an experimental model of PCM [88].

The P10 peptide also elicits the production of type Th-1 cytokines, such as IFN-γ and IL-12, which is important to trigger a host protective immune response and could be used as an adjuvant to treat fungal infections [89]. During

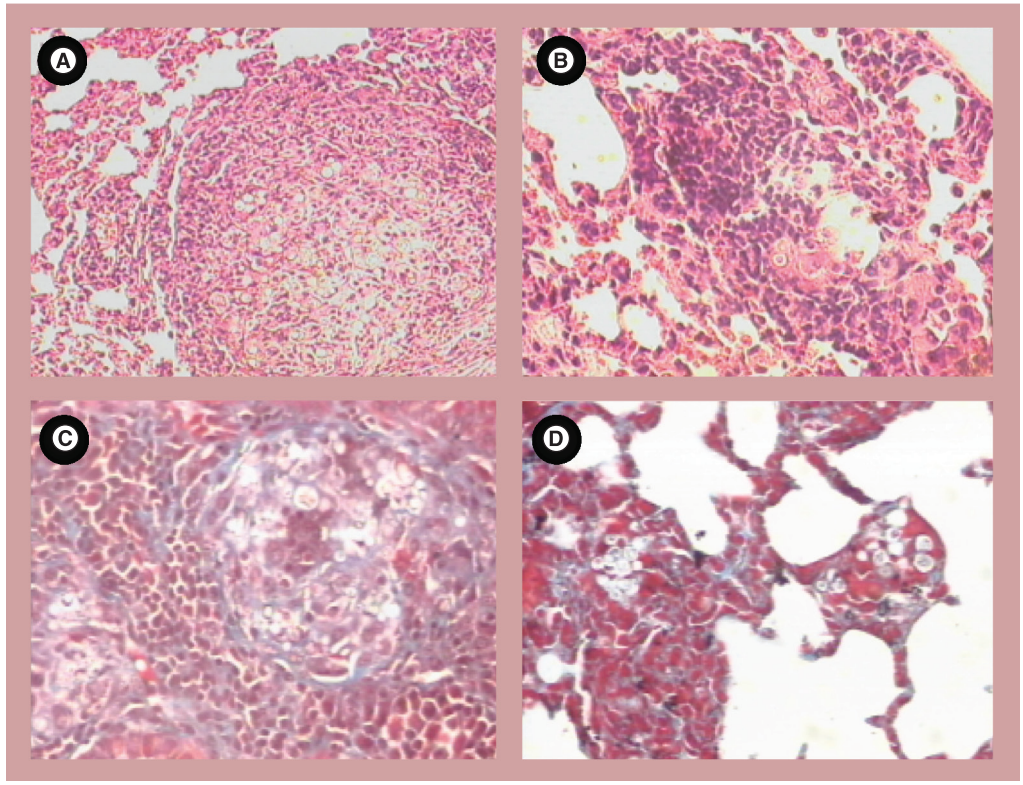

Figure 4. Histopathological analyses of animals from the pVAX1 and DNAhsp65 groups after 30 days of infection with *Paracoccidioides brasiliensis*. (A) Hematoxilin and eosin stain, animal from the pVAX1 group (200×). **(B)** Hematoxilin and eosin stain, animal of the DNAhsp65 group (200×). **(C)** Masson's stain (collagen), animal from the pVAX1 group (200×). **(D)** Masson's staom (collagen), animal from the DNAhsp65 group (200×).

experimental PCM, the immunization with P10 peptide reestablished the cellular immune response, increased IFN-γ and IL-12 levels and reduced fungal burden. Until now, none of the experimental strategies described above has been assayed in a clinical trial.

Strategies for antifungal therapy

The increase in cases of immunocompromised patients could be related to the increased number of infections caused by fungi in addition to the increased resistance to conventional antifungal agents [89,90]. There is a great demand for the development of safer alternative therapies that are able to overcome resistance. This scenario has worsened in recent years owing to the slowing of new drug launches, which is a result of the high costs of research and technological development required to bring new drugs to the market [91]. The long approval period required by regulatory agencies for preclinical and clinical trials is also a factor [90].

One new strategy is the development of more specific drugs through genomic studies. Recently, the comparative genomics analysis from *P. brasiliensis* selected ten genes that were present in eight pathogenic fungi and are relevant for fungal survival, but are absent in the human genome. The authors have proposed four new potential drug targets that could decrease the fungal burden and minimize toxic side effects [92]. Another promising strategy to overcome these bottlenecks and improve antifungal therapy is the combination of conventional drugs with sophisticated delivery systems in the nanometer scale, such as liposomes and polymeric nanoparticles. These structures modify the kinetic or dynamic properties to improve the pharmacological response [93]. These delivery systems are able to reduce the toxicity of certain drugs, they can also carry a larger quantity of drug than necessary to achieve the therapeutic response, thus reducing the applications for the patient, by allowing a slow and gradual drug release [94,95]. There are currently three nanostructured lipid formulations available for clinical use for the antifungal amphotericin B.

Nanostructured formulations for antifungals

One of the most successful examples of the application of nanotechnology to ameliorate the

antifungal efficacy is amphotericin B as liposomal formulations [96]. Despite the undoubted efficacy of this drug, it is associated with severe adverse side effects during its clinical administration [89].

Owing to its severe toxicity, several studies using nanotechnology have attempted to reduce the clinical side effects associated with the application of amphotericin B [97–100]. Formulations prepared using liposomes have demonstrated efficacy in decreasing the toxicity of this drug, in addition to increasing its activity against parasitic diseases in comparison with its conventional formulation [101]. Amphotericin B is also entrapped within polymeric nanoparticles (poly[lactic-co-glicolic acid]; PLGA) and has demonstrated antifungal activity *in vitro* and *in vivo* [102].

The association of amphotericin B within polymeric nanoparticles prepared with a polymeric blend of lactic and glycolic acid PLGA and covered with 2–3-meso-dimercaptosuccinic acid (DMSA) were tested in animals infected with the fungus *P. brasiliensis* to mimic PCM [98]. The efficacy comparison between PLGA–DMSA and conventional amphotericin B formulations indicated therapeutic efficacy comparable to the conventional formulation but with the advantage of sustained release and protection from genotoxic and cytotoxic effects caused by amphotericin B. Observing animal appearance in the PLGA–DMSA–amphotericin B-treated group revealed homogeneity, smooth coat and brightness, which was contrary to the animals treated with deoxycholate amphotericin B. Similar results of amphotericin B nanoformulation efficacy with PLGA was demonstrated in *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum in vitro* and in an acute *A. fumigatus* mouse model when compared with Fungizone® (Bristol-Myers Squibb, NY, USA) and AmBisome® (Gilead Science Inc., IL, USA) [103].

The same type of PLGA–DMSA polymeric nanoparticles were examined in another study of immunoprotective peptide delivery [102]. P10 potentiates the activity of some antifungal agents and its adjuvant effect is believed to be owing to the production of the Th-1 immune response. Immunization with P10 in addition to antifungal chemotherapy helps the organism to combat the infection of *P. brasiliensis* [104]. Based on these results and on the fact that peptides are easily degraded in the biological milieu, we incorporated P10 with PLGA–DMSA and tested it *in vivo* in association with sulfametoxazole/trimethoprim chemotherapy [102]. By incorporating P10 within polymeric nanoparticles it was possible to reduce the free peptide amount by at least 20 times to elicit the same auxiliary antifungal response. Despite the low cost and low incidence of adverse effects, sulfametoxazole/trimetoprim chemotherapy requires a long period of treatment to avoid recurrance [105]. Our results demonstrated that even interrupting the treatment, the therapeutic strategy using PLGA–DMSA nanoparticles for P10, prevented disease recurrence after 30 days of treatment. The peptide P10 was also evaluated as a DNA vaccine (pcDNA3-P10), resulting in protection of mice in therapeutic and prophylactic strategies against *P. brasiliensis*. The results have shown that long-term protection is elicited by the pcDNA3-P10 vaccine against this pathogen as well as a less aggressive infection (observed by minimal inflammation in lung histopathology) [106].

Since liposomes and PLGA particles can lead the antigen to be readily taken up into the APCs and then gradually stimulate the immune system [107], we decided to test these two nanoparticles to deliver the DNA–hsp65 vaccine. After mice treatment with both lipid and polymeric preparations, containing much less DNA than free DNAhsp65 formulation, reduced fungal growth was observed, recovering their lymphoproliferative ability and increasing the production of Th-1 cytokines and IgG2a antihsp65 specific antibodies [86].

Itraconazol is another antifungal drug that has been used in PCM therapies and it has recently been entrapped in PLGA [108] or poly(ethylene glycol)/polylactic acid nanoparticles [100] and showed increased antifungal activity compared with free itraconazol. The *in vitro* activity of PLGA–itraconazole against *P. brasiliensis* was evaluated and showed a good antifungal inhibition and lower cytotoxicity than the free drug [109].

Conclusion & future perspective

Although PCM is a disease that does not have the same global impact when compared with other infections, gaining a better understanding of the pathology may help us to address antifungal resistance in the future. Epidemiological studies as well as the species origin of *Paracoccidoides* should be elucidated by new strategies, such as the direct detection of isolates recovered from armadillos and identifying *Paracoccidioides* sp. in aerosol samples, which may allow us to overcome the uncertainty regarding host migration and the long latency of PCM. Moreover, the high genetic divergence described in the genus *Paracoccidioides* and its implications on diagnosis and treatment should be pondered by clinicians and medical

staff. New strategies, such as a standard exoantigen produced by different strains of the genus *Paracoccidioides*, should be developed in order to increase the serological test specificity.

Considering their particular characteristics, this disease and its etiologic agent can be explored in experimental models of fungal infections to assist with the development of new therapies, such as DNA vaccines and nanostructured drugs, but also to elucidate immunological aspects involved in the host–parasite relationship. In the future, we expect more efficient therapies to be available that are capable of reducing the treatment time and toxic side effects. However, efforts must still be made to resolve the sequelae caused by this infection, such as pulmonary fibrosis. Owing to observed differences in the treatment of PCM caused by distinctive species of the *Paracoccidioides* genus, efforts among regional hospitals and research center staff will improve disease knowledge.

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Executive summary

Epidemiology & ecology

- Paracoccidioidomycosis (PCM) is caused by the human fungal pathogens *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. PCM has been the greatest cause of mortality among systemic mycoses in Brazil and the eighth most important cause of mortality from chronic infectious diseases.
- The disease is geographically restricted to central and South America, mainly affecting individuals who live in rural areas and engage in agricultural activities.
- The species from the *Paracoccidioides* genus are thermodimorphic fungi that live as saprobic mycelium in soil, decaying organic material as a source of nutrients. When infectious propagules are inhaled, the morphology of the fungus shifts to a pathogenic yeast form by raising the temperature to 36–37°C.

The Paracoccidioides *genus & species recognition*

- The genus *Paracoccidioides* is placed in the thermodimorphic fungal pathogens from the family Ajellomycetaceae, order Onygenales.
- *P. brasiliensis* and *P. lutzii* can be discriminated by genomic and morphological characters and the main differences are exposed.
- *P. brasiliensis* harbors a complex of at least four different cryptic species (S1, PS2, PS3 and PS4) that are characterized by different evolutionary traits and geographic distributions.

Clinical aspects & treatment

- The clinical forms of PCM disease are classified as the acute/subacute form (juvenile type) and chronic form (adult type).
- The acute form is characterized by depression of the cellular immune response with low levels of IFN-γ production.
- Considering the *Paracoccidioides* genus, there is not a standard exoantigen in serological tests.
- The therapeutic options to treat PCM patients are sulfone derivatives, amphotericin B, azoles and terbinafine. New experimental approaches have been tested including DNA vaccine and nanostructured formulations.
- The PCM treatment occurs over long periods with high incidences of noncompliance. The elevated frequency of relapses and sequelae are an important concern.

Conclusion & future perspective

- Owing to the observed differences in the treatment of PCM caused by distinctive species of *Paracoccidioides* genus it is expected in the future, more efficient serological diagnostics and therapies capable of reducing the treatment time, toxic side effects and decrease the sequelae caused by this infection will be developed.

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▪ **Postgenomic analysis providing relevant information on future drug development.**

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