Héctor M. Mora-Montes
Leila M. Lopes-Bezerra *Editors*

Current Progress in Medical Mycology

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Preface

It is estimated that the Kingdom Fungi groups have about five million species, and they significantly contribute to the biological interactions that sustain and shape the different ecosystems on Earth. The heterotrophic nature of fungi forces them to interact with other living organisms, and besides some extremophile species, all organisms, unicellular or multicellular, are susceptible to be a source of nutritive macromolecules for the fungal cell. In most of the cases such interaction is established with death cells and tissues, and fungi play a beneficial role helping to clean the environment and recycling the basic building blocks of biomolecules. However, when the interaction occurs with other organisms this could lead to the establishment of mutualistic, commensalism or parasitism. The latter can potentially affect the health and physiological status of the host, leading to the establishment of a disease. Fungi can parasite other fungi, plants, and animals, including the human being. The number of fungal species that cause infections in humans are just a handful, if compared with the fungal biodiversity currently known. It is important to notice that new emerging species adapted to mammalian parasitism are increasing. After viral infections, mycoses are the most frequent infections in humans and are of outstanding interest because the morbidity and mortality associated to them. Depending on the tissues affected, we can classify mycoses as superficial, subcutaneous and systemic or deep-seated infections; and they can be caused by primary pathogens or opportunistic species. Opportunistic fungal infections are of special interest because they are a consequence of a temporal or permanent immunodeficiency, and are associated to high mortality rates. Despite we have several antifungal drugs to treat fungal infections, the diversity is not enough, as some fungal species are naturally resistant to the therapeutic alternatives, or they develop or acquire drug resistance faster than the development of new drugs to combat these pathogens. This book offers a comprehensive overview of medical mycology.

Written by scientists and physicians, it addresses basic aspects of the causative agents and the clinical manifestations of the most common and relevant human mycoses, as well as their diagnosis and the therapeutic alternatives. Further, it discusses the immune response, virulence factors and the development of modern molecular diagnostic tools.

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Chapter 1
Introduction to Medical Mycology

Arunaloke Chakrabarti and Nandini Sethuraman

Abstract Fungi are ubiquitous and nearly 1.5 million fungal species exist in the universe, but only few fungi were known to be pathogenic to humans. The temperature of 37 °C, low redox potential in tissues and immune barrier prevent majority of fungi from invading human hosts. However with the change of host environment due to co-morbidities, several saprophytic fungi get the opportunity to adapt to human tissue. The impact of modern medical interventions and fungi adapting to this environment are reasons why many fungi are now known to cause invasive human disease. Fungal infection in humans varies from superficial colonization to invasive diseases and allergic manifestations; each group associated with unique risk factors. Impact of the global burden of fungal infections is often overlooked and estimates are often extrapolations of limited available data. There is also wide variation in the geographical niches occupied by fungi across the world for reasons largely unknown. Natural disasters also contribute to the upsurge of invasive fungal infections. The severity of infection closely parallels a suppression of immunity. Invasive fungal infections are important causes of morbidity and mortality of hospitalized patients. Diagnosis of these fungal infections is often a challenge due to poor sensitivity, specificity and long turnaround times of conventional diagnostic methods. Availability of advanced, rapid technologies is sparse in the majority of laboratories in developing countries. Several biomarkers and molecular techniques are being studied to reduce these turnaround times. In spite of availability of new antifungals, therapy is often empirical due to rapid progression of disease and non-availability of early diagnosis. There is no ideal antifungal agent. Limited spectrum, drug-interaction, toxicity and cost of antifungals are limiting factors. A brief description of these challenges is presented in this introductory chapter.

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1.1 Definition and Structure

Fungi are aerobic, eukaryotic, heterotrophic, spore-bearing, non-motile saprobes that range from microscopic unicellular forms known as yeasts to multicellular moulds to complex forms that give rise to edible mushrooms. True to their widely used description of being ubiquitous, they can be found in virtually any habitat ranging from dry to humid, tropical to temperate, deserts to deep sea and symbionts to parasites. They reproduce by sexual as well as asexual means. The sexual stage may be seen in a few homothallic fungi, but is largely unknown in majority of fungi (mitosporic fungi). A characteristic feature unique to fungi is the presence of a rigid cell wall composed mainly of chitin and glucans that makes them impenetrable by many substances including antibacterial compounds. There are approximately 1.5 million species of fungi of which around 500 species have the propensity to cause disease in humans and are studied under the term 'medical mycology'.

Morphologically, fungi are broadly divided into two groups:

1. Yeasts: The unicellular forms that reproduce by budding, also known for blastospore formation. Some species produce pseudohyphae, which are elongated, jointed yeast cells with no cell-to-cell continuity or communication. The pseudohypha is differentiated from true hyphae by the presence of a constriction at the junction of the two cells and branching at the constricted point. Macroscopically, yeasts produce moist and creamy colonies. The most common medically important yeasts belong to *Candida* and *Cryptococcus*.
2. Moulds: Filamentous, multicellular forms that exhibit complex structures depending on their sexual or asexual mode of reproduction. The basic unit of a mould is a hypha, which is a filamentous structure that may or may not be divided by septa. A group of hyphae is known as mycelium. Macroscopically mycelia exist both basally (vegetative) and aerially. Asexually reproducing fungi form fruiting bodies that arise from the vegetative mycelia as conidiophores that end up forming conidia. The method of conidiation, structure and arrangement of conidia are useful tools for morphological identification of many moulds. Sexually reproducing fungi produce additional spore sacs denoted by various names, all of which help in morphological identification of fungi.

   Moulds, which are broad ribbon like and lack septa or sparsely septate, are the agents that cause mucormycosis and entomophthoramycosis. They reproduce sexually as well as asexually by way of zygospore and sporangiospore formation respectively. Moulds with septate hyphae are classified under *Ascomycota* and *Basidiomycota*. The majority of the medically important fungi belong to *Ascomycota* with few exceptions. The evolutionary advantage of septations is that even if a part of the hypha is damaged, the rest of the hypha survives as against primitive aseptate hyphae which are continuous and are completely damaged.
3. Dimorphic fungi: Apart from the above two broad categories, certain fungi exist as yeasts in human tissues and specialized media at 37 °C, whereas in the environment at room temperature, exist as moulds. These are phenotypically classified as dimorphic fungi (Fig. 1.1).

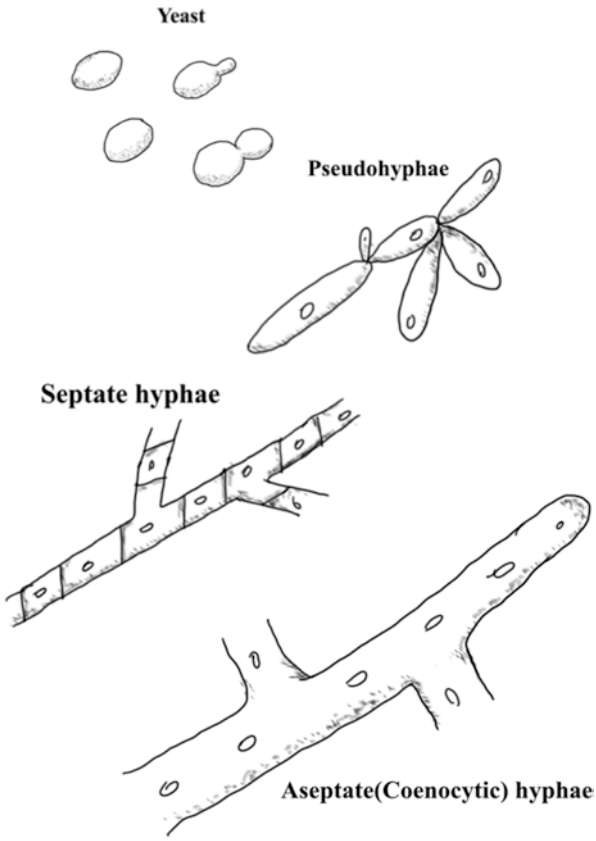

Fig. 1.1 Fungal cell morphology

1.2 Emergence of Challenge Due to Fungal Infections

The progress in modern medicine closely parallels the emergence of invasive fungal infections. Historically, with the exception of dermatophytes and pathogenic dimorphic fungi, few other fungi were known to be pathogenic in immunocompetent individuals. The scenario changed over the last few decades, with the entry of AIDS, immunosuppressive and immunomodulatory therapy, solid and stem cell transplants, frequent use of broad-spectrum antibiotics, aging population staying longer in hospital with many co-morbidities, extensive abdominal surgeries and use of invasive devices and lifestyle diseases such as diabetes. Fungi could access these patients easily. Many new saprophytic fungi could adjust to earlier known hostile human tissue environments and possibly acquired virulence factors while passing through predator soil amoebae [1]. This has led to tremendous rise in number of opportunistic fungal infections.

We face several challenges while managing opportunistic fungal infections: (1) patients do not have characteristic signs or symptoms suggesting fungal infec-

tions; (2) localization of infection often requires sophisticated radiological and laboratory support; (3) awareness about invasive fungal infections among clinicians is limited to specialized units only; (4) appropriate sampling of infected tissues often requires invasive procedures that may be difficult in the immunocompromised cohort; (5) conventional diagnosis like direct microscopy, culture and histopathology have poor sensitivity and high turnaround time; (6) although there is transformational change in diagnostics over the last decade (MALDI-TOF, sequencing, beta glucan assay, galactomannan, etc.), the availability of these advanced tests is patchy in developing countries; (7) there are limited antifungal drug target sites as fungi are eukaryotic and there is non-availability of many antifungal drugs in developing countries; (8) the majority of antifungal agents are fungistatic and cannot get rid of fungi from tissue in absence of host immunity in immunosuppressed patients; (9) antifungal drug dosing needs strict titration as therapeutic window is often narrow with most of the available antifungal agents; (10) antifungal drugs like azoles have plenty of drug interaction issues and requires regular therapeutic drug monitoring; (11) toxicity of antifungal drugs; (12) the intrinsic antifungal resistance in many species and recent development of acquired resistance; (13) the emergence of multi-drug resistant superbugs like *Candida auris* across globe; (14) the cost of antifungal therapy and poor antifungal stewardship programs which leads to heavy economic burden while managing these patients.

1.3 Spectrum of Fungal Infections

1.3.1 Superficial Mycoses

1. Cutaneous—affecting skin and mucous membranes. Cutaneous infections are usually caused by dermatophytes, *Candida* and *Malassezia* species (Pityriasis versicolor) in healthy and compromised individuals. The infection occurs in moist folds of the skin in tropical climates and is treatable with long-term topical and occasional systemic antifungals.
2. Subcutaneous tissue—These are commonly acquired during traumatic implantation (implantation mycoses). Common diseases include sporotrichosis, chromoblastomycosis, phaeohyphomycosis and mycetoma. The lesions are often disfiguring and multiple in individuals who are immunosuppressed. Sporotrichosis is caused by a dimorphic fungus- *Sporothrix schenckii* sensu *lato*. Chromoblastomycosis and phaeohyphomycosis are caused by melanized fungi. Mycetoma can be either bacterial (actinomycotic) or fungal (eumycotic) origin. Rhinosporidiosis caused by *Rhinosporidium seeberi* and lacaziosis (lobomycosis) caused by *Lacazia loboi* are also grouped under subcutaneous fungal infections due to similarity of infection, though the causative agents are no longer classified under Kingdom Fungi.

1.3.2 Invasive/Opportunistic Mycoses

In general, it is believed that invasive fungal infections or opportunistic mycoses occur when patient's immunity is compromised. Both innate and acquired immunity play important roles against fungi. When cell-mediated immunity is compromised (as in HIV infection), infections like cryptococcosis, endemic fungal infections are prevalent. On the other hand during the fall of innate immunity or neutropenia (patients with malignancies undergoing chemotherapy, transplant recipients, etc.), invasive candidiasis, aspergillosis, mucormycosis and several other mould infections prevail. These infections may present in the form of fungaemia, invasive pulmonary infections, central nervous system infections, disseminated infections, etc.

Many saprophytic fungi (considered non-pathogenic) are also reported to cause human infection even in apparently healthy hosts. These new fungal diseases revealed many new immunodeficiencies. The deficiencies like CARD9 or STAT1in signal transduction pathway lead to frequent deep dermatophytosis, central nervous system candidiasis, disseminated phaeohyphomycosis, aspergillosis and mucormycosis (primary immunodeficiency). The use of anti-TNF-α agents has increased due to the dramatic response in autoimmune disorders, but it leads to increased occurrence of endemic mycoses, aspergillosis, pneumocystosis and cryptococcosis (acquired immunodeficiency).

1.3.3 Endemic Mycoses

These are a group of infections caused by a distinct set of fungi which have restricted geographical endemicity and common morphological characteristics of exhibiting thermal dimorphism, i.e. the ability to switch from yeast or spherule form in the tissue phase at 37 °C to mould form at room temperature. They also share the ability to cause invasive infections even in immunocompetent individuals. This group includes blastomycosis, coccidioidomycosis, histoplasmosis, paracoccidioidomycosis and penicilliosis. A new endemic mycosis, emmonsiosis has emerged in AIDS patients of South Africa and sporadic cases have been reported from Italy, India, China and Germany [2, 3]. With the application of molecular tools, the taxonomy of some of these agents has altered or new cryptic species have been explored. The agent causing penicilliosis is now called *Talaromyces marneffei* and many cryptic species have been identified under *Blastomyces* and *Sporothrix.*

1.3.4 Allergic Manifestations

Allergic mycoses occur as a result of hypersensitivity to many fungi. The systemic manifestations range from fungal asthma including allergic bronchopulmonary aspergillosis and severe asthma with fungal sensitization, allergic alveolitis, allergic fungal rhinosinusitis and localized or systemic allergy to many fungi.

1.4 Classification

Fungi were originally classified along with plants in kingdom *Plantae* when Carolus Linnaeus in 1735 classified all living material in two kingdoms. As the understanding of their distinct nature developed, they were placed separately as a fifth kingdom by Whittaker in 1969 [4], on the basis of their mode of reproduction, heterotrophic nutrition, saprobic (living on dead organic matter) existence, cellular structure and lack of motility [4]. With the current level of understanding of taxonomy and molecular phylogeny, we know that over 1.5 million fungal species exist on the planet. The fungi have been placed as a separate kingdom *Fungi* under superkingdom *Eukaryota* in the Catalogue of Life database, which classifies all life into seven kingdoms [5]. The main characteristic of this Kingdom is the presence of chitin (also found in arthropods) along with beta-glucans (also found in plants as beta 1,4 glucans) in their cell wall. Evolutionarily related parasites belonging to *Oomycota* have been separated and placed in kingdom *Chromista*. A simplified, clinically useful classification of fungi is presented in Table 1.1.

Table 1.1 Clinically useful classification of medically important fungi

Type	Prominent members
Yeasts	*Candida* spp. *Cryptococcus* spp.
Hyaline fungi	*Aspergillus* spp. *Fusarium* spp. *Penicillium* spp. (other than *Talaromyces marneffei*) *Paecilomyces* spp. *Scedosporium* spp.
Black/Dematiaceous fungi • Chromoblastomycosis • Phaeohyphomycosis (subcutaneous and systemic)	*Cladophialophora bantiana* *Phialophora* spp. *Fonsecaea* spp. *Bipolaris* spp. *Curvularia* spp. *Alternaria* spp. *Ochroconis gallapova*
Dimorphic fungi	*Histoplasma capsulatum* *Blastomyces dermatitidis* *Coccidioides* spp. *Paracoccidioides* spp. *Talaromyces marneffei* *Sporothrix* spp.
Coelomycetes (also cause phaeohyphomycosis)	*Pyrenochaeta* spp. *Phoma* spp. *Natrassia mangifera* *Colletotrichum* spp.
Fungus-like organisms	*Pneumocystis jirovecii* (placed in *Fungi*) *Pythium insidiosum* (placed in *Chromista*) *Rhinosporidium seeberi* *Lacazia loboi*

One-Fungus One-Name Concept Fungi with known sexual stage have two names, one for anamorph (asexual) and other for teleomorph (sexual) stage. For example, the anamorph *Cryptococcus neoformans* is also known as *Filobasidiella neoformans*, which is its teleomorph stage. This was convenient as long as microscopy was the basis of classification, though it remained confusing. With the advent of genotypic methods, the dual nomenclature of a single organism is neither necessary nor relevant. The Amsterdam Declaration on Fungal Nomenclature recommended the abolition of Article 59 of the Code of Botanical Nomenclature, the provision that sanctioned multiple names for the same fungus, and a new Code of Nomenclature for Algae, Fungi and Plants was created from 1 January 2013, which does not permit dual nomenclature, advocating the one-fungus one-name concept [6]. Anatomical classification of fungi into *Coelomycetes*, *Hyphomyctes*, Zygomycetes have all become redundant. Another advantage of the phylogenetic approach is that close relatives come together even if they may be morphologically quite different. This may be useful for predictions of pathogenicity or antifungal susceptibility. Conversely, distant relationships are expected to predict large differences in clinically relevant parameters. For example all *Candida* species have been grouped together due to morphological and physiological similarities but have huge evolutionary distance responsible for varying antifungal susceptibilities [7]. However, approximately 2000 genus and 10,000 species names need to be reassessed, though not all names will necessarily change. Hopefully medically important species names will be conserved like *Cryptococcus neoformans*. But it will take many years, cost huge money, as all databases would require validation and updating. Many gaps still exist in the understanding of the 'real' phylogenetic tree of the Fungi, and the nomenclature adaptation will be a continuous process with more understanding of taxonomy. Newer techniques like whole genome sequencing have made the classification of fungi at more stable platform.

1.5 Epidemiology of Fungal Infections

1.5.1 Global Burden of Disease

Burden of fungal infections on human health and economy is often overlooked as is exemplified by the fact that the World Health Organization (WHO) has no program on fungal infections. Estimates of global burden are often extrapolations of data available from limited single centre studies or sentinel surveillance sites. Superficial fungal infections constitute the major share of all fungal infections. It is known that about 25% of the worldwide population suffers from fungal infections of the skin and nails at any given point of their lifetime [8]. These infections refer to conditions commonly known as athletes' foot, ringworm, onychomycosis, etc. caused by dermatophytes and *Candida* species. Superficial acute and chronic mucosal infections of the gastrointestinal and genital tracts are also common. It is known that about 50–75% of all women in the reproductive age group suffer from vulvovaginal candidiasis

(VVC) and about 492 million women have recurrent VVC (RVVC) in their lifetime [9, 10]. With the advent of HIV, the numbers of oral and oesophageal candidiasis have gone up remarkably. While these conditions lead to personal and social stigmata, they are usually manageable in the long term with appropriate therapy.

The challenge due to invasive fungal infections is gaining more importance. The global burden of top ten deep seated fungal infections is given in Table 1.2 (modified from [11, 12]). The top five killers among fungi are *Candida*, *Aspergillus*, *Cryptococcus*, *Mucorales* and *Pneumocystis jiroveci* with an average mortality associated with these conditions being around 50%. The predominant pathogen in fungal sepsis is *Candida* and is responsible for 80% of all nosocomial fungal infections and 15% of nosocomial infections overall [13]. With the advent of fluconazole prophylaxis, the rise in incidence of candidemia has been checked considerably in the USA. However non-albicans *Candida* species with inherent resistance to fluconazole are on the rise. This is especially true for *C. glabrata* and *C. krusei* [14]. In many regions of the world, *C. albicans* candidemia still predominates, but the number of cases with non-albicans *Candida* is increasing. *C. tropicalis* or *C. parapsilosis* are the second leading cause rather than *C. glabrata* in developing countries. In tropical region of Asia, *C. tropicalis* is the most common agent and *C. albicans* has dropped to third or fourth in frequency. The true burden of fungal infections is not known and underestimated in developing countries. There are several conditions that allow these infections to thrive in tropical climate: the large economically deprived populations with malnutrition, limited access to health care, improper antibiotic stewardship practices and steroid use and over the counter availability of these drugs add to this problem. Natural disasters like tsunami, tornado, earthquake, etc., predispose to large land upheavals leading to high spore counts in the atmosphere and implantation of saprophytic fungi from soil due to injuries during disasters.

1.5.2 Predisposing Factors for Fungal Infections

The common risk factors for most invasive fungal infections include:

- Neutropenia
- Cytotoxic chemotherapy
- Immunosuppressive therapy for autoimmune disorders
- Haematological malignancies
- Cancer chemotherapy
- HIV—AIDS
- Haematological and solid organ transplantation
- Extremes of age
- Diabetes mellitus
- Extensive trauma and burns
- Prolonged antibiotic therapy
- Total parenteral nutrition
- Central vascular access devices

Table 1.2 Morbidity and mortality of top ten significant fungal infections (adapted from Brown et al. [11] and Vallabhaneni et al. [12])

Opportunistic mycosis (organism)	Location	Life-threatening new infections per year	Mortality (%)	Comments
Candidemia (*Candida* species)	Worldwide	>400,000	46–75	Incidence of 4–14 per 100,000 population
Aspergillosis (*Aspergillus* species)	Worldwide	>200,000	30–95	Annual incidence in transplant recipients is 0.65%, second only to candidiasis
Mucormycosis (*Rhizopus*, *Lichtheimia, Rhizomucor, Mucor*, *Apophysomyces*, etc.)	Worldwide	>10,000	30–90	Mucormycosis in India is very high in number among the diabetics, which is predicted at 70 times the rate in other countries
Pneumocystosis (*Pneumocystis jirovecii*)	Worldwide	>400,000	20–80	Opportunistic infections mainly in HIV but also in transplant recipients, malignancies, etc.
Cryptococcosis (*Cryptococcus* species)	Worldwide	>1,000,000	20–70	Prevalence is high in HIV infection with 700,000 cases annually in sub-Saharan Africa. *C. gattii* commonly infects immunocompetent hosts
Endemic mycosis				
Histoplasmosis (*Histoplasma capsulatum*)	Worldwide distribution, the endemicity demarcates well in Midwestern United States	~25,000	28–50	African histoplasmosis (*H. capsulatum var. duboisii*) is restricted to Africa and Madagascar
Coccidioidomycosis (*Coccidioides immitis, C. posadasii*)	Southwestern USA, Mexico, some parts of Central and South America	~25,000	<1–70	The numbers increase during earthquake in endemic zone
Blastomycosis (*Blastomyces dermatitidis*)	Midwestern and Atlantic USA	~3000	<2–68	Also reported in Canada. Incidence of 0.3–41 cases per 100,000 population. In Asia, reported from India
Paracoccidioidomycosis (*Paracoccidioides brasiliensis*)	South of Mexico to Argentina, high number in Brazil	~3000	5–27	Incidence of 0.2–2 per 100,000 population in endemic area
Penicilliosis (*Talaromyces marneffei*)	Southeast Asia	>8000	2–75	The number of cases decreased after introduction of anti-retroviral therapy

1.5.3 Geographical Distribution

The distribution of majority of opportunistic fungal infections is independent of geography as they are present ubiquitously in the environment or are commensals in humans. However, due to socioeconomic diversity and better control of lifestyle related diseases like diabetes in the developed world, some infections are more common in Southeast Asia, Africa and Latin America. The incidence of mucormycosis is highest in India especially in uncontrolled diabetic patients and has been reported at 1.6 cases per 1000 consecutive diabetics [15]. Human pythiosis due to opportunistic oomycete *Pythium insidiosum* remains almost exclusive to Thailand and neighboring Southeast Asian countries [16]. Trichosporonosis in Japan and south Asian countries, scedosporiosis in Australia and Spain, fusariosis in Brazil, phaeohyphomycosis due to *Rhinocladiella mackenziei* in the Middle East Asian region are prevalent opportunistic fungal infections in the respective regions [17–19]. Neglected tropical disease like mycetoma is prevalent in Sudan, India and a few Latin American countries possibly due to life style and existence of causative agents in the environment [20]. Endemic mycoses, as the name suggest are endemic with restricted geographic distribution (see Table 1.2).

1.5.4 Community Acquired Fungal Infections

Apart from endemic fungi and superficial infections, community acquired fungal infections are most commonly seen in immunocompromised individuals and are frequently opportunistic in nature. One of the common opportunistic infections seen mainly in HIV patients is cryptococcal meningitis. This is common among developing countries of Africa and Southeast Asia which parallels the burden of HIV. *Pneumocystis jirovecii* is a pathogen that causes opportunistic pneumonia in HIV infected individuals. Community acquired candidemia is rare. However according to the SENTRY surveillance data, about a third of the candidemia in the US is community acquired. This is attributable to the increasing preference for home based health care in developed countries [21]. When immunosuppressed patients undertake gardening without protection, they may acquire *Aspergillus* and many other saprophytic fungi from environment. Construction activities and natural disasters predispose individuals to aspergillosis, mucormycosis and invasive mould infections especially when the individual is immunosuppressed.

1.5.5 Nosocomial Fungal Infections

Though other than dermatophytes and *Malassezia* infection, fungal infections are considered non-contagious, nosocomial transmission of fungal infections is not rare. *Candida* and related yeasts may spread from the hands of health-care providers to the patients. Several outbreaks have been reported in the hospitals across the world.

In a single outbreak in India 379 babies suffered from *Pichia anomala* fungaemia [22, 23]. Analysis of the outbreak revealed that the transmission of this agent from the index case to the subsequent cases occurred through the hands of a resident doctor. Fungi from environment also get access to the patients due to breach of physical barrier (intravascular catheters) or through respiratory route. The most recent nosocomial outbreak involving contaminated methylprednisolone injections, which were directly inoculated intraspinally, led to an outbreak of fungal meningitis claiming over two dozen lives in USA. The agent identified in this outbreak was *Exserohilum rostratum*, an extremely rare black fungus causing fungal meningitis [24, 25].

1.6 Fungal Infection Immunity and Pathogenesis

Fungi are present ubiquitously in the environment. Some may reside on human body as resident or transient commensals. In order to co-exist, the human immune response strikes a balance between invading fungi and host resistance. When the balance is lost, fungal infections occur. Both innate and adaptive immunity play a role in protection against fungal infections.

1.6.1 Innate Immunity

Primary immunity to fungal infections is provided by barriers—both skin and mucosal barriers of respiratory, gastrointestinal and genitourinary epithelia, which are continually in contact with fungi [26]. Cells of innate immunity such as monocytes, macrophages, neutrophils and even epithelial and endothelial cells contribute to fungal clearance by phagocytosis aided by opsonic recognition of cell wall components by defensins, collectins and complement. Neutropenia is one of the most common underlying factors for development of invasive candidiasis, aspergillosis and mucormycosis. The functional neutropenia seen in diabetic ketoacidosis underlies development of mucormycosis prevalent in India.

Host cells express pathogen recognition receptors (PRRs) that specifically recognize pathogen associated molecular patterns (PAMPs) and initiate downstream signalling pathways. The most important PRRs in antifungal immunity are toll-like receptors (TLRs) and C-type lectin receptors (CLRs). Of these, Dectin 1, TLR 2 and 4 play important role in fungal infections (Table 1.4).

1.6.2 Adaptive Immunity

Although both cellular and humoral arms are involved in immune response to fungi, it is the T cell-mediated immunity that is mainly protective (Table 1.3). The capacity of dendritic cells (DCs) to initiate different adaptive antifungal immune responses

Table 1.3 T cell Subsets in Antifungal Immunity

T cell subset	Function	Key cytokines	Susceptibility in deficiency
Th1	Protective	IFNγ	Overwhelming opportunistic fungal infections
Th2	Dampens Th1, promotes fungal allergy and relapses, role in antibody production	IL-4, IL-5, IL-13	Th2 reactivity- allergic broncho-pulmonary aspergillosis, allergic fungal rhinosinusitis
Th17	Promotes Th1 and depresses Th2	IL-17A, IL-22	Th17 hyperactivity-role in chronic fungal infections
Treg	Regulation of other Tcell subsets	TGFβ, IL-10	Fungal persistence to immunosuppression

depends on balanced activation of DC subsets. Inflammatory DCs initiate antifungal Th17 and Th2 cell responses through signalling pathways involving the TLR adaptor MYD88, whereas tolerogenic DCs activate Th1 and regulatory T (TReg) cell differentiation through TRIF (TIR domain-containing adaptor protein inducing IFNβ). The multiple, functionally distinct, receptor signalling pathways in DCs ultimately affect the balance between CD4+ effector T cells and Treg cells and thus are likely to be exploited by fungi to enable them to establish commensalism or infection.

1.6.3 Fungal Virulence

Several mechanisms are involved in fungal pathogenesis. Fungi produce several virulence factors involved in adhesion, tissue invasion and evasion of immune response. The tough fungal cell wall composed of various polysaccharides has many roles to play (Table 1.4) (Fig. 1.2). Primarily it protects the organism from external environmental stresses and also resists turgor pressure generated inside the cell during hyphal growth. Melanin of many pigmented fungi is involved in evasion of immune response by preventing opsonization and binding of C3 to the fungal cell wall, as well as recognition by PRRs [27]. Chitin blocks dectin 1 and the subsequent cytokine production [28].

Tissue invasion is assisted by formation of pseudohyphae and true hyphae in tissues. Elaboration of phospholipases, extracellular proteases such as secreted aspartyl proteinases (SAPs) and other hydrolytic enzymes are important especially for *Candida* species [29]. Rapid growth, angioinvasion and heat tolerance are important features for *Aspergillus* and mucormycosis agents. Cot III (invasin) has been recognized as important virulence marker of *Mucorales*, which act as a ligand for endothelial cell glucose-regulated protein 78 (GRP78) receptor causing angioinvasion. Other specific virulence mechanisms are shown in Table 1.5.

Table 1.4 Important PAMPs and their effects on host immunity

Pathogen associated molecular pattern (PAMP)	Pattern recognition receptor (PRR)	Cytokine signalling/ effect	Cytokines activated	Fungal infection related to deficiency
β-glucans	Dectin-1	CARD9 pathway and RAF pathway	NLRP3 inflammasome-IL-1β, IL-18, ROS	Chronic mucocutaneous candidiasis, invasive aspergillosis
High mannose structures—hyphae > yeasts	Dectin-2	CARD 9 pathway	NLRP3 inflammasome-IL-1β, IL-18, ROS	*C. albicans*, recurrent vulvovaginal candidiasis
Damaged cells, polysaccharides of *Malassezia, Candida*	Mincle	CARD 9 pathway	NLRP3 inflammasome-IL-1β, IL-18, ROS	*C. albicans*, recurrent vulvovaginal candidiasis
N-linked mannans	DC-SIGN	DC endocytic pathway	IL-10, activation of T cell subsets	
N-linked mannans, α-glucans, chitin	Mannose receptor	DC endocytic pathway	Activation of T cell subsets including TH17	*cryptococcosis*
Zymosan, phospholipomannan, *O*-linked mannans and fungal DNA	TLRs- 2,4 and 9	MyD88 pathway	Antigen presentation by DCs and Tcell activation	TLR4- pulmonary aspergillosis, candidemia TLR9- ABPA

1.6.4 Tissue Reaction

Tissue reaction to various fungi depends on the infecting fungus as well as the immune status of the host [30]. It ranges from suppurative changes in acute infections to granulomatous and fibrotic changes in subacute to chronic infections (Table 1.5). Broadly, yeasts or moulds can be visualized on histopathology. Some useful pointers are shown in Table 1.5.

1.7 Diagnosis of Fungal Infections

Superficial infections can be diagnosed easily in the outpatient department as they often produce characteristic lesions. A Wood's lamp examination helps in diagnosis of dermatophytosis and a potassium hydroxide (KOH) mount of skin scrapings helps to identify the hyphal forms. Calcofluor white fluorescent stain improves sensitivity of direct microscopy. The challenge lies in the diagnosis of deep invasive infections whose clinical manifestations are non-specific and require a high index of clinical suspicion. The setting of infection, the immune status of the host, residence,

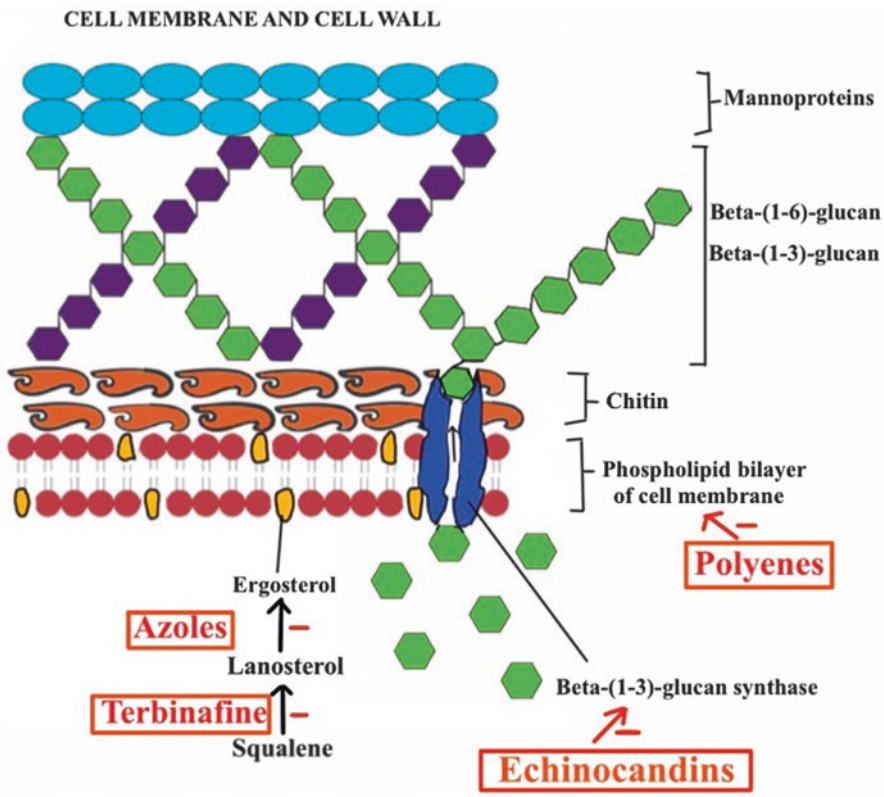

Fig. 1.2 Schematic diagram of the fungal cell wall

travel and occupational history are clinical pointers to the type of infection. Available diagnostic tests are far from perfect and the gold standard tests lack sensitivity and are of long turn-around times. Sample collection from deep sites of infection is also difficult due to thrombocytopenia in these patients. Though European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) a consensus definition has been laid down criteria for proven, probable and possible invasive fungal infections to serve as case definitions in clinical trials [31]. Many clinicians use the same criteria to diagnose invasive fungal infections in their routine clinical practice. Briefly, a proven IFD is defined as histopathological, cytopathological or direct microscopic evidence or culture of yeasts or moulds in a specimen derived from a sterile site. In the absence of these diagnostic criteria, other predisposing host factors, clinical clues and indirect evidences in the form of imaging, serological tests and biomarkers are used to define a probable or possible IFD.

Table 1.5 Pathology of common fungal infections

Organism	Clinical features	Virulence	Host tissue reaction	Morphology on H&E, PAS and GMS
Yeasts				
Candida	Superficial infections to invasive health care associated disease	Adherence and biofilm formation, tissue invasion using secreted aspartyl proteinases, phospholipases. Yeast to hyphal switch	Suppurative inflammation, angioinvasion, necrotizing vasculitis	Small yeasts of 3–5 μm size with or without pseudohyphae and occasionally true hyphae
Cryptococcus	Pneumonia, pleural effusion, chronic meningitis, cryptococcomas	Polysaccharide (glucuronoxylomannan) capsule evades immune response, melanin	Granulomas in lung and skin, various inflammatory reactions depending on immune status, mucinous and occasionally fibrotic granuloma	Narrow based budding yeasts of various sizes between 4 and 10 μm with thick capsule
Dimorphic fungi				
Histoplasma	Acute pneumonia, chronic pneumonia, mediastinitis, disseminated disease	Dimorphism, endotoxin-like substance, collagenases and elastases, siderophores and ability to neutralize acidic pH of macrophages	Nodules with vascular necrosis and lympho-histiocytic vasculitis to granulomatous inflammation with abundant intracellular yeasts	Small yeasts (2–4 μm) with narrow-based budding grouped in clusters inside macrophages
Blastomyces	Acute and chronic pneumonia or disseminated and cutaneous disease	Dimorphism, Blastomyces adhesion (BAD1), α-glucan	Mixed suppurative and granulomatous inflammation	Broad-based budding yeasts (10–15 μm)
Coccidioides	Acute and chronic pneumonia or disseminated and cutaneous disease	Dimorphism	Mixed suppurative (including eosinophils) and granulomatous inflammation with a rim of lymphocytes, Splendore-Hoeppli phenomenon likely	Spherules with multiple endospores of 10–100 μm
Paracoccidioides	Acute and chronic pneumonia or disseminated and cutaneous disease	Dimorphism, α-glucan, chitin	Mixed suppurative and epitheloid granulomatous inflammation with or without fibrosis	Multiple-budding yeast forms, 4–60 μm with pilot wheel appearance

(continued)

Table 1.5 (continued)

Organism	Clinical features	Virulence	Host tissue reaction	Morphology on H&E, PAS and GMS
Talaromyces (Penicillium) marneffei	Cutaneous to disseminated disease	Dimorphism, acid phosphatases, survival in acidic pH of macrophages	Suppurative inflammation with infected macrophages and granulomas	Small yeasts (2–4 μm) with transverse septations inside macrophages
Sporothrix	Cutaneous, lympho-cutaneous and occasionally disseminated disease	Cell wall components	Mixed suppurative (including eosinophils) and granulomatous inflammation, Splendore-Hoeppliphenomenon, asteroid bodies, epidermis with pseudoepitheliomatous hyperplasia	Asteroid bodies surround small round, oval to cigar shaped yeast cells 2 to 6 μm in size with narrow based budding
Moulds				
Aspergillus	Allergic bronchopulmonary aspergillosis and allergic fungal rhinosinusitis	Thermal tolerance, siderophore production, ability to survive in oxygen deprived environments, elaboration of proteases	Eosionphilic mucus, Curshmann's spirals, Charcot-Leyden crystals	Hyaline, septated hyphae with acute angle branching
	Chronic and necrotising pulmonary aspergillosis		Fungus ball with surrounding fibrosis, necrosis, granulation tissue, granulomatous inflammation	
	Invasive disease		Angioinvasion by hyphae with consequent necrosis or hemorrhage of surrounding tissue	
Fusarium, Scedosporium	Locally invasive and disseminated disease	Thermal tolerance, proteases	Angioinvasion by hyphae with consequent necrosis or hemorrhage of surrounding tissue	Hyaline, septated hyphae with acute angle branching[a]
Mucorales	Cutaneous, rhinocerebral, pulmonary, gastrointestinal and disseminated	Thermal tolerance, siderophore production, rapid growth	Angioinvasion by hyphae with consequent necrosis or hemorrhage of surrounding tissue; inflammation is suppurative more than granulomatous	Hyaline, aseptate to sparsely septate ribbon-like hyphae with right-angle branching

Entomophthorales	Muco-cutaneous, subcutaneous and occasionally gastrointestinal tract disease	Proteases and lipases	Fibrosis, granulation tissue, mixed eosinophilic and granulomatous inflammation; Splendore-Hoeppli phenomenon	Hyaline, aseptate to sparsely septate ribbon-like hyphae with right-angle branching
Dematiaceous fungi such as *Cladophialophora, Fonsecaea, Madurella, Curvularia*, etc.	Superficial and deep subcutaneous infections and invasive disease	Melanin	Mixed suppurative and granulomatous inflammation with pseudo-epitheliomatous hyperplasia and draining sinuses	Pigmented irregular hyphae and yeast-like swellings with septations
Others				
Pneumocystis	Atypical pneumonia to occasional disseminated infections	not clearly known	Minimal reaction; rarely fibrosis or granuloma, foamy eosinophilic exudates	Round cysts with GMS staining only the wall, seen in groups
Rhinosporidium seeberi	Nose, nasopharynx	not clearly known	Granulomatous inflammation with fibrosis	Immature trophocytes are spherical measuring 10-100μm whereas mature sporangia are 100-350 μm in diameter, containing many spores
Lacazia loboi	Cutaneous, disseminated disease	not clearly known	Granulomatous inflammation constituted by a dense histiocytic infiltrate with multiple epithelioid and multinucleated giant cells with accentuated fibrosis. Asteroid bodies may be seen	Lemon-shaped budding yeast-like organisms measuring 6–12 μm

[a]At times, *Fusarium* and *Paecilomyces* exhibit adventitious sporulation that may be seen in histologic sections and blood culture positive broths as yeast-like structures which promote their dissemination in blood

1.7.1 Radiological Imaging

Imaging plays a crucial role in early detection of signs attributable to fungal infections. Imaging of lungs, paranasal sinuses, central nervous system (CNS) provide clues for possible fungal infections. Chest radiographs may be normal or non-specific in non-neutropenic patients with pulmonary aspergillosis, however the presence of nodules may be suggestive [32]. *Pneumocystis* pneumonia can seem normal at presentation, followed by typical bilateral, perihilar, diffuse granular, or hazy (ground-glass) opacification, which becomes dense with worsening infection, progressing to areas of consolidation [33].

The computed tomography (CT), multidetector CT (MDCT) and high-resolution CT (HRCT) are preferred imaging techniques. In neutropenic patients, the characteristic halo sign is transitory and an early feature of invasive aspergillosis but more specifically nodules greater than 1 cm suggest fungal infection over bacterial or viral [34]. CT-Angiography may improve the specificity for fungal infections. Air crescent sign appears late in invasive aspergillosis. Reverse halo sign or multiple (>10) nodules suggest mucormycosis. CT and MRI are also crucial for diagnosis of invasive sinusitis and CNS infection. Recently antibody guided PET-CT/MR provides better sensitivity and specificity of fungal diagnosis. While radiology provides room for suspicion of IFD, the changes are still non-specific and need confirmation with mycological diagnosis.

1.7.2 Conventional and Culture Based Diagnosis

Diagnosis of IFDs often requires a biopsy or aspirates from sterile body sites and blood. Haematoxylin and eosin staining is useful for evaluating tissue reaction whereas fungus specific stains such as Gomorri's or Grocott'smethenamine silver (GMS), periodic acid Schiff (PAS) or Giemsa are often required for morphological diagnosis of infecting fungi (Table 1.5).Often, the biopsy material may land only in the histopathology laboratory in 10% formol saline, as the clinician had not suspected a fungal infection. In such situations, an attempt may be made to extract DNA from tissue and identify the pathogen by sequencing of the PCR amplified DNA product.

For samples from non-sterile sites, it is difficult to separate colonizers from true infections. This is especially true for genitourinary and respiratory specimens where *Candida* and occasionally filamentous fungi may be present as commensals. There is no accepted cut-off for candiduria on quantitative cultures although several have been proposed [35]. Conventional blood culture yields for candidemia are typically under 50%. Automated blood cultures have greatly improved this scenario with the availability of specialized containers for fungal blood cultures increasing yield [36]. The volume of blood cultured determines sensitivity. At least 10 ml of blood should be collected from central and peripheral line separately to improve sensitivity and to

Table 1.6 Identification of fungi from positive cultures

Method	Time required	Comment
Non-commercial conventional	3–4 days	Most laboratories in developing countries perform this technique
Commercial phenotypic	1–3 days	Accurate identification depends on database
PNA-FISH	90 min	Mainly *C. albicans*, *C. glabrata*
Sequencing—ITS barcoding and other conserved sequences	1 day	Best method for identification, but technically challenging
MALDI-TOF	Few minutes	Future for identification

establish central line related infections. Lysis-centrifugation (Isolator) tubes increase yield at the cost of introduction of possible contamination during processing. However, sensitivity of blood culture still remains around 50–60%. Moreover, the average time to detection for these systems ranges from 14 to 38 h and may take up to 72 h (Table 1.6). This is unacceptable considering the rate at which mortality increases with delay in start of therapy for candidemia. Recent technique T2 magnetic resonance enabled nano-particle-mediated rapid diagnosis is promising, as it can detect candidemia within 3–4 h compared to 2.6 days by BACTEC or BacT/ALERT [37]. However, this technique can only detect five common *Candida* species and cannot perform antifungal susceptibility testing.

1.7.3 Sepsis Biomarkers

Commonly used biomarkers of sepsis may be used to distinguish candidemia and bacterial sepsis, as the levels do not rise in fungal infections. A low procalcitonin (PCT) value may be interpreted as high likelihood for IFI in critically ill patients with clinical symptoms of sepsis, though some clinicians may not agree for the same. Fungaemia, even severe sepsis or septic shock, does not necessarily elicit a substantial increase in serum PCT. In a meta-analysis of utility of PCT for diagnosis of fungal infections, it was found that he PCT value was markedly lower in the fungal infection group (range, 0.69–1.23) than in the bacteraemia group (range, 4.18–12.9) at the onset of fever [38]. The same analysis found C-reactive protein to be neither sensitive nor specific for fungal infections.

1.7.4 Serology

Several serological tests have been validated and introduced into routine clinical practice. They are useful compared to culture in that the turnaround time is few hours, and they are positive weeks before culture positivity. The β-D-glucan (BDG) assay is a useful adjunct to culture for diagnosis of most of the invasive fungal

infections including *Pneumocystis*. The BDG is a major cell wall component of fungi found in sera of patients with IFDs. It is negative in mucormycosis and yeast stage of blastomycosis. It may also be negative in cryptococcosis. BDG can be measured in serum prior to clinical symptoms and facilitates early initiation of antifungal therapy. Serial measurements are associated with improved sensitivities and predict response to therapy. However cut-offs for measuring BDG still need evaluation and standardization. It is associated with false positivity especially in patients with haematological malignancies who may be heavily colonized by many fungi [39]. Dialysis membranes, *Pseudomonas* bacteraemia among other bacteraemias may also lead to a false positive BDG. BDG in respiratory samples, though appears more sensitive, requires standardization. The test is expensive and can be performed by highly skilled personnel in specialized laboratories only.

Galactomannan (GM) measurement is another useful tool for early detection of invasive aspergillosis (Table 1.7). It is US FDA approved for serum and brochoalveolar lavage (BAL) samples for the presumptive diagnosis of invasive aspergillosis. It has good positive and negative predictive values in haematology–oncology units where patients are frequently neutropenic. Sensitivity is lower in non-neutropenic patients. GM test also suffers from limitations of cost and high turnaround time. Other available serological techniques are presented in Table 1.7.

1.7.5 Molecular Detection

Molecular detection directly from samples or cultures is challenging mainly due to the thick cell wall of fungi. Extraction of nucleic acids and intrinsic proteins often require specialized harsh methods for cell wall lysis, distinct from viruses or bacteria. The fungal burden may be less per gram of tissue or fluid and thereby lower DNA quantities, requiring larger volumes of samples for DNA extraction. Environmental contamination can lead to false positive amplification of fungi. PCR is also hindered by presence of inhibitors such as heparin, haemoglobin and lactoferrin. Contamination is often difficult to avoid as about 20% of commercially available blood collection tubes may have fungal DNA [41]. Also there is lack of standardization as to which component of blood to use for maximal fungal recovery. European *Aspergillus* PCR Initiative standardized a test protocol in a multicentric study. It recommends large volume of sample should be used with small elution volume, and mechanical breakage rather than enzymatic disruption should be practiced for extraction of DNA. Plasma is best sample though serum and whole blood may also be used [42].

As fungi are eukaryotic, target selection for PCR amplification needs to be distinct from human genomic DNA and preferably in multiple copies due to low fungal burden. The usual target for nucleic acid amplification are 18S, 28S and internal transcribed spacer (ITS) region. Nested PCRs are preferred, as they increase specificity of target amplified. However there is a chance of introducing contamination. Use of panfungal primers with post-amplification processing for identification by

Table 1.7 Serological diagnosis of fungal infections

Technique	Antigen/Antibody	Organisms	Sensitivity (%)	Specificity (%)	Comments
Latex agglutination	Glucuronoxylomannan antigen detection	*Cryptococcus*	97–100	97–100	False positives are seen in trichosporonosis and *Capnocytophaga* infections
ELISA	Galactomannan antigen	*Aspergillus*	72–90	84–92	Detectable GM precedes clinical infection. BAL GM precedes serum GM. Useful in monitoring response to therapy
	Antigen	*Cryptococcus*	97–100	97–100	
		Histoplasma	87–94	88–92	Antigenuria more sensitive than antigenaemia
		Blastomyces	90–93	90–93	
		Candida (mannan)	58–60	90–95	
	Antibodies	*Coccidioides, Blastomyces, Histoplasma, Candida* (antimannan)	88–100	95–99	
		Sporothrix [40]	89	82	*In-house* ELISA using purified cell wall antigen SsCBF (*S. schenckii* ConA binding fraction)
Immunodiffusion	Antibodies	*Histoplasma* and *Blastomyces*	60–70	100	
Complement fixation	Antibodies	*Histoplasma, Coccidioides*and *Blastomyces*	45–70	100	*Histoplasma* antibodies persist for years after infection. *Coccidioides* antibody titre used to monitor treatment response

(continued)

Table 1.7 (continued)

Technique	Antigen/Antibody	Organisms	Sensitivity (%)	Specificity (%)	Comments
Lateral flow assay	Antigens	*Cryptococcus*	97–100	97–100	More sensitive than presently used tests, can be used in field, takes less time
		Aspergillus	48–100	100	Still presents lot of variability, needs standardization, respiratory samples may be better
Immunofluorescence	Antigen	*Candida* germ tube assay (CAGTA)	77–89	91–100	
Beta-glucan test	All fungi except *Mucorales* and *Cryptococcus*	BDG assay	77–81	87–94	Apart from serum, shown to be highly specific in CSF for fungal meningitis and in BAL for pneumocystosis

sequencing or hybridization provides highly specific results. Quantitative real time PCRs are required for differentiation of colonization and infection especially in case of *Pneumocystis jirovecii* pneumonia.

A variety of other molecular methods have been studied. Fluorescence in situ hybridization (FISH) is a technique that uses fluorescent probes to identify target areas on the genomes of microbial pathogens in human samples, which can then be detected by fluorescence microscopy. This method has been proven to be accurate for the identification of *Candida* spp. infections from blood culture bottles. Nucleic acid sequence-based amplification (NASBA) is a method that amplifies mRNA using an RNA polymerase and is isothermal. A rapid, affordable loop-mediated isothermal amplification (LAMP) assay has been validated for *Histoplasma* with high sensitivity and specificity in urine specimens [43].

MALDI-TOF MS, is based on mass spectrometry to identify the intrinsic ribosomal protein fingerprints of different microorganisms. By direct comparison of the spectral pattern of the organism with databases of known patterns from different microorganisms, it is possible to identify the microbe at the genus, species and even strain level. It is a rapid and cost effective method in comparison with nucleic acid based techniques.

1.8 Treatment

Superficial infections are usually treatable with long-term topical and oral antifungals in patients with extensive or recurrent disease. Disseminated or invasive infections require systemic antifungal therapy. Delay in initiation of therapy is associated with significant mortality risks. According to one study, there is a 20% increase in mortality if empiric therapy is initiated beyond 12 h after the first blood culture sample is drawn [44]. Choice of empiric therapy is also challenging as the spectrum of agents in particular cohort is changing and several species acquire resistance or have intrinsic resistance profiles. Currently available systemic and important topical antifungals are described in detail in Chap. 2. Strategies for therapy based on available evidence of fungal infection are presented in Table 1.8.

Choice of antifungal therapy depends not only on the species but also formulations available, toxicity profiles and pharmacokinetic parameters. Lipid formulations of amphotericin B allow higher dosage of antifungal agent with lesser toxicity, but impose significantly higher costs. Therapeutic drug monitoring is recommended especially for voriconazole, itraconazole and posaconazole to ensure adequate plasma levels for efficacy and to avoid toxicity, and for flucytosine to monitor toxicity.

Antifungal resistance in *Candida* and mycelial fungi is an emerging problem. Fluconazole resistance in *Candida albicans*, though rare, is increasing in certain reports. The problem is more severe for non-albicans *Candida* species where fluconazole resistance has been reported between 5 and 65% [46]. This is a serious problem especially in countries where fluconazole is the only drug available to treat

Table 1.8 Strategies of antifungal therapy

Strategy of therapy	Definition	Recommended population	Drugs used	Comments/Pitfalls
Prophylaxis	Generally in patients at high risk (>10%) of invasive fungal infections before developing signs and symptoms	Patients with haematological cancer, recipients of haematopoietic stem cell transplantation and other immunosuppressed states; intraabdominal candidiasis especially in patients with acute pancreatitis or repeated bowel leak	The IDSA recommends fluconazole prophylaxis when invasive candidiasis rate exceeds 5% in the ICU [45]. Fluconazole for patients with AML, MDS and recipients of SCT.	Fluconazole prophylaxis leads to emergence of non-albicans *Candida* species such as *C. krusei* and *C. glabrata* with high-level resistance to fluconazole.
			Voriconazole or posaconazole for invasive aspergillosis. Posaconazole recommended in haematological malignancies and myelo-dysplastic syndrome	Voriconazole treatment may lead to breakthrough mucormycosis
Empiric	Patients at high risk of IFI with established clinical signs and symptoms of infection, but without a known source	Patients with acute leukaemia or myelodysplastic syndrome during remission induction chemotherapy with prolonged neutropenia	L-AmB at 3 mg/kg OD i.v. if candidemia suspected, caspofungin at 70 mg OD loading dose and 50 mg OD maintenance or voriconazole if aspergillosis suspected	
Pre-emptive	Patients at high risk of IFI with radiographic signs and/or biomarker positivity suggesting IFI, without definitive histopathological and/or cultural pathogen identification	Candida colonization scores, fungal beta-D glucan	Echinocandins	
		Positive serum/BAL galactomannan/beta-D glucan, halo sign or radiological changes in chest	Voriconazole	
Targeted	Diagnostic criteria permit pathogen identification	Proven invasive fungal disease by microscopy/culture/ histopathology	Specific therapy based on identification and susceptibility	Therapy based on histopathological report may also lead to inappropriate therapy for example of specimens showing hyaline septate hyphae presumed to be aspergillosis which may in fact turn out to be invasive fusariosis or scedosporiosis

Candida infections. Resistance to echinocandins, the most frequently used drugs for invasive candidiasis occurs to a much lesser degree, although 6–10% resistance in *C. glabrata* is seen in United States and Denmark. Azole resistance in *Aspergillus fumigatus* has recently gained importance especially in patients with chronic exposure to azoles such as for chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillois or due to agricultural use of azoles. The rates are much higher in UK and Netherlands (around 10–15%), but around 0–5% in rest of the world.

Antifungal susceptibility testing of fungi is technically challenging and there is a lack of availability of interpretive breakpoints for majority of moulds and non-*Candida* yeast. Microbroth dilution MIC is the CLSI or EUCAST recommended method of choice for most organism-drug combinations, although disk diffusion can be done for few yeasts with azoles and echinocandin. Automated commercial systems [Sensititre Yeast one (Trek Diagnostic System, USA); ASTY micro-dilution panel (Kyokuto Pharamaceutical Industrial Co. Ltd, Japan); Fungitest, (Biorad); ATB Fungus 2 (bioMérieux), VITEK 2 (bioMérieux)] are available for performing susceptibility to yeasts. The commercial systems are based on agar diffusion principle or colorimetric method. The tests have reasonably good agreement (≥92%) with standard micro-broth dilution method for itraconazole, flucytosine, amphotericin B against yeasts, but not so good for fluconazole (82%), voriconazole (85%), caspofungin [46]. Categorical agreement is lowest for *C. glabrata* and *C. tropicalis,* and significant variation may be seen in the end point obtained.

1.9 Fungal Vaccines

While effective vaccines have been developed for viral and bacterial infections, no vaccine has received formal approval to use against fungal infections, though many researchers are working in this field. With improved understanding of the host–fungus relationships, and the spectacular advances in genomics and proteomics, considerable progress has been made in fungal vaccine research. Some vaccines induce the generation of directly fungicidal antibodies; others are protective in animals carrying major risk factors for fungal infections, such as CD4+ T cell-deficiency or neutropenia. A common immunological pathway studied includes activation of cell-based, pro-inflammatory, Th1 or Th17 immune response to improve phagocytic killing of fungi. Despite the advancement of research, the lack of complete understanding of the market potential for such vaccines has resulted in the non-availability of capital to develop these vaccines. Continued education about the economic importance of vaccines for invasive fungal infections, combined with continued development of well-defined antigens and effective adjuvants with a track record of safety, should enable such vaccines to enter clinical testing in the coming decade.

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Chapter 2
Antifungal Drugs

Beatriz Bustamante, Jose A. Hidalgo, and Pablo E. Campos

Abstract Fungal infections have increased globally due to the increment of the size of population at risk for fungal infection, which is a consequence of the increased use of immunosuppressive drugs and invasive techniques for advanced life support and extended life expectancy among other reasons. Although invasive fungal infections currently are a significant cause of mortality among critically ill patients, development and approval of new systemic antifungal drugs have not occurred at the same rate as the increase in the number of fungal infections. Only one new class of systemic antifungal drugs, Echinocandins, has been included in the antifungal armamentarium in the last 20 years.

The purpose of this chapter is to review the systemic antifungal drugs currently in use, including new insights on pharmacologic and pharmacokinetics properties, clinical indications, adverse events, and resistance mechanisms. Resistance to antifungal drugs is particularly important because it has increased for every drug, including the echinocandins class. New formulations of triazol drugs and combination therapy is also highlighted.

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2.1 Polyenes (Amphotericin B Deoxycholate and Its Lipid-Associated Formulations)

2.1.1 Amphotericin B Deoxicholate

Amphotericin B (Amph B), derives from *Streptomyces nodosus*, belongs to a group of polyene macrolides characterized by a macrocyclic ring of lactone. Due to its low water solubility, oral bioavailability is low. This drug can be administered intravenous, intrathecal, intraarticular, intravesical, and in surgical sites. This is one of the oldest antifungal drugs but is still used in the fungal therapy because of its broad activity spectrum.

Although amph B is fungicidal in vitro, it may be fungicidal or fungistatic in vivo depending on the concentration of the drug achieved in body fluids and the susceptibility of the fungus [1].

2.1.1.1 Chemical Structure

Amph B is a heptaene macrolide compound comprising of seven conjugated double bonds within the main ring. This drug has an amino sugar called mycosamine, which in the pyranose form is linked to the hydroxyl group at C-19 of the macrolactone ring of amph B through a glycoside side chain, and a free carboxyl group on the macrocycle. Amph B has a molecular formula of $C_{47}H_{73}NO_{17}$ and a molecular weight of 924.09 [2, 3] (Fig. 2.1).

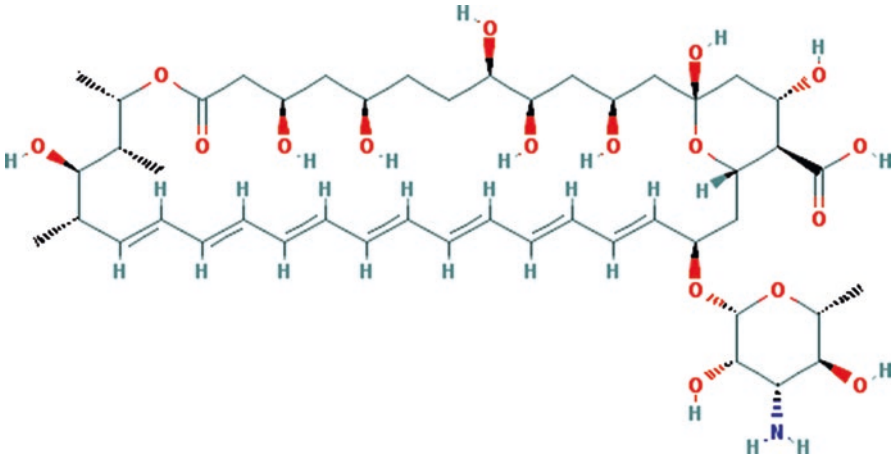

Fig. 2.1 Chemical structure of Amphotericin B deoxicholate. Source: Reference [4]

2.1.1.2 Mechanism of Action

Amph B exerts its antifungal action by binding to ergosterol in the fungal cytoplastic membrane resulting in the formation of pores that causes an increase in their permeability with leakage of small molecules from the cytoplasm, leading to fungal death. This drug may have other effects as an oxidative damage to fungal cell or by immunomodulatory properties on the host cells [5–7].

2.1.1.3 Pharmacokinetics and Pharmacodynamics

Amph B is not appreciably absorbed when taken orally or subsequent to aerosol administration. Then, the intravenous formulation should be used for fungal systemic diseases. After IV administration, this drug is highly bound (>90%) to plasma proteins mainly to albumin, is weakly dialyzable and it is taken up by reticuloendothelial organs, especially the liver, spleen and lung [8–10].

The activity of amph B depends on its concentration and prolonged post-antifungal effect [11]. Its elimination is biphasic. Initially, it is quickly removed with a half-life of about 24 h, while the second elimination phase has a half-life of up to 15 days [12]. In vitro evaluations have shown evident concentration-dependent killing and maximal antifungal activity at concentrations exceeding the MIC by two- to tenfold [11, 13]. In vivo time-kill studies with amph B against different *Candida* species have also shown an improved rate and extent of killing with increasing drug concentrations [14, 15].

Additionally, an in vivo study using a rabbit model of invasive pulmonary aspergillosis to evaluate the pharmacokinetics and pharmacodynamics of amph B deoxycholate, amph B lipid complex (ABLC) and liposomal amph B (L-AmB) found that all formulations of amph B induced a dose-dependent reduction in markers of lung injury and circulating fungus-related biomarkers. Dosing of L-AmB of 3 mg/kg/day predicts complete suppression of galactomannan and (1, 3)-D-glucan levels in the majority of patients [16].

The amph B concentration in urine is low, finding only 3% of the dose, and its penetration in cerebrospinal fluid is limited (2–4% of serum concentrations) [17]. Amph B goes through minimally into the vitreous humor and normal amniotic fluid. In the peritoneal fluid, pleura, or joint, fewer than 50% of the serum levels are obtained [18]. Serum level of the drug is not influenced by hepatic or renal function, or by hemodialysis or peritoneal dialysis. Even in anuric and nephrectomized patients, the drug serum levels and its elimination are just the same as in healthy patients. It seems that amph B is eliminated principally by metabolic conversion or by the bile [9, 19]. However, the metabolites are not yet well known.

2.1.1.4 Spectrum of Activities and Resistance

Amph B has activity against a wide range of fungi including most yeasts, and hyaline and dematiaceous molds. Among the yeast, *Candida* isolates resistant to polyenes are still infrequent. *C. lusitaniae* and *Trichosporum beigelii* are two fungi that

have intrinsic resistance, and recently some *C. krusei* and *C. glabrata* strains with high MICs to amph B has been reported [20–22]. In the group of molds, *Aspergillus terreus, Scedosporium apiospermum, Scedosporium prolificans,* and *Fusarium* species are usually resistant to amph B [23].

Although breakpoints for polyenes are not available, most microbiologist use a MIC of ≥1 μg/mL to determine if an isolate is not susceptible to amph B. Reduction or absence of ergosterol in the fungal cell membrane has been associated with resistance. These alterations could be due to mutations in genes coding some of the enzymes involved in the synthesis of ergosterol. The mechanisms involved in the resistance of *C. albicans* isolates to the amph B include a double loss in function of both ERG3 (C-5 sterol desaturase) and ERG11 (lanosterol 14α demethylase) [24, 25]. It has also been identified isolates amph B resistant lacking ERG2, encoding C-8 sterol isomerase, and ERG6, encoding C-24 sterol methyltransferase [26–28].

2.1.1.5 Clinical Uses

Despite the emergence of new antifungal drugs in the last decades, amph B deoxycholate or its lipid formulations are still recommended as primary treatment for several severe or refractory fungal infections or as alternative therapy for other forms of these diseases. According to the Infectious Diseases Society of America (IDSA) guidelines for the management of candidiasis [29], amph B deoxycholate is recommended for the treatment of neonates with disseminated candidiasis or candidiasis in central nervous system, for patients with asymptomatic candiduria who undergo urologic procedures, and for symptomatic candida cystitis or symptomatic ascending candida pyelonephritis due to fluconazole-resistant *C. glabrata* or to *C. krusei*. This drug is recommended as alternative in the treatment for fluconazole-refractory oropharyngeal/esophageal candidiasis. Amph B deoxycholate or lipid formulations are also indicated for the treatment of cryptococcal meningitis, mucormycosis, moderately severe to severe forms of blastomycosis and histoplasmosis [30–33].

Aerosolized amph B, in their different formulations, is utilized as prophylaxis (either alone or in combination with systemic antifungals) in patients at highest risk of invasive fungal infections. A recent meta-analysis presented evidence supporting the concept that the prophylactic use of aerosolized amph B effectively reduces the incidence of invasive pulmonary aspergillosis among high-risk patients [34]. The current IDSA guidelines for the diagnosis and management of aspergillosis stated that aerosolized formulations of amph B may be considered for prophylaxis in patients with prolonged neutropenia and in lung transplant recipients (weak strength recommendation; low-quality evidence) [35].

Because of the low intraocular levels attained with systemic administration, intravitreal injection of amph B is useful to reach high confined antifungal activity for the treatment of severe macular involvement and vitritis. The IDSA guidelines suggest treatment with systemic antifungal drug plus local amph B deoxycholate, 5–10 μg/0.1 mL sterile water, for *Candida* chorioretinitis without vitritis and with

macular involvement, and for *Candida* chorioretinitis with vitritis [29]. In addition, IDSA guidelines recommend intraocular amph B with partial vitrectomy as primary treatment of *Aspergillus* endophthalmitis and keratitis [34]. There are controversial reports on toxicity of intravitreal injection of amph B deoxycholate in humans, some of them have described low toxicity and others have reported toxic uveitis [36–41].

There is not a good evidence to recommend bladder irrigation with amph B deoxycholate for treatment of cystitis but, when this infection is due to fluconazole-resistant species, such as *C. glabrata* and *C. krusei*, this method of delivering the antifungal drug directly to the affected site could be used [29, 42].

2.1.1.6 Dosing

Dosing of amph B deoxycholate in adults with normal renal and hepatic function is 0.5–1 mg/kg daily for candidemia, other invasive candidiasis and for endemic dimorphic fungal infections; 1–1.5 mg/kg daily for invasive aspergillosis; 0.3 mg/kg daily for esophageal and oropharyngeal candidiasis; 0.7 mg/kg daily for empiric treatment of febrile neutropenia; and 0.7–1 mg/kg daily (usually with 5-flucytosine) for cryptococcal meningitis in the induction phase of the therapy.

2.1.1.7 Adverse Events and Toxicity

Acute toxicity related to amph B administration is due to stimulation by the antifungal drug of inflammatory cytokine production from innate immune cells via an interaction that requires CD14 and Toll-like receptors [43]. The most frequent acute effects are nausea, vomiting, rigors, fever, hypertension/hypotension, and hypoxia. Other acute adverse events with rare presentation are ventricular arrhythmias, bradycardia, and severe hypertension [44–46]. In addition, two cases with fatal leukoencephalopathy associated with the intravenous administration of the amph B has been reported [47].

Chronic toxicity is referred commonly to nephrotoxicity, but there are other events such as hypomagnesemia, hypokalemia, hyperphosphatemia, anemia, and rare cases of hyperbilirubinemia [48–51]. Reports of anemia of any degree of severity ranges from 33 to 63% in different studies [52–54].

The incidence of nephrotoxicity due to amph B is high, ranging from 49 to 65% [55, 56]. This toxicity is result of renal vasoconstriction producing reduction in glomerular filtration rate, and of direct effect on tubular epithelial cell membranes forming pores. The modification in the permeability of the tubular cell membrane will allow the back diffusion of hydrogen ions thereby weakening the acid elimination [57]. There are some factors that influence the nephrotoxicity of amph B such as cumulative dose, average daily dose, abnormal baseline renal function, concomitant nephrotoxic drugs, and patient's risk category (bone marrow transplant, solid organ transplantation) [58]. Nephrotoxicity is cumulative and dose dependent but it is

reversible after amph B treatment is stopped [58, 59]. This toxicity can be decreased by hydration and electrolyte supplementation before amph B infusion [60–63]. In the case of aerosolized amph B, the most common side effects are cough, bad taste, nausea and vomiting; most of them mild or moderate severity [64–67].

2.1.1.8 Contraindication

Amph B is contraindicated in those patients who have known hypersensitivity to this antifungal drug, and because the increment risk of nephrotoxicity and hearing impairment, its use is contraindicated with the simultaneous administration of the following drugs: amikacin, cidofovir, cyclosporine, ioversol, neomycin, streptozocin, and tacrolimus. Amph B should be discontinued before iodinated contrast media administration [68].

2.1.1.9 Drug Interactions

Corticosteroids, thiazide, loop diuretics, and neuromuscular blockers increase risk of amph B-induced hypokalaemia. Amph B can also increase the risk of digoxin toxicity [69]. Use of alternative drugs or monitoring of amph B associated adverse events is indicated when some of the above drugs needs to be used. Administration of amph B with most of the antiretrovirals does not cause interactions, but concomitant or sequential use with tenofovir increases the risk of nephrotoxicity [70]. In the same way, concomitant use of zidovudine and amph B results in increased risk of anemia and neutropenia [71].

2.1.1.10 Use in Special Population

Amph B and their lipid formulations do not need dose adjustment for patients with decreased renal function or in patients receiving hemodialysis or continuous renal replacement therapy or for those with moderate or severe hepatic disease.

Children In pediatric population, pharmacokinetic (PK)/pharmacodynamic (PD) indices of amph B are not validated; therefore, optimal dosing of this drug has not been defined. Amph B pharmacokinetic is very variable in neonates, which may lead to treatment failure or toxicity, and the lack of maturity of the blood-brain barrier in premature infants can be the basis of a better penetration of the antifungal to the CSF [72]. Compared with adults, amph B has a lower volume of distribution and faster clearance in children [72–76]. Even though most of PK studies in children encourage a dosage of 0.5–1 mg/kg/day, a population PK analysis suggested that younger children receiving 1 mg/kg/day might be underexposed, while older children may be overdosed at the same dose [75]. Another study showed that amph B doses of 0.25–1 mg/kg daily to infants causes lower serum concentrations compared with older children and adults [73, 76].

The preferred amph B deoxycholate pediatric dosing is 1–1.5 mg/kg/day. The infusion adverse events and nephrotoxicity due to amph B in children are similar to adults, but fever has been rarely described in infants [77, 78]. Children usually can tolerate higher doses than adults [79].

Pregnancy and Lactation Amph B is considered as category B (B: animal studies no risk, but human studies not adequate, or animal toxicity but human studies no risk) by the US FDA [80]. ABLC has been evaluated in animals without having found harms to the fetus [81]. Although, there are not well-conducted evaluations of amph B use in pregnancy, its use in pregnant women has been described repeatedly without confirmation of teratogenesis in their neonates [82–96].

This is the only systemic antifungal drug that is safe to use during the pregnancy and lactation period, although there are not published data in the last condition. Due to the properties of amph B such as its large molecular size, poor absorption, and high protein binding ability, breast milk amounts are probable insignificant [97]. There is limited information on the use of ABLC and L-AmB in pregnancy [98, 99].

It is known that amph B crosses the placenta and reaches fetal tissues where can persist some weeks after the mother has stopped the drug or given birth. This characteristic possibly would be due to placental deposit or deferred removal by the fetal kidneys [100].

2.1.2 Lipid Formulations of Amphotericin B

Lipid formulations of amph B have higher hydrophobicity, lower nephrotoxicity, and are distributed more efficiently to the sites of fungal infection or inflammation than amph B deoxycholate. The two lipid formulations available in most countries are liposomal amphotericin B (L-AmB) and amphotericin B lipid complex (ABLC), commercially available under the name AmBisome® (Gilead Sciences Inc., Foster City, CA, USA) and Abelcet (Enzon Pharmaceuticals Inc., Bridgewater, NJ, USA; Sigma Tau, Gaithersburg, MD; and Cephalon Limited, Welwin Garden City, UK) respectively. A third lipid formulation, amphotericin B colloidal dispersion (ABCD), is no longer commercially available.

The lipid compositions and particle size are different between the amph B lipid formulations, producing distinct pharmacokinetic parameters and tissue distribution. Then, the lipid formulations of amph B cannot be used interchangeably to treat patients [101–103].

ABLC consists of amph B complexed to two phospholipids (l-α-dimyristoylphosphatidylcholine and l-α-dimyristoylphosphatidylglycerol in a 7:3 molar ratio) in a 1:1 drug-to-lipid molar ratio to form a ribbon-like structure with a diameter of 1600–11,000 nm, making it the largest of the lipid formulations [104]. ABLC is taken by macrophages and becomes sequestered in the liver and spleen. It has lower circulating amph B serum concentrations, when it is compared with the amph B deoxycholate, but larger volume of distribution and clearance. Lung concentration of ABLC is higher than the obtained with L-AmB or amph B deoxycholate.

By contrast, molecules of L-AmB are smaller and no captured by the mononuclear phagocyte system. L-AmB consists of amph B integrated into the lipid bilayer of small unilamellar liposomes, which are composed of hydrogenated soy phosphatidylcholine, cholesterol and distearoyl phosphatidylglycerol [105]. After a dose of L-AmB, it reaches a higher peak plasma level (Cmax) than amph B deoxycholate and a larger area under the concentration–time curve. The highest amph B concentrations with the L-AmB administration are found in the liver and in the spleen, followed by kidneys and lungs. Levels in myocardium and brain tissues are low [106].

The ABLC mechanism of action requires that the fungal lipase, a heat labile extracellular product, produces a lipid breakdown within the ribbon-like material with subsequent discharge of amph B into the tissues [107]. The suggested mechanism of action of L-AmB states it binds to the fungal cell wall and disintegrates itself, discharging amph B that binds to ergosterol in the fungal cell membrane [108, 109]. Table 2.1 summarizes the characteristics of the different amphotericin B formulations.

Dissimilar results have been reported in relation to in vitro susceptibility of amph B deoxycholate and lipid formulations. Montagna et al. found great correlation between the in vitro activities of amph B deoxycholate and L-AmB against 604 clinical yeast isolates [125], while Johnson et al. found that MIC 50 and MIC 90 of ABLC were the same to or lower than those of amph B deoxycholate when they were tested against 190 isolates from different fungal species, including *Aspergillus fumigatus*, *Candida* spp., and *Cryptococcus neoformans* [126]. In the later study, the L-AmB was the least active of the different amphotericin B preparations tested, showing 2–4 dilutions higher in their MICs than those of amph B deoxycholate. Carrillo-Muñoz, studying the in-vitro susceptibilities of 120 clinical isolates of yeasts (including different species of *Candida*, *Rhodotorula rubra*, *Trichosporon* spp., *Cryptococcus laurentii* and *C. neoformans*), found no statistical significance among MICs of amph B deoxycholate, ABLC, L-AmB and other antifungal drugs when evaluating all strains. However, there were differences in the activity of these drugs for individual species. Amph B deoxycholate and ABLC had high MIC values for an isolate of *C. laurentii*, while L-AmB, had moderately low MIC for the same isolate. ABLC and L-AmB had higher mean MICs against *Trichosporon* spp., and L-AmB had high MIC90 values for *C. glabrata* than amph B deoxycholate [127].

In addition, the manufacturer of AmBisome® (L-AmB) claims that it has in vitro activity equivalent to amph B deoxycholate against the following fungi: *Aspergillus* species, *Candida* species, *C. neoformans*, *Fusarium* species and *Blastomyces dermatitidis* [69].

Experimental studies carried out in animals demonstrated that it is necessary higher doses of the two lipid formulations to achieve the same or greater antifungal effect than the obtained by the amph B deoxycholate [123, 128–130]. Although it is also true in the clinical setting, the toxicity, mainly nephrotoxicity, is lower when lipid formulations are used [117, 123, 131–139]. There is some evidence of greater effectiveness of the lipid formulations of amph B in relation to amph B deoxycholate. When used as induction therapy, L-AmB has

Table 2.1 Comparative characteristics of amphotericin B formulations

Characteristics	Amphotericin B deoxycholate	ABLC	L-AmB
Structure	Micelle	Ribbon-like structures	Small spherical unilamellar lipid vesicles
Size (nm)	0.035	1600–11,000	Mean diameter: <100
Composition	Amph B deoxycholate	1:1 molar ratio of amph B/phospholipid (DMPC and DMPG)	HSPC, cholesterol, DSPG and amph B
Mode of action	It binds to ergosterol (fungal membrane) resulting in increased permeability, output of intracytoplasmic compounds and finally in fungal death	Release of amph B from complexes, possibly host macrophage- and phospholipase-mediated	Liposome targeting to fungal cell wall with release of amph B into the fungal cell
Nephrotoxicity	+++	++	+
Infusion-related toxicity	+++	++	+
Pharmacokinetic parameters (mean ± standard deviation)			
Cmax (μg/mL) at steady state	1.1 ± 0.2	1.7 ± 0.8	83 ± 35.2
AUC_{0-24} (μg h/mL)	17.1 ± 5.0	14.0 ± 7.0	555 ± 311
Terminal elimination half-life (h)	91.1 ± 40.9	173.4 ± 78.0	6.8 ± 2.1
Vd (L/kg)	5.0 ± 2.8	131 ± 57.7	0.11 ± 0.08
Clearance (mL/h/kg)	38.0 ± 15.0	436 ± 188.5	11.0 ± 6.0
Distribution			
Protein binding, %	>95	>95	>95
CSF concentration relation to serum, %	0–4	3 (in animal)	<1
Urine concentration relation to serum, %	3–20	<5	4.5
Metabolism	Minor hepatic	Unk	Unk
Elimination	Feces	Unk	Unk
Comparative tissue concentrations of amphotericin formulations			
Liver	++	+++	+++
Spleen	+	+++	+++
Lung	+	++++	+
Kidney	+	±	+
Brain	−	±	±

Source: Data derived from [69, 81, 110–124]

ABLC amphotericin lipid complex, *L-AmB* amphotericin B lipid complex, *HSPC* hydrogenated soy phosphatidylcholine, *DSPG* distearoyl-phophatidylglycerol, *DMPC* dimyristoyl phosphatidylcholine, *DMPG* dimyristoyl phosphatidylglycerol, *Cmax* maximum concentration of drug in serum, *Cmax* peak drug concentration, *AUC* area under the concentration curve, *Vd* volume of distribution

Note: pharmacokinetic parameters after multiple doses of 0.6 mg/kg/day of amph B deoxycholate or 5 mg/kg/day of L-AmB or ABLC

demonstrated faster culture conversion in cryptococcal meningitis, and was associated with improved survival and lower toxicity in AIDS patients with moderate to severe disseminated histoplasmosis in comparison to amph B deoxycholate [140, 141]. L-AmB (5 mg/kg/day) also showed superior clinical efficacy to amph B deoxycholate (1 mg/kg/day) in the treatment of neutropenia-associated invasive fungal infections [139].

Any of the lipid formulations of amph B are recommended by the IDSA guidelines for the treatment of non-neutropenic patients with suspected azole- and echinocandin-resistant candidemia, candida suppurative thrombophlebitis, candida endocarditis, and chronic disseminated (hepatosplenic) candidiasis. In addition, they proposed these drugs as an alternative treatment for candidemia in neutropenic and non-neutropenic patients, candida septic arthritis, or osteomyelitis, and as alternative empiric treatment of non-neutropenic patients in the intensive care unit with suspected invasive candidiasis and with intolerance to other antifungal drugs. The same guidelines recommend L-AmB for the initial treatment for central nervous system candidiasis and for candida chorioretinitis without vitritis due to fluconazole/voriconazole-resistant isolates. L-AmB is also suggested as an alternative treatment for central nervous system candidiasis in neonates [29].

For aspergillosis infections, the IDSA guidelines recommend amph B deoxycholate and lipid formulations as alternative treatments for initial or salvage therapy. This document also suggests lipid formulations for refractory and progressive aspergillosis or for empiric and preemptive therapy in allogeneic hematopoietic stem cell transplant recipients and patients treated for acute myelogenous leukemia with prolonged neutropenia who remain persistently febrile despite broad-spectrum antibiotic therapy [35]. The IDSA guidelines also recommend lipid formulations of amph B instead of amph B deoxycholate for patients with cryptococcal meningitis with or predisposed to renal dysfunction and for the treatment of pulmonary, meningitis and disseminated sporotrichosis [30, 142].

2.1.2.1 Dosing

In general, for all fungal infections but CM, the recommended dose of lipid formulations of amphotericin B is 3–5 mg/kg daily for treatment and 1–3 mg/kg daily for prophylaxis. The dose for CM is 3–4 mg/kg per day of L-AmB and 5 mg/kg per day of ABLC, both of them with or without flucytosine, for at least 2 weeks [30, 143]. The pediatric dosing is 5 mg/kg/day of ABLC or L-AmB [144].

2.1.2.2 Adverse Events and Toxicity

In comparison with amph B deoxycholate, rates of infusion related acute reactions are similar with ABLC (60%) but lower with L-AmB (48%) [103, 117]. To reduce infusion-related reactions, it is recommended the use of low-dose hydrocortisone

(1 mg/kg), diphenhydramine (25–50 mg), meperidine (0.5 mg/kg), and nonsteroidal anti-inflammatory agents as premedication [118, 135, 145].

Numerous studies have shown that lipid formulations are less nephrotoxic than amph B deoxycholate. The Barrett et al.'s systematic review found that ABLC and L-AmB decreased all-cause risk of mortality and renal toxicity, compared with amph B deoxycholate [146]. Martino's systematic review also found that ABLC is significantly less nephrotoxic than amph B deoxycholate and can be administered securely to patients with preexisting renal injury [147]. L-AmB used in the treatment of fever in neutropenic patients with cancer showed similar efficacy but significantly less infusion-related reactions and nephrotoxicity than amph B deoxycholate [138]. In comparative trials, adverse events requiring discontinuation of the drug have occurred less frequently when patients received L-AmB (12%), followed by those receiving ABLC (32%) and amph B deoxycholate (44%) [103]. Wade et al. have also reported considerably lower rates of nephrotoxicity, infusion reactions and hypomagnesemia among patients with renal dysfunction and invasive fungal infections receiving L-AmB compared with those receiving ABLC [115].

L-AmB used in 33 consecutive patients at least 65 years old as empirical therapy for the treatment of invasive fungal infections showed equivalent safety and efficacy to those observed in younger patients, but higher incidence of severe hypokalemia when used for extended periods. The incidence of grade III or IV hypokalemia was similar in the older and younger groups [148]. A characteristic triad of acute infusion-related toxicity to L-AmB has been described. The following symptoms alone or in combination of 1 of 3 symptoms can be present: chest pain, dyspnea, and hypoxia; severe abdomen, flank or leg pain; and flushing and urticaria. These reactions occur within the first 5 min of infusion and disappear with administration of diphenhydramine. A multicenter analysis found a mean overall occurrence of 20% (range: 0–100%) of this specific toxicity among 64 centers [149].

2.2 Triazole Antifungal Agents

2.2.1 General Properties

Azoles are a group of antifungals of great importance in the treatment of systemic mycoses that share a common basic chemical structure and mechanism of action, inhibition of membrane sterol synthesis. A major change occurred with the identification of increased antifungal activity of the N substitution in the imidazole ring, which led to the development of current triazoles.

A first generation of triazoles were developed in the eighties and have been in clinical use since then, with limited (itraconazole) or no activity (fluconazole) against filamentous fungi. A second generation of drugs developed in late nineties and more recently is now available (voriconazole, posaconazole, isavuconazole), with improved activity against *Aspergillus* and other filamentous

fungi. Clinical studies have shown their effectiveness in the management of multiple fungal infections.

Triazoles are widely used due to their broad spectrum of antifungal activity, availability of both IV and oral formulations, and safety profile. It is expected that in the future newer agents and more indications will be identified [150, 151].

2.2.1.1 Chemical Structure

The imidazole nucleus is a 5-atom heterocyclic structure with 3 C and 2 N (see Fig. 2.2). This structure is present in nitroimidazoles, utilized in antianaerobic and antiparasitic therapy (metronidazole), and azole antifungals (clotrimazole, miconazole, ketoconazole), currently used for local therapy of superficial infections. The substitution of a C for an N atom originates the term "triazoles" (three N atoms in the ring), and it is associated with significant changes in the antifungal activity and pharmacokinetic properties in relation to older azoles (i.e. ketoconazole). The triazoles have a more specific binding to fungal enzymes than to mammal enzymes for sterol synthesis, more potent antifungal activity, and broader spectrum, less metabolic side effects, and better bioavailability and tissue distribution than older azoles.

2.2.1.2 Mechanism of Action

The structural target for triazoles in the fungal cell is the cellular membrane. Action at this level explains part of older azoles limitations, since their activity would not be completely specific for fungal organisms. Older azoles have also some inhibitory activity on steroid metabolism in mammalian cells; for example, ketoconazole was used in the management of primary Cushing syndrome because of its significant inhibition of corticoid synthesis [157].

Triazoles are inhibitors of the enzyme lanosterol 14-α-demethylase, coded by the gene CYP51A, involved in the synthesis of ergosterol. This enzyme catalyzes the oxidative elimination of 14-methyl group from fungal cell lanosterol (monooxigenase P450 activity). Its inhibition causes the accumulation of lanosterol precursors (methyl-esterols) and changes the proper composition of the cell membrane, which produce structural and functional consequences (lanosterol deficiency and decreased membrane fluidity), primarily in cellular reproduction and death. Triazoles block the activity of the enzyme by binding to the active site, and their increased affinity to this site due to the third N atom present in the imidazole ring produces higher antifungal activity [158].

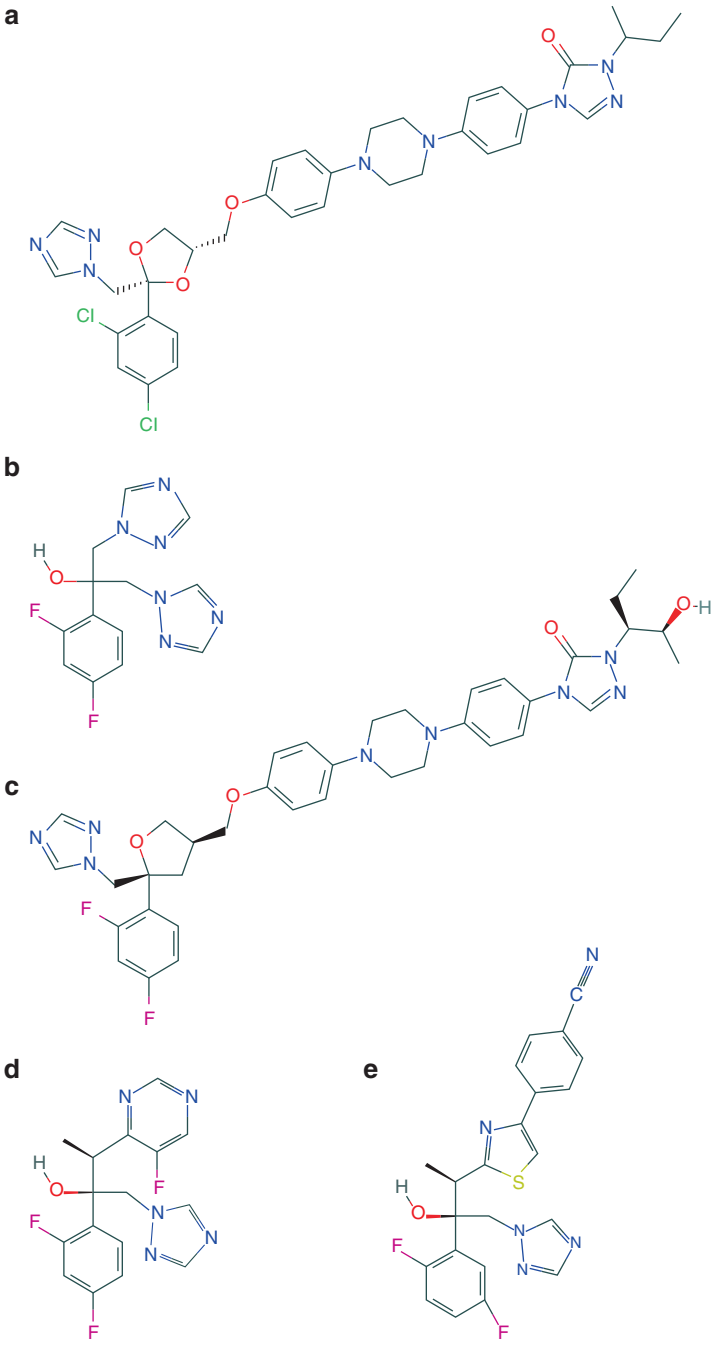

Fig. 2.2 Chemical structure of triazoles. (**a**) Itraconazole, (**b**) Fluconazole, (**c**) Posaconazole, (**d**) Voriconazole, and (**e**) Isavucunazole. Source: References [152–156]

2.2.1.3 Pharmacokinetics

Main pharmacokinetic characteristics of triazoles are displayed in Table 2.2. In summary, these drugs have good oral absorption (with special ingestion requirements for certain formulations of itraconazole and posaconazole), a prolonged half-life (which allows once or twice daily dosing regimens in most cases), good distribution in body tissues with clinical use in different types of invasive disease, and are available in both oral and parenteral formulations [160].

Fluconazole and voriconazole reach higher concentrations in tissues because of their smaller molecular size. Fluconazole shows better concentration in cerebrospinal fluid, and all azoles reach good concentrations in brain tissue. Posaconazole reaches the highest concentration in alveolar cells and voriconazole in bone tissue. Triazoles concentrate similarly well in liver and kidneys [113].

Due to a less predictable absorption, interactions with other drugs, and subject variability of metabolism, therapeutic drug monitoring is recommended to optimize regimens and minimize side effects for itraconazole, voriconazole, and posaconazole [161–163]. Trough levels should be tested within 30 min before patient dosing. The recommended target trough plasma levels for triazoles and their recommended day of testing after initiation of therapy are shown in Table 2.3.

2.2.1.4 Spectrum of Activity and Resistance

All members in the class show good activity against most *Candida* species. Fluconazole is effective against most clinically significant *Candida* sp. and against *Cryptococcus* sp., and to a lesser extent against most dimorphic endemic fungi (*Histoplasma, Blastomyces, Coccidioides*, and *Paracoccidioides* spp). Itraconazole has a broader spectrum that includes *Sporothrix schenckii*, *Aspergillus* spp. and some other filamentous fungi such as dematiaceous fungi and mucorales. Its mold activity is lower than newer triazoles. Voriconazole is active against dose-dependent and fluconazole-resistant *Candida* species (*C. glabrata* and *C. krusei*, respectively). It also shows increased activity against molds, particularly *Aspergillus* spp., *Fusarium* spp., *S.apiospermium,* and dematiaceous fungi. Posaconazole and isavuconazole add to voriconazole spectrum their activity against Mucorales [167, 168].

Resistance to triazoles is well described. Mechanisms of acquisition of resistance include overexpression with increased activity of efflux pumps (ATP binding cassette, ATP and major facilitator superfamily -MFS), and point mutations that cause changes in tridimensional structure or activity of C-14α demethylase [169]. The point mutations cause structural changes in the active site of the demethylase, decreasing the affinity to its ligands. *Candida* species can be intrinsically resistant as is *C. krusei* to fluconazole, show dose-dependent susceptibility as *C. glabrata* versus fluconazole or acquire resistance like *C. albicans* to azoles, largely by changes in the activity of efflux pumps.

Aspergillus resistance to triazoles with mold activity has also been reported in some places. Specific alterations in coding regions of the enzyme in the CYP51A

Table 2.2 Main pharmacologic characteristics of triazoles in clinical use

Characteristics	Itraconazole	Fluconazole	Voriconazole	Posaconazole	Isavuconazole
MW	705.6	306.2	349.3	700.7	814[a]
Half-life (h)	19–22	27–34	6	20–30	56–77
Plasma protein binding (%)	>95	11–22	58	98	>99
Oral bioavailability (%)	55 (capsule with food)	>80	96	See text	98
Volume of distribution (L/kg)	796	46	1.2–4.6	1774	450
Liver failure dose adjustment	Yes	No	Yes	No	No
Metabolism	Liver		Liver	Liver (limited proportion)	Liver
Elimination	Feces	Kidney (60–75% unchanged)	Kidney (2% unchanged)	Feces (mostly unchanged)	Feces
Kidney failure dose adjustment	No	Yes	No (oral)	No	No
Therapeutic drug monitoring	Recommended		Recommended	Recommended	
Most significant toxicities	Nausea, gastrointestinal symptoms	ALT elevation (10%)	Visual disturbances, photosensitivity, confusion	Gastrointestinal symptoms, headache	Gastrointestinal symptoms
Comparative tissue concentrations of triazoles					
Liver	+	+	++	ND	ND
Spleen	+	++	+	ND	ND
Lung	+	+	+	++	ND
Kidney	+	+	+	ND	ND
Brain	+	++	+	+	ND

Source: References [113, 150, 151, 159]
ND No data
[a]MW (Molecular Weight) of isavuconazonium

Table 2.3 Recommended target trough plasma levels for triazoles

Antifungal drug	Target concentration during prophylaxis mg/L	Target concentration during treatment of IFD mg/L	Day of test after initiation of drug
Itraconazole	>0.5	>1	>7 days
Voriconazole*	>1	1–5.5	After 3–5 days
Posaconazole	>0.35 after the first 48 h ≥0.9 after day 7**	>1.8	>7 days

Source: Data from [161, 164–166]
IFD invasive fungal disease
aIrrespective of formulation
bFor the tablet and intravenous formulations TDM is not required when they are used for prophylaxis, as target levels are anticipated to be reached in nearly all the patients

gene have been associated in some cases with itraconazole and posaconazole resistance and with cross-resistance to all triazoles [170].

Identification of triazole resistance is increasingly available with standardization of methods, identification of clinically relevant breakpoints, and consensus from major international institutions (CLSI, EUCAST) in recent years [171].

2.2.1.5 Clinical Utility

Triazoles have a wide spectrum antifungal activity, which includes both yeast and filamentous fungi. Much more limited in the case of the older generation, but with significant increase of activity in the case of the newer products, such as posaconazole and voriconazole.

Fluconazole continues to be of major importance in the management of different clinical presentations of candidiasis in multiple groups of patients [168], and for consolidation treatment of cryptococcosis. Itraconazole is recommended in the management of dimorphic fungi and to some extent in filamentous organisms.

Voriconazole, posaconazole, and isavuconazole have evolving roles in the management of severe fungal infections by filamentous organisms in the most immunosuppressed individuals. Voriconazole is considered a first choice for invasive aspergillosis in most clinical situations. Posaconazole has been used preferentially in antifungal prophylaxis and Mucor infections, and isavuconazole has shown high efficacy in the treatment of aspergillosis and Mucorales [172].

2.2.1.6 Adverse Events and Drug Interactions

Compared to older azoles (i.e. ketoconazole), currently available drugs show much lesser hormonal inhibition, gastrointestinal side effects, and hepatotoxicity. Triazoles as a group are deemed relatively safe drugs.

Itraconazole most commonly causes nausea (10%) and gastrointestinal symptoms (diarrhea, 8%; vomiting, 6%; abdominal discomfort, 6%). Hyperbilirubinemia and liver enzyme elevation is reported in about 5% [173].

Fluconazole used at doses higher than 400 mg/day can cause headache in 2%, anorexia in 3% of patients and transient ALT elevation in 10% of patients.

Reversible visual disturbances (30%), photosensitivity (20%), hallucinations and confusion (15%) have been described with voriconazole use. Recent reports have associated presentation of skin cancer in immune-suppressed patients with use of voriconazole, which needs further clarification [174, 175].

Side effect profile of posaconazole is very similar to that of fluconazole. Experience with isavuconazole in clinical trials has shown that it is largely well tolerated.

Prolongation of the QTc has been observed with triazoles, including posaconazole [176, 177], and this can cause sudden death. The I_{Kr} channel, one of the membrane channels responsible for potassium outflux movement, is inhibited by fluconazole and other triazoles. This inhibition is associated to ventricular repolarization changes that increase vulnerability to cardiac arrhythmias. Patients with other drugs that can cause QTc prolongation, severe bradycardia, hypokalemia, or hypomagnesemia should be cautiously prescribed when used concomitantly with azoles. Contrary to other drugs in the group, isavuconazole has been associated with QTc shortening, of unclear clinical significance.

Triazoles present a significant number of drug interactions [178, 179]. Interactions with immunosuppressants, rifamycins, anticonvulsants, omeprazole, warfarin, statins, and antiretrovirals, amongst others, are particularly important. See Table 2.4 [181].

Interactions of triazole antifungals can be divided into the following categories: modifications of antifungal pharmacokinetics by other drugs, modifications of other drug pharmacokinetics by antifungals, and two-way interactions. The mechanisms involved include azole inhibition of drug metabolizing enzyme cytochrome (CYP) P450 isozymes, such as CYP3A4/5, CYP2C9 and CYP2C19 in varying degrees [182]. Triazoles can also inhibit drug transporter P-glycoprotein (P-gp) within the gastro-intestinal tract and the liver, for which immunosuppressants are substrates.

The degree of these interactions varies greatly, as azole inhibition of relevant enzymes can be dose-dependent and differs in potency and selectivity. Itraconazole and voriconazole are reported to be more potent inhibitors of CYP3A4 than posaconazole and fluconazole. In terms of inhibition of P-gp, itraconazole and posaconazole have the more significant activity [183].

Triazoles are used frequently in transplant patients, either for prevention or treatment of suspected or proven fungal infections, and can interfere with the metabolism and transport of immunosuppressants (i.e. cyclosporine, tacrolimus, sirolimus, and everolimus), which are drugs of narrow therapeutic margin.

Triazoles can increase exposure to immunosuppressant, consequently increasing risk for side effects of these drugs. On the other hand, discontinuation of azoles without dose adjustment of the immunosuppressant drugs may lead to sub-therapeutic immunosuppressant exposure and risk of transplant rejection or graft-versus-host-disease.

Table 2.4 Recommendations for selected drug interactions of triazoles

Drug	Itraconazole	Fluconazole	Voriconazole	Posaconazole	Isavuconazole
Antacids	Avoid				
Atazanavir				Monitor for atazanavir toxicity	
Atorvastatin	Monitor for atorvastatin toxicity				
Carbamazepine			Avoid		Avoid
Cyclosporin A	Monitor for CsA toxicity	Monitor for CsA toxicity	Monitor for CsA toxicity	Monitor for CsA toxicity	Use with caution
Efavirenz	Avoid		Efavirenz dose reduction	Increase dose of posaconazole if need as per therapeutic drug monitoring	
Midazolam	Monitor for midazolam toxicity	Monitor for midazolam toxicity	Monitor for midazolam toxicity	Monitor for midazolam toxicity	
Omeprazole	Avoid capsules, use solution	Omeprazole AUC increased >100%, consider omeprazole dose reduction	Omeprazole AUC increased >100%, consider omeprazole dose reduction		
Phenytoin	Avoid	Monitor for phenytoin toxicity		Avoid	
HIV protease inhibitors (other than atazanavir)	Avoid higher doses of itraconazole		Avoid		
Rifampin	Avoid	Consider fluconazole dose increase	Avoid		Avoid
Sirolimus			Avoid	Avoid	Use with caution
Tacrolimus	Monitor for tacrolimus toxicity	Monitor for tacrolimus toxicity	Monitor for tacrolimus toxicity	Monitor for tacrolimus toxicity	Use with caution

Source: Data from [178–180]

As a potent inductor of CYP enzymes, rifampin significantly increases the metabolism of azoles. Concomitant use of rifampin with itraconazole, voriconazole, or isavuconazole should be avoided, and consideration to increasing fluconazole dosing is required if concomitant use is necessary [184].

In the management of HIV-infected patients, fungal infections are frequent and important complications. Efavirenz should not be coadministered with itraconazole or posaconazole. Voriconazole and itraconazole doses >200 mg/day are not advised in patients receiving protease inhibitors. Posaconazole can increase >100% AUC of atazanavir (boosted or unboosted). Fluconazole use does not generally require adjustment of dosing and can be used with most antiretrovirals [181].

2.2.1.7 Dosing and Administration. Use in Special Populations

Dosing criteria for triazoles are already established for general use in adult populations. However, the newer drugs (posaconazole, isavuconazole) still miss clinical pharmacokinetic data for groups of patients such as neonates, younger children, and pregnant women.

Neonates and Children As more experience is accumulated, use of older triazoles in children is recommended for their routine indications [185, 186]. FDA labeling of voriconazole and posaconazole still restricts their use to older children [187]. In spite of being an older drug, itraconazole has not been developed for pediatric use and does not have formal indications [188]. Recommendations for the use of triazoles in the management of aspergillosis are similar in children and adults, although recognizing that doses of voriconazole are higher in younger children (<12 years) and in younger adolescents with a weight < 40 kg. In these patients, loading dose is 9 mg/kg twice, followed by 4–8 mg/kg (higher dose for invasive molds and more serious infections) [35, 189].

Pregnant Women According with the FDA classification of drugs and risk category in pregnancy, fluconazole status varies with the dose used. It is considered C when a single 150 mg-dose is indicated, but is D for higher doses. This in based on observation of birth defects in five children exposed in utero to fluconazole, and animal experiments showing teratogenic potential. Fluconazole remains contraindicated in pregnancy with the exception of the lower dose. Itraconazole, isavuconazole and posaconazole are currently classified as category C, while voriconazole is in category D. Current consensus is to restrict use of triazoles in pregnancy, in particular in the first trimester [190].

Renal Failure Dose adjustment is recommended for fluconazole. Daily dose should be reduced by 50% with a creatinine clearance lower than 50 mL/min. For end-stage renal disease patients (ESRD) on hemodialysis (HD) dose is normal and goes after dialysis. Parenteral voriconazole preparation with cyclodextrin is not recommended in renal failure due to potential accumulation and toxicity of cyclodextrin. Patients who are already on some form of renal replacement therapy do not

have any concern as cyclodextrin is efficiently removed by dialysis. Because cyclodextrin contained in Itraconazole oral solution is metabolized by amylase, patients with renal failure can use it without dose adjustment [191].

Liver Failure Voriconazole has more extensive liver metabolism and in moderate to severe liver failure, its maintenance dose should be reduced to 50% after a regular loading regimen.

Obesity Fluconazole should be dosed by total body weight to achieve AUC/MIC ratios that have been associated with better outcomes. Posaconazole dose should not be corrected for increased body weight. This has still to be evaluated for the newer formulations (tablet, IV solution). Voriconazole dosing using total body weight can reach supratherapeutic concentrations. Its dose should not be changed for increased body weight or BMI in the case of oral voriconazole. Until further studies are performed, use of either the adjusted body weight or ideal body weight when dosing weight-based IV voriconazole could be justified [192].

2.2.2 Individual Agents

2.2.2.1 Itraconazole

Itraconazole is available as a 100 mg capsule and an oral suspension in cyclodextrin (100 mg/10 mL). Cyclodextrin makes itraconazole soluble and enhances its absorption. Oral absorption of the capsule depends on food intake, although absorption of the solution is best on an empty stomach. Interestingly, coadministration with a cola beverage increases the AUC of the capsule formulation. Absorption of the capsule is decreased with hypochlorhydria, mucositis, and graft-versus-host intestinal changes, conditions that can be present in AIDS patients or bone marrow transplant recipients.

Therapeutic drug monitoring is useful to adjust proper dosing, taking in consideration the method used for adequate interpretation. Tissue, pus and bronchial secretion concentrations of itraconazole are higher than plasma levels. The drug is metabolized in the liver and excreted in feces; prolonging its half-life in cirrhosis. When administered via oral, minimal amount of active itraconazole is eliminated in urine with most of cyclodextrin (>99%) excreted intact in feces.

Itraconazole most frequent side effects are nausea and abdominal discomfort. Frequency of gastrointestinal symptoms is higher with the solution presentation (osmotic effect). Hypokalemia and edema can be seen with higher doses. Itraconazole is contraindicated in pregnancy and in nursing mothers.

Itraconazole is useful in the treatment of dimorphic organisms (*Blastomyces* spp., *Histoplasma* sp., *Coccidioides* spp., *Paracoccidioides* spp., *Sporothrix* spp.), especially in less severe forms, less immunocompromised individuals or as consolidation treatment. Because activity against *Aspergillus* is lower in comparison to newer agents (second generation), indications of itraconazole in aspergillosis are more limited. It is indicated for the management of allergic

bronchopulmonary aspergillosis or as an alternative therapy in aspergilloma [35]. Other uses include phaeohyphomycosis, ringworm, onychomycosis, tinea versicolor, and occasionally candidiasis.

Usual daily dose for cutaneous conditions, including sporotrichosis, is 200 mg. An initial loading dose of 200 mg tid for the first 3 days when used in the treatment of deep mycoses is recommended to ensure adequate serum and tissue levels in the short term. Recommended dose for treatment of invasive mycoses, selected forms of aspergillosis or prophylaxis of invasive aspergillosis is 400 mg daily, divided every 12 h.

2.2.2.2 Fluconazole

Fluconazole is an imidazole analogue to ketoconazole with more specific fungal sterol synthesis inhibition and increased antifungal activity. It has good bioavailability (>90%), which is a significant difference with itraconazole, good distribution in fluids and tissues, long serum half-life (approximately 30 h), and relatively low (11–12%) binding to plasma proteins. Its elimination is mostly renal. The molecule has a second triazole ring that decreases lipophilicity and increases unbound drug in blood.

Fluconazole for systemic use is available in capsules, tablet, powder for oral suspension, and injectable form for IV infusion at a concentration of 2 mg/mL. Tolerance to fluconazole is considered good. Liver toxicity is a concern, especially when higher doses are used for treatment of cryptococcosis or disseminated candidiasis.

Fluconazole is active against yeasts and inactive against molds. It can be used to treat mucosal candidiasis (oro-pharyngeal, esophageal, vaginal), disseminated and invasive candidiasis, cryptococcosis, and systemic dimorphic mycoses (histoplasmosis, coccidioidomycosis, paracococcidioidomycosis, and sporotrichosis). Depending on the disease severity, immunologic status or comorbidities of the patient, and available of other antifungals, fluconazole can be an initial treatment, or a consolidation phase treatment in these indications [193].

Fluconazole has had an important role in the management of serious forms of infection by *Candida* [194], although this role is changing in recent years with newer recommendations favoring use of echinocandins in most seriously ill patients [29, 195, 196]. In the case of invasive candidiasis and candidemia, fluconazole should be considered for patients not critically ill and infected by fluconazole-sensitive organisms, using a loading dose of 800 mg followed by 400 mg daily [29, 195]. In the treatment of mucosal candidiasis, lower doses of fluconazole are appropriate. A single 150 mg is indicated for vulvo-vaginal candidiasis, and daily doses of 100 mg are used for oro-pharyngeal and esophageal candidiasis.

Fluconazole at doses of 800 mg/day combined with amphotericin B is recommended as an alternative regimen for induction treatment in cryptococcal meningitis or disseminated disease in HIV patients when flucytosine is not available. In these patients, doses of 400 or 200 mg daily are used in the consolidation and maintenance phases respectively [30]. Dose of 400 mg daily of fluconazole are recommended as initial

antifungal treatment of immunosuppressed and immunocompetent patients with mild-to-moderate pulmonary cryptococcosis [30].

2.2.2.3 Voriconazole

Voriconazole is a second generation triazole that was approved for clinical use in 2002. In voriconazole, the second triazole ring has been replaced with a fluoropyrimidine nucleus, which explains its broader spectrum. The main advantage of Voriconazole over first-generation triazoles is its activity against filamentous fungi, including *Aspergillus* sp., *Fusarium* spp., and *S. apiospermium* [197–199]. Despite its broad-spectrum activity against yeast and molds, voriconazole is not active against Mucorales.

Currently, voriconazole is considered the drug of choice for the treatment of invasive aspergillosis [35, 200]. Reported clinical experience shows some promising results with combination of voriconazole and echinocandins as a rescue regimen [201–204].

Voriconazole is available for oral and parenteral use. It should be taken with empty stomach because food and high content of fat decrease Voriconazole absorption, reducing bioavailability in 20%. Half-life is 6 h, requiring twice daily administration. Parenteral formulation of voriconazole also contains cyclodextrin, which could accumulate in renal failure. Its use in patients with renal impairment should be individualized.

In general, voriconazole is well tolerated. Reversible disturbances in vision (impaired color discrimination, blurring, and photophobia) is reported in about 25–30% of patients and are not seen with other triazoles. Skin rashes, photosensitivity, facial erythema, hallucinations and confusion are other significant side effects. Periostitis has been associated to prolonged use, and serious EKG alterations (torsade de pointes, QTc prolongation) have occurred in patients with predisposing factors to arrhythmia.

For invasive aspergillosis and serious mold infections, a loading dose of 6 mg/kg twice daily is recommended in the first day, followed by 4 mg/kg bid. For the treatment of invasive *Candida* infections dose should be lower, at 3 mg/kg bid. Oral dosing (tablets) is 400 mg bid the first day and then 200 mg bid for persons of >40 kg. For persons under 40 kg, the recommended dose is 200 mg bid for the first day, followed by 100 mg bid. Intake should be 1 h apart from meals.

2.2.2.4 Posaconazole

This triazole has a broad spectrum of antifungal activity, including *Aspergillus* and *Candida* [167]. It can be considered a derivative of itraconazole with structural modifications that enhance its activity and tissue concentration (lipophilic molecule with high concentration in tissues). Oral absorption and bioavailability of posaconazole suspension are difficult to predict, because they are significantly affected by factors like meal ingestion or presence of mucosal lesions in the gastrointestinal

tract [205–207]. Posaconazole suspension should be taken with high-fat meals to enhance absorption. Although its half-life is long and suggests the possibility of once daily dosing, AUC is higher with bid or tid dosing, which is recommended for the oral suspension.

The newer delayed-release tablet has better absorption, improved bioavailability, and a fourfold increase in maximum concentration, a threefold increase of the area under the curve, and more prolonged presence in plasma. In contrast to the suspension, the effect of food or drugs that may alter gastric acidity is moderate. Additionally, patient intervariability is reduced. All this favorable pharmacokinetic changes allow for once daily dosing [161, 208, 209].

Posaconazole is usually well tolerated. In clinical trials the most common side effects have been gastrointestinal symptoms including nausea and abdominal pain. Initially available only for oral administration, its indications have been mainly referred to prophylaxis of fungal infection in patients at high risk (prolonged neutropenia, acute myeloid leukemia, post-transplant), in particular for filamentous fungal complications [210, 211]. The oral suspension has been evaluated for refractory aspergillosis at a daily dose of 800 mg with a 42% rate of global response [212]. Currently, a formulation for parenteral administration is also available and undergoing phase III clinical trial in the treatment of invasive aspergillosis, with completion estimated for July 2018 [213].

Posaconazole has good activity against *Mucor* spp. and has a role in the treatment of mucormycosis in combination with surgical procedures, usually after an initial period with amphotericin B [214].

Oral suspension and delayed-release tablet have different dosing regimens. The dose of the suspension is 200 mg tid with food for prophylaxis or 400 mg bid with meals when indicated for treatment [215–217], while the 300 mg tablet is administered once a day. Intravenous dosing of 300 mg daily is approved for prophylaxis.

2.2.2.5 Isavuconazole

It is the newest member of the second-generation triazole antifungal approved by the US FDA [159, 168, 180]. It has been approved for the treatment of both invasive aspergillosis and invasive mucormycosis [218]. It is also under investigation for the treatment of candidemia and invasive candidiasis, cryptococcosis, and dimorphic fungi [219].

Isavuconazole is administered as the hydrosoluble prodrug isavuconazonium, which is available in tablets and for parenteral administration. In preclinical and clinical studies, it has shown significant antifungal potency against a broad range of yeasts, dimorphic fungi, and molds. Isavuconazole has a broad spectrum of antifungal activity, similar to amphotericin B.

Clinical experience so far has revealed that isavuconazole may be associated with less toxicity than voriconazole, even when administered without therapeutic drug monitoring. Additionally, the oral formulation is highly bioavailable and the parenteral presentation is b-cyclodextrin–free (due in large part to the presence of aromatic

moieties in the molecule). These are interesting properties that will increase interest on isavuconazole as a new addition to the triazole class of antifungals. Isavuconazole dosing is similar when administered either intravenously or orally. In both cases, the loading dose is 200 mg every 8 h for six times, followed by 200 mg daily.

2.2.3 Newer and Investigational Agents: Efinaconazole, Albaconazole, Ravuconazole and Others

A numerous group of newer triazole molecules are currently under different stages of development [220]. Preliminary clinical studies are already available for ravuconazole, albaconazole, and efinaconazole (available as a topical agent).

Ravuconazole is related to fluconazole and voriconazole. It has activity against yeasts (*Candida* spp. and *Cryptococcus* spp.), dimorphic fungi and filamentous organisms (dematecious, mucorales). It is not active in vitro against *Fusarium* spp. Clinical studies for onychomycosis have been reported with a mycological cure rate of 59% and clinical response of 56% [221]. It is still to be determined what potential indications may have and what results are obtained in clinical trials for systemic mycoses.

Albaconazole shows low MICs against *Candida* spp. and has been clinically studied in the treatment of vulvo-vaginal candidiasis and onychomycosis, and experimentally against *S. prolificans*. Its long half-life allows for weekly dosing.

Efinaconazole is a potent antifungal drug against *T. rubrum*, *T. mentagrophytes* and *C. albicans*, approved in 2014 for the treatment of onychomycosis. It also has activity against other species of fungus, including some nondermatophytes molds (*Acremonium* spp., *Fusarium* spp., *Paecilomyces* spp., *Pseudallescheria* spp., *Scopulariopsis* spp., and *Aspergillus* spp.), *Cryptococcus* spp., *Trichosporon* spp., and other species of *Candida* different to *C. albicans* [222].

The list of newer compounds includes RI26638, KP103, T8581, TAK187, FX0685, ZJ522, TAK456, Syn2869, and additional molecular modifications for dioxantriazoles, triazole-quinoxalines, and triazole-benzimidazoles.

The search for newer clinically active compounds might lead to the availability of triazole derivatives with increased antifungal spectrum and effectiveness, as well as better tolerance.

2.3 Echinocandins

Echinocandins are the newest members of the antifungal armamentarium and the first ones targeting the fungal cell wall [223, 224]. Currently, three semi-synthetic echinocandin derivatives have received FDA approval for clinical use: caspofungin (2001), micafungin (2005), and anidulafungin (2006). A fourth compound, the CD101, is under development.

2.3.1 Chemical Structure

Echinocandins are semisynthetic lipopeptides antibiotics, composed of cyclic hexapeptides with modified N-linked acyl lipid side chains [225, 226] (Fig. 2.3).

2.3.2 Mechanism of Action

Echinocandins competitively inhibit the beta-1,3-D-glucan synthesis, a polysaccharide which is an essential component of the fungal cell wall of many fungi. Beta-glucans represent between 30 and 60% of the cell wall mass in yeasts, and its depletion results in fungicidal activity for *Candida* spp. and fungistatic effect for *Aspergillus* spp. [230, 231].This mechanism of action is different from the one of other drugs, allowing a potential use of echinocandins in combination therapy [232], and because the target of echinocandins is unique to fungi, then absent in human cell, these drugs cause less toxicity and have fewer drug–drug interactions. In addition, some evidence from in vitro studies and murine models supports an immunomodulatory effect of echinocandins. They can unmask highly antigenic epitopes and amplify the host immune response [233].

2.3.3 Pharmacokinetics and Pharmacodynamics

Although pharmacokinetic and pharmacodynamic characteristics of echinocandins are similar, they differ in dosing, metabolic elimination pathways, and drug interaction profile. Like other large lipopeptide antibiotics, these drugs are poorly absorbed through the gastrointestinal system and must be administered by intravenous infusion. Due to their long half-life (10–26 h), they are dosed once daily, and because echinocandins are highly bound to plasma proteins, administration of a loading dose is recommended for caspofungin and anidulafungin, although it is not yet clear for micafungin. Also, high binding to plasma protein limit distribution of echinocandins to the cerebrospinal fluid and the eye, making them inadequate treatment for infections of these compartments [234–236].

Echinocandins are primarily eliminated through nonmicrosomal metabolism nonenzymatic degradation to inactive products, and then their urinary concentration is very low [234, 237]. They are not significantly metabolized by the cytochrome P450 enzymes nor are they substrates or inhibitors of P-glycoprotein pumps. As consequence, they have less drug–drug interactions in comparison with others antifungal drugs. However, caspofungin must be used with caution when severely impaired hepatic function is present.

Caspofungin shows a net terminal half-life of 27–50 h, and degrades spontaneously and is metabolized via hydrolysis and N-acetylation to two inactive

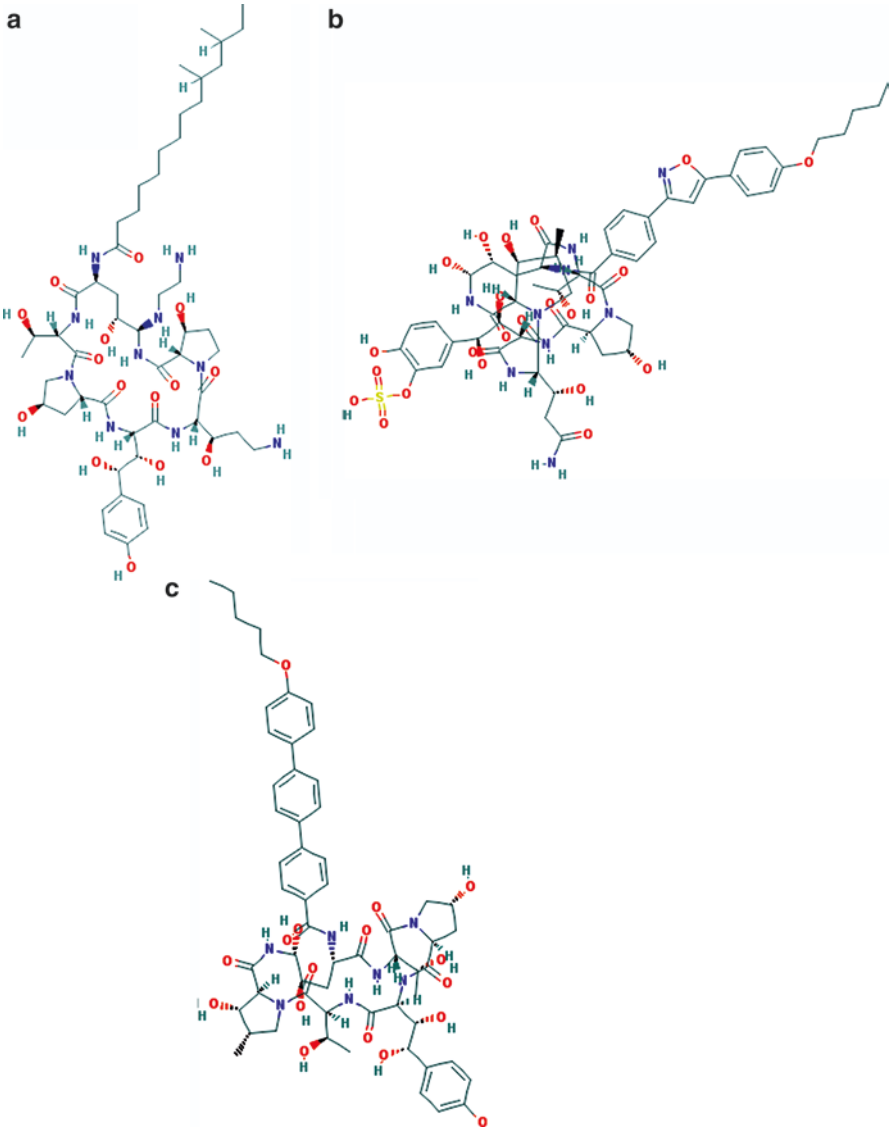

Fig. 2.3 Chemical structure of Echinocandins (**a**) Caspofungin, (**b**) Micafungin, and (**c**) Anidulafungin. Source: References [227–229]

metabolites; micafungin has a terminal half-life of approximately 15 h in adults, and is metabolized hepatically by arylsulfatase, catechol *O*-methyltransferase, and hydroxylation; while anidulafungin shows a terminal half-life of 40–50 h, and is not metabolized but instead eliminated by slow spontaneous degradation. All three echinocandins are nondialyzable, and their breakdown products are excreted predominantly by the fecal route, with only low concentrations of active drugs excreted by urine (less than 2%) [226, 231, 238].

In vitro studies showed that the fungicidal effect of echinocandins against *Candida* spp. is proportional to the maximum plasma drug concentration, that this effect persist after falling of drug concentration below MICs, and that it seems to correlate with the area under time-concentration curve to MIC ratio [237, 239]. However, similar information related to killing or inhibition of Aspergillus spp. is not completely defined yet [239, 240]. In addition, there are not established strategies to conduct therapeutic drug monitoring for echinocandins [237, 239].

2.3.4 Spectrum of Activity and Resistance

Because echinocandins show a similar spectrum of activity, they could be interchangeable specially when treating candidiasis infections. They have potent activity against many Candida spp. (*C. albicans*, *C. glabrata*, *C. dubliniensis*, *C. tropicalis*, and *C. krusei*), and although MICs againts *C. parapsilosis* and *C. guilliermondii* are often higher, they are useful drugs against these candida species [241, 242]. The fungicidal activity against *Candida* spp., including fluconazole-resistant *C. glabrata* and *C. krusei*, is the main advantage of echinocandins [226].

Even though echinocandins inhibit growth of *Aspergillus* species at very low echinocandin levels, their activity against *Aspergillus* spp. is only fungistatic [223, 243–245], this is explained because in *Aspergillus species*, higher activity of cell wall remodeling and beta-glucan synthase is localized in apical and sub-apical branching points. In guinea pig models, echinocandins seem to potentiate the activity of triazoles against *Aspergillus* spp. [219, 246].

Although beta-1,3-D-glucan synthase from Cryptococcus spp. is highly inhibit by caspofungin, echinocandins have not activity against *C. neoformans* and *Cryptococcus gattii*, neither against *Trichosporun* spp. [225, 226]. Echinocandins are not effective drugs to treat mycosis produced by endemic dimorphic fungi (*Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Coccidioides* spp.), due to their modest activity against the mycelial phase of them. In addition, echinocandins have not significant activity against non-*Aspergillus* molds (Mucorales, *Fusarium* spp., or *Scedosporium* spp.) [226, 247–249], and only modest in vitro activity, without clinical utility, for some phaeohyphomycetes [250, 251]. Echinocandins are effective agents for prophylaxis of *Pneumocystis jirovecci* pneumonia although less effective for established pneumonia in experimental models [252, 253].

In contrast with what happens with amphotericin B and triazoles, activity of echinocandins are not affected by presence of biofilm; echinocandins MICs are

minimally affected when tested under biofilm conditions. *C. albicans* inoculum embedded in biofilm is almost completely cleared at the usual echinocandin serum levels [254, 255]. When evaluating activity against *C. tropicalis* biofilm, micafungin showed high activity while liposomal amphotericin B performed poorly [256]. This unique characteristic of echinocandins makes them particularly useful for the treatment of prosthetic device and catheter-associated infections.

Overall resistance to echinocandins of *Candida* spp. has been reported in up to 4%, and results from mutations in conserved regions of the gene-encoding glucan synthase (FK1 and FK2) [257, 258], and resistance to echinocandins has been documented for *C. albicans*, *C. glabrata*, *C. lusitaniae*, *C. tropicalis*, and *C. parapsilosis* [259, 260]. Previous exposure to an echinocandin had been associated with echinocandin resistance on multivariative analysis [254].

Resistance of *C. glabrata* is of particular concern, because it is now reported from around the world, at rates between 3 and 15%, and because isolation of strains with resistance to fluconazole and voriconazole and to echinocandins [259, 261–264]. Among the 162 fluconazole-resistant *C. glabrata* strains isolated between 2006 and 2010 in the US, resistance to any echinocandin was demonstrated in 18 (11%), while there was no resistance to echinocandins among 110 fluconazole-resistant strains isolated between 2001 and 2004 [262]. All the 18 resistant isolates contained an FKS1 or FKS2 mutation.

Resistance to echinocandins is associated with treatment failure and relapse or recurrence if there was an initial response and with higher rates of mortality at days 14 and 30 [261, 265, 266].

In an organ transplant recipient with persistent candidemia, Imbert and colleagues demonstrated that switching from both azole and echinocandin therapy to liposomal amphotericin B, produced that resistant *C. glabrata* isolate lost the FKS2 S663P alteration, regaining full susceptibility to echinocandin, while maintaining their pan-azole resistance. Based on this observation, authors suggest that more restricted use and/or a discontinuous administration of echinocandins may limit the spread of clinical resistance to these drugs [267].

2.3.5 Clinical Uses

Echinocandins are extensively used for prevention and empiric treatment of fungal infection, and for treatment of invasive candidiasis, especially in critically ill and neutropenic patients. The three echinocandins have FDA approval for the treatment of esophageal candidiasis and invasive candidiasis in adults. Micafungin has FDA approval to be used as prophylaxis of *Candida* infections in hematopoietic cell transplanted adults, while caspofungin is approved as empiric treatment for neutropenia febril, and for esophageal candidiasis and invasive candidiasis in children older than 3 months [29, 268, 269]. Echinocandins had demonstrated improved survival when compared to amphotericin B and triazoles in the treatment of candidemia and invasive candidiasis [191, 270, 271] and, similar efficacy to amphotericin

B and fluconazole in the treatment of oropharyngeal or esophageal candidiasis. However, they are not frequently used for these latter indications due to their parenteral-only presentation [272–277].

Although echinocandins are not the choice to treat aspergillosis, they had shown useful for the treatment of refractory aspergillosis, when used in combination with voriconazol or with amphotericin lipid formulations [278–280]. Caspofungin has FDA approval as salvage therapy of invasive aspergillosis, and current IDSA guidelines stated that caspofungin or micafungin can be used to treat aspergillosis in settings in which azole and polyene antifungals are contraindicated [35]. There is also limited evidence supporting the use of echinocandins in combination therapy for the initial treatment of aspergillosis. Association of anidulafungin to voriconazole therapy had shown improved outcome in comparison to monotherapy, although without statistical significance [203, 232, 281].

Because their low urinary excretion rate, echinocandins are not considered for the treatment of UTIs. However, patients with fluconazole-resistant *Candida* spp. or with hepatic injury and fluconazole-sensitive *Candida* spp. have been successfully treated with caspofungin [282].

2.3.6 Adverse Events and Toxicity

Due to the target of echinocandins is absent in human cells, these drugs cause less toxicity. Mostly, echinocandins are well tolerated and their adverse events are mild and similar for all the three drugs currently in use. Serious adverse events requiring treatment discontinuation are fewer with these drugs than with other systemic antifungals. Most common adverse events are gastrointestinal symptoms (diarrhea, nausea, vomiting, abdominal pain, abdominal distention, and constipation), laboratorial alterations (increment of aminotransferases and alkaline phosphatase and bilirubin, hypokalemia, among others) and general disorders and administration site conditions (pyrexia, edema peripheral, Infusion-related reaction, pain at the site of infusion). Table 2.5 summarizes the most frequent adverse reactions, with frequency of at least 5% in any of the groups under evaluation, reported in clinical trials testing echinocandins [283–285].

Asymptomatic elevation of liver enzymes, 5–13% for aminotransferases and 12% for alkaline phosphatase, is less frequent in patients treated with echinocandins in comparison with azoles and amphotericin B. Because hepatitis, hepatomegaly, hyperbilirubinemia, and hepatic failure have been rarely reported, monitoring of hepatic enzymes is recommended when using echinocandins [226, 283–285]. Renal adverse event reported with the use of echinocandins involved mild decrease of serum potassium, reported between 11 and 20% in clinical trials, without significant drug related toxicity observed [226, 283–285]. Occurrence of anemia, neutropenia, and thrombocytopenia have been reported between 6 and 15% in clinical trials, but again hematologic toxicity attributed to echinocandins is infrequent [226, 283–285].

Table 2.5 Adverse reactions[a] in patients who received ECHINOCANDINS in clinical trials[b], incidence 5% or greater in any of the groups under evaluation

	Caspofungin (n = 1951)		Micafungin (n = 479)		Anidulafungin (n = 131[d])	
Adverse reactions[c]	*n*	%	*n*	%	*n*	%
With at least one adverse reaction	**1665**	**85**			**130**	**99**
Investigations	**901**	**46**	**191**	**40**	**66**	**50**
Alanine Aminotransferase Increased	258	13	45	10	7	5
Aspartate Aminotransferase Increased	233	12				
Blood Alkaline Phosphatase Increased	232	12			15	12
Blood Potassium Decreased	220	11			33	25
Blood Bilirubin Increased	117	6				
Urine output decreased			18	4		
White blood cell increased					11	8
Blood creatinine increased					7	5
Hypomagnesemia					15	12
Hypoglycemia					9	7
Hyperkalemia					8	6
Hyperglycemia					8	6
General disorders and administration site conditions	**843**	**43**	**256**	**53**	**70**	**53**
Pyrexia	381	20	103	22	23	18
Chills	192	10				
Edema Peripheral	110	6			14	11
Infusion-related reaction			24	5		
Chest pain					7	5
Gastrointestinal disorders	**754**	**39**	**285**	**60**	**81**	**62**
Diarrhea	273	14	106	22	24	18
Nausea	166	9	91	19	32	24
Vomiting	146	8	91	19	23	18
Abdominal Pain	112	6	76	16	8	6
Abdominal Distension			29	6		
Constipation					11	8
Blood and lymphatic system disorders			**161**	**34**	**34**	**26**
Thrombocytopenia			70	15	8	6
Neutropenia			61	13		
Anemia			63	13	12	9
Febrile neutropenia			23	5		
Leukocytosis					7	5
Infections and infestations	**730**	**37**			**82**	**63**
Pneumonia	115	6			8	6

(continued)

Table 2.5 (continued)

Adverse reactions[c]	Caspofungin (n = 1951)		Micafungin (n = 479)		Anidulafungin (n = 131[d])	
	n	%	n	%	n	%
Bacteremia					23	18
Urinary tract infection					19	15
Sepsis					9	7
Respiratory, thoracic, andmediastinal disorders	**613**	**31**	**194**	**41**	**67**	**51**
Dyspnea					15	12
Pleural effusion					13	10
Respiratory distress					8	6
Cough	111	6			9	7
Epistaxis			45	9		
Cardiac disorders			**97**	**20**		
Tachycardia			47	10		
Renal and urinary disorders			**78**	**16**		
Hematuria			18	4		
Psychiatric disorders			**80**	**17**	**48**	**37**
Anxiety			35	7		
Insomnia					20	15
Confusional state					10	8
Depression					8	6
Skin and subcutaneous tissue disorders	**520**	**27**	**197**	**41**		
Rash	159	8	55	12		
Erythema	98	5				
Pruritus			54	11		
Urticaria			24	5		
Decubitus ulcer					7	5
Nervous system disorders	**412**	**21**				
Headache	193	10			11	8
Vascular disorders	**344**	**18**			**50**	**38**
Hypotension	118	6			19	15
Hypertension					15	12
Deep vein thrombosis					13	10
Musculoskeletal and connective tissue disorders					**27**	**21**
Back pain					7	5

Source: References [283–285]

[a]Defined as an adverse reaction, regardless of causality, while on echinocandins or during the 14-day post-echinocandins follow-up period

[b]Incidence among individuals who received at least 1 dose of trial drug

[c]Within any system organ class, individuals may experience more than 1 adverse event

[d]Patients receiving 100 mg for the treatment of Candidemia/other Candida Infections

Infusion of echinocandins produces several histamine-release symptoms, including rash, pruritus, hypotension, bronchospasm, angioedema, and may be some acute cardiovascular events. Their occurrence is associated with the infusion rate and in most patients is enough to slow it to obtain improvement. In the case of anidulafungin, the infusion rate should not exceed 1.1 mg/min [226, 283–285]. In addition, rare cases of anaphylaxis, erythema multiforme, Stevens–Johnson syndrome, and skin exfoliation have been associated with the use of echinocandins, although a causal relationship has not been established [283–285].

2.3.7 Drug Interactions

Because echinocandins are not significant inhibitors or inducers of the CYP450 enzymatic pathways or p-glycoprotein drug efflux transporters, they have very few drug–drug interactions when compared with other systemic antifungals [226].There are mild interactions of caspofungin with the immunosuppressant tacrolimus and cyclosporine. In the case of tracolimus, standard drug monitoring of tracolimus is recommended. The concomitant use of caspofungin with inducers of hepatic CYP enzymes is expected to reduce the plasma concentration of caspofungin. Then adult patients receiving rifampin, which is a potent inducer of CYP3A4, should receive 70 mg/day and pediatric patients 70 mg/m^2/day of caspofungin. The same dosing should be considered when patient receive other inducers such as efavirenz, nevirapine, phenytoin, dexamethasone, or carbamazepine [283].

There is no drug–drug interaction of micafungin with mycophenolate mofetil, cyclosporine, tacrolimus, prednisolone, fluconazole, and voriconazole.

In the case of nifedipine and itraconazole, the concomitant use of micafungin increment their AUC and C_{max}, while sirolimus AUC was increased but its C_{max} not. It is recommended that patients receiving micafungin with sirolimus, nifedipine or itraconazole should be monitored for these drugs, which dose should be reduced if necessary [284].There is not drug–drug interaction of anidulafungin with cyclosporine, voriconazole, tacrolimus, rifampin, or amphothericin B liposomal [285].

2.3.8 Use in Special Population and Dose Adjustments

Pediatric Caspofungin and micafungin have FDA approval for use in children. Larger doses based on milligrams per kilogram are prescribed for both children and infants because the increased rate of clearance of these drugs among neonates, infants, and younger children compared with adolescents and adults [226, 286]. Caspofungin is considered safe and effective for pediatric patients older than 3 months, having the same indications as adults, with dosing based on body surface

area [283]. Micafungin is approved for pediatric patients older than 4 months and is dosed in mg/kg [284].

Pregnancy and Nursing Mothers All three echinocandins are class C agents in the pregnancy category. They should be used only if the potential benefit justifies the risk to the fetus. In animal studies, echinocandins caused embryofetal toxicity, including skeletal changes, increment of abortions and visceral abnormalities. Echinocandins could be detected in the plasma of the fetus, indicating they cross the placental barrier in rats. It is unknown if echinocandins are excreted in human breast milk, but they could be detected in the milk of lactating rats. Again, they should be administered to nursing mothers only if the potential benefit justifies the risk [226, 283–285].

Dose Adjustments As described above, a 70 mg/day dose of caspofungin is recommended when adult patients use rifampin concomitantly, while the pediatric dose is 70 mg/m^2/day. The same dosing should be considered if there is concomitantly use of other inducer of CYP450, such as carbamazepine, dexamethasone, efavirenz, nevirapine, or phenytoin [283–285].There is no need of dose adjustment in presence of renal insufficiency, including patients in hemodialysis or continuous renal replacement therapy [226, 283–285]. In the case of adults with mild hepatic insufficiency, maintenance dose of caspofungina is the same. This should be reduced to 35 mg/day in the case of moderate hepatic insufficiency (Child-Pugh score 7 to 9). There is no recommendation available for dosing caspofungin in adults with severe hepatic insufficiency or pediatric patients with any degree of hepatic insufficiency [283]. There is no need of dose adjustment of micafungin or anidulafungin in presence hepatic insufficiency of any degree [284, 285].

Obesity Because clearance of echinocandins increment with body weight and there is no difference in outcomes of obese and nonobese patients receiving the same dose of caspofungin, it is recommended an increment between 25 and 50% of the daily dose only for patients weighting 75 kg with severe infection [226, 287].

2.3.9 Adult Dosing

The dosing of echinocandins is slightly variable according with the indication. See Table 2.6.

2.3.10 New Echinocandin

Currently, a new echinocandin, named CD101/Bifungina, is under development for topical and weekly IV administration. It exhibits prolonged stability in plasma and aqueous solutions up to 40 °C [288], and has shown in vitro activity against resistant *Candida* spp. and *Aspergillus* spp. strains. There are two phase II studies currently enrolling patients.

Table 2.6 Dosing of echinocandins for adults by indication

Indication	Caspofungin	Micafungin	Anidulafungin
Esophageal candidiasis	Loading dose 70 mg IV, then 50 mg IV daily	150 mg IV daily; no loading dose is required	200 mg IV daily
Invasive candidiasis	Loading dose 70 mg IV, ten 50 mg IV daily	100 mg IV daily; no loading dose is required	Loading dose 200 mg IV, then 100 mg IV
Salvage therapy for invasive aspergillosis[a]	Loading dose 70 mg IV, then 50 mg IV daily. Daily dose can be increased to 70 mg if response is inadequate	150 mg IV daily; no loading dose is required	Loading dose 200 mg IV, then 100 mg IV
Neutropenic fever (empiric therapy)	Loading dose 70 mg IV, then 50 mg IV daily		
Candida prophylaxis in hematopoietic cell transplant recipients		50 mg IV daily; no loading dose is required	

Source: References [283–285]
[a]Only caspofungin has US Food and Drug Administration approval for this indication

2.4 Flucytosine

2.4.1 Chemical Structure

Flucytosine (5-fluorocytosine or 5-FC) is a synthetic nucleoside analogs chemically related to anticancer drugs (fluorouracil and floxuridine). Its molecular formula is C4H4FN3O with a MW of 129.1 (Fig. 2.4).

2.4.2 Mechanism of Action

Flucytosine is transferred into fungal cells by cytosine permeases, where it is converted into 5-fluorouracil and phosphorylated to 5-fluorodeoxyuridine monophosphate. This compound inhibits thymidylate synthase, a crucial enzyme in the synthesis of 2′-deoxythymidine-5′-monophosphate that is an essential precursor for DNA biosynthesis, therefore disturbing DNA synthesis [290]. In addition, the 5-fluorodeoxyuridine monophosphate can be further phosphorylated and be incorporated to RNA, disrupting protein synthesis [291].

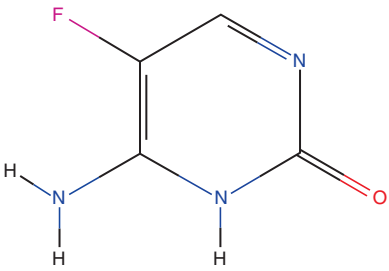

Fig. 2.4 Chemical structure of flucytosine. Source: Reference [289]

2.4.3 *Pharmacokinetics and Pharmacodynamics*

A feature of the drug is its almost complete and fast absorption after oral administration, having a bioavailability of 76–89% [292]. The AUC is 62 mg·h/L and the maximal concentration is 80 μg/mL [290]. Flucytosine (5-FC) achieves fungistatic levels quickly and distributes extensively throughout the body fluids, including eyes and the cerebrospinal fluid, where it reaches approximately 75% of serum levels.

The 5-FC half-life in humans with normal kidney function is 3–5 h, but it is considerably delayed to 30–250 h in renal insufficiency [293, 294]. Only 2–4% of 5-FC is protein binding, between 80 and 90% is eliminated unchanged in the urine, and the liver metabolizes only a minimal amount. Flucytosine is removed by hemodialysis in 66–75%, but peritoneal dialysis is not as effective as hemodialysis [292, 295].

2.4.4 *Spectrum of Activities and Resistance*

Flucytosine is active against *C. neoformans* and *Candida* species except *C. krusei*, but isolates of *Aspergillus* species are usually nonsusceptible to 5-FC in vitro. Exist synergy with amphotericin B, which modifies the permeability of the fungal cell membrane allowing greater penetration of 5-FC.

Fungi with primary resistance to 5-FC are rare. A mutation in the *FCY2* gene, which encodes the cytosine permease, affects the absorption of the drug diminishing accumulation of the drug within the cell [296, 297]. Secondary resistance develops during therapy, especially during monotherapy, and it is based on inactivation of enzymes of the pyrimidine pathway. Mutations in the *FCY1* gene that encodes for the cytosine deaminase, or mutation in the *FUR1* gene that encodes for the uracil phosphoribosyl transferase induce acquired resistance by interference in the conversion of 5-FC to 5-fluorouracil, or from 5-fluorouracil to 5-fluorouridine monophosphate respectively [296–300].

Other resistance mechanisms have been suggested for *C. glabrata*. It was found that in the presence of 5-FC the fungal cell wall showed higher resistance to lyticase, suggesting that cell wall alteration occurs in response to 5-FC. Genes CgFPS1 and CgFPS2 of *C. glabrata*, encoding a plasma membrane aquaglyceroporin, are

recognized as factors of 5-FC resistance. Both genes facilitate resistance by declining 5-FC accumulation in *C. glabrata* cells. Unlike, the deletion of CgFPS2 and particularly of CgFPS1 was found to improve the susceptibility to 5-FC registered for the parental strain [301].

2.4.5 Clinical Uses

Flucytosine should be used in combination therapy, generally with amphotericin B (Amph B), to decrease development of resistance. This combination is recommended as primary therapy for cryptococcal meningitis, severe pulmonary cryptococcosis and cryptococcocemia [30]. Additionally, 5-FC in combination with Amph B is used for patients with refractory *Candida* infections, such as endocarditis, meningitis, or endophthalmitis and it is also recommended for the treatment of symptomatic ascending *Candida* pyelonephritis due to fluconazole-resistant *C. glabrata* [29].

The ESCMID and ECMM guidelines for the management of rare invasive yeast infections recommend amph B alone or in combination with 5-FC for infections due to *Geotrichum candidum* or *Rhodotorula* spp. They suggest the combination of amph B and 5-FC for infections due to *Saccahromyces cerevisiae,* and the combination of triazole plus echinocandin plus 5-FC to treat cerebral abscess due to dematiaceous fungi when surgery is not possible [30, 302].

2.4.6 Adverse Events and Toxicity

The toxicity to 5-FC is dose-dependent. The most frequent adverse events with this drug are bone marrow depression (leukopenia, anemia, and thrombocytopenia) and gastrointestinal disturbances (nausea, vomiting, diarrhea, abdominal pain, anorexia, dry mouth, and duodenal ulcer) [303–308]. Although bone narrow toxicity can occur with lower serum concentrations of 5-FC, it is more frequent when the concentration is greater than 100 μg/mL [305, 309]. For this reason, it is necessary to monitor the 5-FC serum concentrations to be sure they range between 25 and 100 μg/mL [310].

Less frequently, toxicity occurs in the central nervous system (headache, drowsiness, confusion, vertigo, and hallucinations) or manifest as liver function test abnormalities (jaundice, bilirubin elevation, increased hepatic enzymes, and acute hepatic injury). Colitis is reported infrequently, with toxicity related to local cytotoxic effect on protein synthesis [311–313].

Recently, a study performed in mice suggests that therapy with amph B combined with 5-FC originates a synergistic inflammatory activation in a dose-dependent way in hepatic tissues. Caution when using this antifungal combination is required, particularly for patients with hepatic deficiency [314].

2.4.7 Drug Interactions

Use of clozapine or deferiprone concurrently with 5-FC is not advised. They procainamide. Use of cytosine arabinoside could deactivate the antifungal action of 5-FC by competitive inhibition [290].

It was also noted that drugs which decrease glomerular filtration may extend the half-life of 5-FC [315]. Amph B-associated nephrotoxicity will delay elimination of 5-FC, causing an increase in serum 5-FC concentrations, may increase the risk and severity of bone marrow toxicity. Others agents that can increase the myelotoxic risk and therefore caution should be exercised in their use concomitantly with 5-FC are antineoplastic drugs (cyclophosphamide, doxorubicin, methotrexate, paclitaxel, vinblastine), antiviral agents (gancyclovir, foscarnet), antirretrovirals (zidovudine, lamivudine, didanosine, stavudine), chloramphenicol, dapsone, interferon alfa, linezolid, pentamidine and procainamide.

2.4.8 Use in Special Population

Dose adjustment is necessary in patients with renal dysfunction. In that case, the dose interval has to be extended (see Table 2.7). With a creatinine clearance below 10 mL/min, 5-FC serum levels should be monitored, doing appropriate dose adjustments so not to exceed 80 μg/mL. Because 5-FC is dialyzable, the daily dose must be administered post hemodialysis.

Because of reduced renal function in neonates with a very low birth weight, use of 5-FC in this population should be done with very close monitoring of serum drug levels to avoid large accumulation of 5-FC in plasma [72]. Flucytosine is considered as category C according to the FDA pregnancy category (animal studies show toxicity, human studies inadequate but benefit of use may exceed risk). It is contraindicated during early pregnancy (first trimester) because the drug crosses the human placenta and for its known teratogenic effect in rats and its interference with DNA synthesis in the growing fetus [320, 321]. The delivery of 5-FC in human milk is unknown, and its use during breastfeeding is not recommended. Dosing of 5-FC in obese patients is that for the ideal body weight.

Table 2.7 Flucytosine dosing in adult people according to glomerular filtration rate (standard dose of 100–150 mg/kg/day)

Renal clearance mL/min	Dose	Period (h)
>50	25–37.5 mg/kg	Every 6 h
10–50	25–37.5 mg/kg	Every 12–24 h
<10	25–37.5 mg/kg	Every 24–48 h
Hemodyalisis	25–50 mg/kg	Dose post-dialysis, every 48–72 h
Peritoneal dialysis	0.5–1.0 g	Every 24 h

Source: Data from [316–319]

2.5 Terbinafine

Terbinafine is a drug that belongs to the allylamine group, which includes also the topical antimycotic naftifine. Terbinafine is potent inhibitors of ergosterol biosynthesis, available as tablets, spray, cream, and gel formulations.

2.5.1 Chemical Structure

Chemically, terbinafine is (E)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1- naphthalenemethanamine hydrochloride with a MW of 327.90 (Fig. 2.5).

2.5.2 Mechanism of Action

Terbinafine acts as antifungal drug by noncompetitive inhibition of squalene epoxidase, an enzyme that converts the squalene into 2,3-oxidosqualene that then form lanosterol, which is a precursor of ergosterol, an essential constituent of fungal membrane. At that point, the intracellular accumulation of squalene, which is toxic to fungal cells, and a deficiency in ergosterol cause a quick cell death [323, 324].

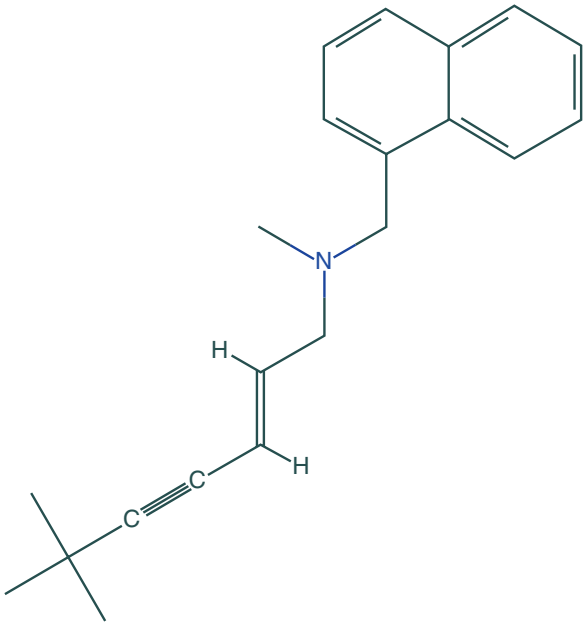

Fig. 2.5 Chemical structure of terbinafine. Source: Reference [322]

2.5.3 Pharmacokinetics and Pharmacodynamics

Terbinafine is well absorbed from the gastrointestinal tract, with a bioavailability ranging from 70 to 85%, which varies discretely when it is taken with foods no requiring dose modification. It has a low affinity for muscle, spleen, and brain tissues, but it has high affinity for the skin and adipose tissues, which are the largest depot for the antifungal drug [325, 326].

Gastric acidity does not seem to influence the absorption. Terbinafine reaches maximal plasma concentrations approximately 2 h after a single dose, getting high concentrations in the adipose tissue, stratum corneum, dermis, epidermis, sebum, nails, and in the hair follicle [327–329]. Penetration of the antifungal drug into the brain ranges between 6 and 43% of the plasma concentration, and is undetectable in CSF [325].

Linear dose-proportional pharmacokinetic has been proven after a range of single doses from 125 to 750 mg of terbinafine [330]. Steady-state plasma levels of terbinafine are attained after 10–14 days of treatment decreasing rapidly after the end of treatment [331, 332]. Plasma half-life ranges from 16 to 26 h after administration of 250 mg of terbinafine in healthy volunteers. A supplementary elimination phase with a half-life of more than 90 h was detected after administration of radiolabelled terbinafine [333–336].

This antifungal drug is extremely lipophilic, 99% protein bound and it has a good penetration in the keratinized tissue, reaching active concentrations in nail in 1–2 weeks. A characteristic of this drug is to remain in the nail in therapeutic concentrations for a long time after discontinuing the drug. Terbinafine is not detected in sweat [328, 329, 335].

Terbinafine is metabolized by the liver. Several cytochrome isoenzymes are involved in the main pathways of terbinafine metabolism. Some of them are CYP2C9, CYP2C8 and CYP1A2 (N-demethylation), CYP3A4 (deamination), CYP2C9, CYP2C8, CYP2C19, and CYP1A2 (alkyl side chain oxidation) and, CYP2C9 and CYP1A2 (dihydrodiol formation). The multiple cytochrome P-450 s implicated in the metabolism of this drug indicates a reduced potential for drug–drug interactions [337, 338]. Nonetheless, terbinafine may inhibit the metabolism of CYP2D6 substrates. It could be important for the interaction with some drugs that are known substrates of CYP2D6 such as amitriptyline, carvedilol, codeine, haloperidol, metoprolol, paroxetine, risperidone, and tramadol. The coadministration of terbinafine with these drugs with could cause a prolonged increase in their plasma levels [339].

Terbinafine is excreted mainly in the urine (80%) and in small amounts in feces (20%) [335]. In children, a dosage adjustment according to bodyweight is recommended because AUC values are significantly higher than in young adults [340].

2.5.4 Spectrum of Activities and Resistance

Terbinafine has potent antifungal activity against a diversity of dermatophytes isolated from patients worldwide, including *Trichophyton tonsurans*, *Microsporum canis*, *M. gypseum*, *T. verrucosum*, *T. violaceum*, *M. audouinii*, *T. rubrum*, *T. interdigitale*, *T. mentagrophytes,* and *Epidermophyton floccosum* [341–343]. Also, in vitro studies have shown that terbinafine is highly active against *Sporothrix brasiliensis*, *S. schenckii*, and *S. globosa* isolated from clinical samples in Brazil [344, 345], and for a wide variety of agents of chromoblastomycosis [346]. Terbinafine is only moderately active against *Madurella mycetomatis* isolates [347].

2.5.5 Clinical Uses

Terbinafine is available in different formulations, tablets, and topical preparations such as cream, jelly, spray, and solution. Topical formulations are used to treat superficial fungal infections due to dermatophytes or Candida species.

Terbinafine is the first line treatment for toenail onychomycosis due to dermatophytes [348, 349]. This antifungal drug presents the greatest rate of mycological cure according to results from a systematic review and a network meta-analysis conducted to compare the relative efficacy of treatments for onychomycosis due to dermatophyte [350]. Oral terbinafine has been also proved effective in treating onychomycosis due to *Scopulariopsis* species [350–352].

Terbinafine is also indicated for tinea capitis caused by *Trichophyton* species in children. A meta-analysis of randomized, controlled trials comparing griseofulvin and terbinafine for the treatment of tinea capitis has shown that terbinafine is superior for tinea capitis caused by *Trichophyton* spp., whereas griseofulvin is superior when *Microsporum* spp. is the etiological agent [353, 354]. For other tinea infections (corporis, cruris, or pedis) without response with cream/gel, oral terbinafine may be used.

For cutaneous and lymphocutaneous sporotrichosis, terbinafine administered at a dosage of 500 mg orally twice daily is considered a safe alternative treatment (A-II) to itraconazole [142]. Based in reports showing successful outcome using combination therapy including terbinafine for treating *Scedesporium* infections, the ESCMID and ECMM joint guidelines proposes the use of caspofungin plus terbinafine as salvage therapy for *Scedosporium* spp. infections in cystic fibrosis patients with lung transplantation (quality of evidence and strength of recommendation: CIII) and voriconazole plus terbinafine for patients with lung infections, osteomyelitis/septic arthritis and for disseminated infection due to *S. prolificans* (quality of evidence and strength of recommendation: BIII) [355–359].

ESCMID also recommends use of an azole or terbinafine plus surgery (AIII) for the treatment of eumycetoma or combination therapy with azoles plus terbinafine or flucytosine for refractory eumycetoma cases (B III). High-dose of terbinafine (1000 mg/day) alone for 24–48 weeks had shown to be clinically effective and well tolerated when used to treat patients with eumycetoma, whose only therapeutic option is surgery. Among 23 eumycetoma patients, 16 out of 20 who completed the study showed improvement or cure [360].

Few case reports about successful outcome of the use of terbinafine plus amphotericin B in the treatment of disseminated fusariosis had been published [361, 362].

Terbinafine can be used as alternative to itraconazole (BIII) in the treatment of chromoblastomycosis, and in combination with itraconazole for cases of refractory disease [302]. Terbinafine dosing for the most common indications in adult and pediatric patients are shown in Tables 2.8 and 2.9.

2.5.6 Adverse Events and Toxicity

Terbinafine is an antifungal drug well tolerated even in people over 65 years, many of whom may be taking antihypertensives, antidiabetics, or lipid-lowering agents concomitantly [363]. Adverse reactions related to terbinafine are usually mild in

Table 2.8 Terbinafine dosing for the most common indications in adult patients

Fungal disease	Doses (mg)	Duration	Comments
Tineas corporis or cruris	250 qd	2–4 weeks	
Tinea pedis	250 qd	2–6 weeks	
Tinea capitis	250 qd	6 weeks	Dosing for patients with more than 40 kg
Onychomycosis	250 qd	6 weeks (fingernails) or 12 weeks (toenails)	Sometimes a treatment for >6 months may be necessary
Sporotrichosis	500 bid	Until 2–4 weeks after clinical cure	Usually for 3–6 months

Source: References [142, 332, 348–350, 363]

Table 2.9 Pediatric dosing according body weight in patients with onychomycosis

Body weight (kg)	Doses (mg)	Comments
10–20	62.5	6 weeks (fingernails) or 12 weeks (toenails)
20–40	125	6 weeks (fingernails) or 12 weeks (toenails)
>40	250	6 weeks (fingernails) or 12 weeks (toenails)

Source: Reference [348]

severity. Gastrointestinal complaints are common events [364]. Patients on treatment with this drug may complain of nausea, diarrhea, bloating, dyspepsia, epigastric pain, and other less-frequent gastrointestinal symptoms [365]. More rare effects are skin reactions, cholestatic hepatitis and taste loss [366–369]. The frequency of taste loss ranges between 0.1 and 1% [369]. Risk factors associated with taste loss due to terbinafine are age (65 years and older, OR: 4.4, 95% CI: 1.4–16.1) and body mass index below 21 kg m^2 (OR:4.4, 95% CI: 1.6–14.2). The OR of taste loss in patients 55 years and older with a BMI below 21 kg m^2 is 12.8 when comparing with patients below 35 years old (95% CI: 1.9–88.6) [370].

Cutaneous adverse effects of terbinafine have a wide spectrum of presentation and are infrequent, having been reported in less than 2% of the patients. Recently, a case report of terbinafine-induced lichenoid drug eruption in a patient receiving the antifungal drug for 2 weeks was published. Lesions disappeared totally after 8 weeks of drug withdrawal [371]. Other rare cutaneous side effect induced by terbinafine is pityriasis rosea [372]. Also, the induction of subacute cutaneous lupus erythematosus and exacerbation of systemic lupus erythematosus by terbinafine have been reported [373, 374].

The incidence of serious side effect is less than 1%. Cases of Stevens-Johnson syndrome, neutropenia/agranulocytosis, thrombocytopenia, and aplastic anemia have been rarely reported [375–384]. Hepatotoxicity (including acute hepatitis, cholestasis, acute liver failure and vanishing bile duct syndrome) due to terbinafine has an incidence of 0.5–3/100,000. In most of these events improve after discontinuation of the drug [385–387]. A mixed hepatitis-cholestatic liver injury has also been reported [388].

2.5.7 Drug Interactions

Terbinafine does not inhibit or induce CYP 3A4, but it inhibits CYP2D6. Then, interactions with drugs that are metabolized by CYP 2D6 such as tricyclic antidepressants (amitriptyline, amoxapine, clomipramine, doxepin, imipramine, nortriptyline), ß-blockers (atenolol, metoprolol, propranolol, timolol, carvedilol), type B monoamine oxidase inhibitors (rasagiline and selegiline), some antipsychotic (chlorpromazine, haloperidol, risperidone, thioridazine), certain arrhythmics (lidocaine, procainamide), several medications for attention deficit hyperactivity disorder (atomoxetine, methamphetamine, methylphenidate), chloroquine, mirtazapine and dextromethorphan can potentially occur, increasing risk of side effects of these drugs. Concomitant use of tamoxifen with terbinafine should be avoided because it causes a decrease of endoxifen, one of the most important metabolites of tamoxifen, thereby decreasing the effectiveness of tamoxifen [389]. Terbinafine also mildly inhibits the metabolism of cyclosporine with little clinical significance [390].

2.5.8 Use in Special Population

No dosage modification is necessary in elderly patients. Terbinafine is contraindicated for patients with chronic or active hepatic disease [391]. Also, it is not recommended for patients with creatinine clearance lower than 50 mL/min because there are not satisfactory studies to confirm its safety in this population. Terbinafine has not been associated with any teratogenic toxicity in animals. Although it is not known whether terbinafine crosses the human placenta, it is categorized as a Pregnancy Category B drug by the FDA. The product labeling recommends against its use during pregnancy for both, topical and oral formulations. Small amounts of terbinafine are excreted in breast milk contraindicating its use in breastfeeding mothers [392].

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Chapter 3
Candida and Candidiasis

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Abstract *Candida* species comprehend a wide group of opportunistic pathogens that usually form a part of the microbiota on human linings. Under specific conditions such as immune depression, tissue invasion and infection may occur. *Candida* infections are ranked as the sixth most common cause of nosocomial infections according to studies by the Centers for Disease Control and Prevention, thus constituting a serious public health problem. *Candida albicans* has been the most studied member of this species; nevertheless, non-*albicans* species have risen as emergent pathogens in many regions across the globe. This text presents an outlook of *Candida* and its infections, including the historical aspects of the fungus, epidemiology and clinical manifestations of the infection, diagnostic techniques as well as treatment guidelines.

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3.1 Introduction

Candidiasis or candidosis is the name of a group of varied infections caused by *Candida* species, ranging from colonization of the mucosa to invasive diseases. When the fungus reaches the bloodstream, the infection is called candidemia, being one of the most important opportunistic mycosis in the world, especially among hospitalized patients [1]. *Candida albicans* and related species are ranked as the sixth cause of most common nosocomial infections according to studies by the Centers for Disease Control and Prevention [2]. In this regard, invasive fungal infections have reached high morbidity and mortality rates, thus constituting a serious public health problem [3].

3.2 History of *C. albicans*

C. albicans remains among the most widespread fungal species implicated in human infections, and the history of its discovery extends from the ancients Greeks to modern-day researchers. Table 3.1 summarizes some of the discoveries about this organism.

From 1940 to the present there are 35,037 papers listed in PUBMED (https://www.ncbi.nlm.nih.gov/pubmed/) that have studied *C. albicans*. Table 3.2 compiles some of the works described from 2000 to 2017.

3.3 Etiology

The genus *Candida* comprises around 150 different species [50], of which approximately 10% are considered human pathogens. *C. albicans* is the most frequently isolated species in the blood cultures of patients with candidiasis, with an incidence of up to 70% [51]. However, in the last three decades, other species of *Candida* have been found to cause candidiasis in human patients such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* [3]. Other species such as *C. dubliniensis*, *C. orthopsilosis*, *C. guilliermondii*, *C. metapsilosis*, *C. inconspicua*, *C. lusitaniae*, *C. norvegensis*, *C. nivariensis*, *C. bracarensis,* or *C. auris* have been isolated as well from some patients with candidiasis [52–54].

The genus *Candida* has a characteristic oval morphology of 2–7 μm in diameter, it reproduces by blastoconidia and is able to assimilate and differentially ferment carbohydrates [55]. In healthy people, it inhabits the skin, mucosa, upper respiratory tract, and the genitourinary and digestive tracts. However, *Candida* can become an opportunistic pathogen, causing different types of candidiasis when the human host immune system is compromised.

Table 3.1 History of *C. albicans*

Event		Refs.
460–370 B. C.	The term "aphthous" was originated by **Hippocrates** in reference to "Mouths affected with aphthous ulcerations" the currently recognized oral candidiasis in the form of "thrush".	[4, 5]
1764	Thrush was first described by **Rosén von Rosenstein**, who mentions a disease of the mouth, more severe if it spreads into the lungs.	[6]
1839	**B. Langenbeck** was the first to describe in detail what is now recognized to be septate hyphae, branched pseudohyphae, and blastoconidia "Under the microscope magnified, the pseudomembranes consisted of an immense number of fungi grown in confusion".	[7]
1849	**J. S. Wilkinson** described thrush in the female genital tract when he found the fungus, which he called epiphytes in the vaginal discharge of a 77 years old woman.	[8]
1853	**C. P. Robin** called the fungus causing thrush as *Oidium albicans*. *Oidium* reflecting the egg shape of the yeast cell.	[9]
1864	**M. Burchardt** noted the filaments were branched and were periodically blocked by internal barriers called septa. Such septate filaments are now known as hyphae. *Candida* has pseudohyphae which did not have these septa.	[8]
1868	**C. Quinquaud** renamed the fungus as *Syringospora robinin*.	[10]
1869	**J. Parrot**, found that this fungus was not restricted to the mouth and could invade other organs (linings of the stomach, intestines, larynx, trachea, and bronchi). This is now called invasive candidiasis.	[8]
1877	**P. Grawitz**, supported the idea that this fungus was dimorphic. He cultivated the thrush fungus in acidic conditions and grows only as unicellular yeast cells and not as filaments. At the same time, in an independent work **M. Reess** described the hypha was capable of penetrating epithelial cells.	[8, 10]
1887	**C. Audry** showed that thrush was due to a single fungus, which he called *Saccharomyces albicans*, and its form varies according to the medium on which it was grown.	[8]
1889	**E. Laurent** classified the fungus as *Dematium albicans*.	[11]
1890	**W. F. Zopf** classified the fungus as *Monilia albicans*.	[12]
1918	**Mello and L.G. Fern** classified the fungus as *Parasaccharomyces albicans*.	[13]
1920	**Brownlie and Shattock** classified the fungus as *Blastomyces albicans*.	[14]
1923	**C. M. Berkhout** proposed the genus *Candida*, separating this genus from the universal *Monilia* genus that affects fruit and vegetables**.**	[15]

Table 3.1 (continued)

Event		Refs.
1928	**M. Ota** classified the fungus as *Myceloblastanon albicans.*	[16]
1931	**N.M Dekker** classified the fungus as *Endomycopsis albicans.*	[17, 18]
1931	**R. W. Benham** found that corn-meal agar is an important medium in studying the morphology of the *Mycotoruloideae*, especially for the development of the diagnostic chlamydospores of *Candida albicans.*	[19, 20]
1935	**C. W. Dodge** classified the fungus as *Syringospora albicans.*	[21]
1938	**M. Langeron and P. Guerra**, in France listed 102 synonyms for the thrush fungus *Candida albicans.*	[22]
1953	**W.J. Nickerson**, when inoculated onto two different agar media containing a complex bismuthyl-polyhydroxy-polysulfite (termed Bi-SO_3), *Candida albicans* develops black colonies.	[23, 24]
1954	**Eighth Botanical Congress** officially endorsed the binomial *Candida albicans* as the *nomen conservandum* formally ending the 200-year uncertainty over the etiology and taxonomy of *Candida.*	[8]
1980	**Hans Rieth** started the campaign of anti-*Candida* diet, promoting food with low-sugar content.	[25]
1985	**B. Slutsky, J. Buffo and D. R. Soll** discovered that *C. albicans* switches heritably and at high frequency between at least seven general phenotypes identified by colony morphology on agar.	[26]
1996	**Stanford Genome Technology Center**, supported by grants from the NIDCR, NIH, and the Burroughs Wellcome Fund, began whole genome shotgun sequencing of strain SC5314 of *C. albicans*.	http://www.sequence.stanford.edu/group/candida/index.html
1998	**H. Chibana, B. B. Magee, S. Grindle, Y. Ran, S. Scherer and P. T. Magee**, described the complete physical map of chromosome 7 from *C. albicans.*	[27]
1999	**C.M. Hull and A.D. Johnson** performed the identification of a mating-type locus in the asexual pathogenic yeast *C. albicans.*	[28]

Table 3.2 Discoveries of *C. albicans* (2000–2017)

Date	Report	Refs.
2000	**H. Chibana, J. L. Beckerman, and P. T. Magee** found four types of events to explain the genomic diversity in *C. albicans*: (1) Chromosome length polymorphism results from expansion and contraction of the RPS; (2) reciprocal translocation occurs at the Major Repeat Sequence loci; (3) chromosomal deletion; and (4) trisomy of individual chromosomes.	[29]
2000	**R. Kandasamy, G. Vediyappan, and W. L. Chaffin** demonstrated the existence of β1,3- glucan linked proteins in *C. albicans*, which are related to Pir family proteins of *S. cerevisiae.*	[30]
2000	**P. L. Jr. Fidel, J. Cutright and C. Steele** reported that estrogen but not progesterone, is an important factor in hormone-associated susceptibility to *C. albicans* vaginitis**.**	[31]
2001	**J. B. Anderson, C. Wickens, M. Khan et al.** described the infrequent genetic exchange and recombination in the mitochondrial genome of *C. albicans.*	[32]
2004	**T. Jones, N. A. Federspiel, H. Chibana, J. Dungan et al.** published a whole-genome description of heterozygosity in strain SC5314.	[33]
2004	**P. W. de Groot, A. D. de Boer, J. Cunningham et al**. identified by proteomic analysis several proteins of *C. albicans*: (1) five CWPs are predicted carbohydrate-active enzymes (Cht2p, Crh11p, Pga4p, Phr1p, and Scw1p); (2) Als1p and Als4p are believed to be adhesion proteins; (3) Sod4p/Pga2p is a putative superoxide dismutase and is possibly involved in counteracting host defense reactions.	[34]
2005	**H. Chibana, N. Oka, H. Nakayama et al.** reported the sequence finishing and gene mapping for *C. albicans* chromosome 7 and systemic analysis against the *Saccharomyces cerevisiae* genome.	[35]
2006	**D. P. Thomas, S. P. Bachmann, J. L. Lopez-Ribot** reported the use of proteomics for the comparative analysis of subcellular fractions obtained from *C. albicans* biofilm and planktonic cultures, including cell surface-associated proteins and secreted components present in liquid culture supernatants (for planktonic cultures) and exopolymeric substance (for biofilms).	[36]
2007	**M. van het Hoog, T. J. Rast, M. Martchenko et al.** published the assembly of the *C. albicans* genome into sixteen supercontigs aligned on the eight chromosomes.	[37]
2007	**Oliveira, et al.** showed in their study an association between clinical forms of oral candidiasis and the number of colonies of *C. albicans* in saliva, and that a systemic immune response characterized by the production of TNF-alpha and IFN-gamma is observed in patients with oral candidiasis.	[38]
2007	**K. M. Yeater, J. Chandra, G. Cheng et al.** used microarrays to identify changes in gene expression associated with *C. albicans* biofilm development.	[39]
2009	**G. Butler, M. D. Rasmussen, M. F. Lin et al.** reported the evolution of pathogenicity and sexual reproduction in eight *Candida* genomes.	[40]
2009	**M. Martínez-Gomariz, P. Perumal, S. Mekala et al.** published the proteomic analysis of cytoplasmic and surface proteins from *C. albicans*	[41]
2011	**S. Nicholls, et al.** demonstrated that Hsf1 activation, and thermal adaptation, contribute significantly to the virulence of *C. albicans*.	[42]
2013	**D. Muzzey, K. Schwartz, J. S. Weissman, and G. Sherlock** published the assembly of a phased diploid *C. albicans* genome that facilitates allele-specific measurements and provides a simple model for repeat and indel structure.	[43]

(continued)

Table 3.2 (continued)

Date	Report	Refs.
2013	**T. F. Bartelli, R. C. Ferreira, A. L. Colombo et al.** sequenced and assembled, with eightfold coverage, the mitochondrial genomes of two *C. albicans* clinical isolates (L296 and L757) and compared these sequences with the genome sequence of reference strain SC5314 and revealed 372 polymorphisms (230 coding/142 noncoding).	[44]
2015	**Maiti, et al.** performed the mapping of functional domains and characterize the transcription factor Cph1 that mediates morphogenesis in *C. albicans*.	[45]
2016	**R. Rajendran, A. May, L. Sherry, R. Kean et al.** integrated the *C. albicans* metabolism with biofilm heterogeneity by transcriptome mapping.	[46]
2016	**M. D. Leach, et al.** described a mechanism by which *C. albicans* responds to temperature via Hsf1 and Hsp90, to orchestrate gene expression and chromatin architecture, thereby enabling thermal adaptation and virulence.	[47]
2016	**R. Cevik, R. Tekin, and M. Gem** reported an unusual manifestation of *Candida* arthritis in a patient diagnosed with spondyloarthritis.	[48]
2017	**P. R. Zhou, H. Hua, X. S. Liu** reported that the value of 270 CFU/mL was considered a threshold for distinguishing oral candidiasis from healthy carriage.	[49]

C. albicans is a dimorphic species that can grow as hyphae or yeast (Fig. 3.1), is one of the few species that form true hyphae and chlamydoconidia [56]. Its genome consists of 8 pairs of homologue chromosomes with a total size of 16 Mb [29]; it is the most prevalent and pathogenic species of the genus *Candida*, since it causes between 37% and 70% of the fungal infections caused by this genus [3, 57].

C. parapsilosis sesu lato is responsible for 15.5% of candidiasis in North America, 16.3% in Europe and 23.4% in Latin America [58], mainly affecting neonates and immunocompromised patients [59]. This emerging pathogen is in fact a complex composed of three genetically distinct species: *C. parapsilosis sensu stricto*, *C. metapsilosis,* and *C. orthopsilosis* [60]. The genome of *C. parapsilosis sensu stricto* harbors 5733 genes, with a total size of 13.1 Mb [40].

C. tropicalis is one of the three most commonly isolated non-*albicans* species [61–63]. This is a diploid, dimorphic yeast that exists as either ellipsoidal budding cells or as a pseudomycelium, consisting of long, branched elements bearing conidia, in short chains or clusters [64]. In rare cases, *C. tropicalis* can form true hyphae [65]. Additionally, *C. tropicalis* has the ability to ferment and assimilate sucrose and maltose [66]. The number of chromosomes and the genomic size of *C. tropicalis* are not known precisely, but pulse-field gels reveal approximately 5–6 pairs of homologous chromosomes and a genome size of ~30 Mb [67].

C. glabrata is a nondimorphic species that grows as a yeast, but does not form hypha; and has been reported as the third cause of candidiasis in human patients [68], accounting for 18–26% of all systemic infections caused by *Candida* in the USA [69–71]. This pathogen presents a natural resistance to azole compounds, which are widely used in the treatment against candidiasis; therefore, infections caused by *C. glabrata* are difficult to treat due to inadequate patient management, which can lead to death, causing high mortality rates in immunocompromised and hospitalized patients [72]. The *C. glabrata* genome has 13 chromosomes, with a total size of 12.3 Mb [73].

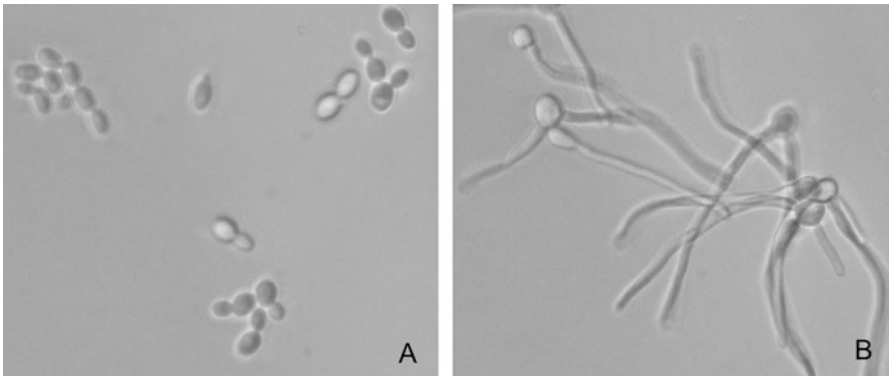

Fig. 3.1 Cellular morphology of *Candida* sp. (**A**) Yeast form and (**B**) hyphal form, cultured in liquid media, observed under 40× objective by means of bright field microscopy (Source: The authors)

3.4 Epidemiology

Candidiasis is a mycosis caused by the *Candida* genus, which has been detected on all continents since the 1980s. It has been classified as one of the most common causes of bloodstream infections [74]: incidences of 6–10 cases in 100,000 people have been reported in most population-based studies in the USA recent study in this country analyzed data from 33 states collected during 2002–2012, and found the average annual rate of invasive candidiasis was 5.3 hospitalizations per 100,000 population ratio [75]. In most European surveys, incidences of 1.4 to 5.7 cases per 100,000 habitants have been reported [76].

Several *Candida* species are commensal and colonize the skin and mucosal surfaces of humans. Critically ill, or otherwise immunocompromised patients, are prone to develop both superficial and life-threatening *Candida* infections [77].They also constitute the most common fungal infections in Acquired Immune Deficiency Syndrome (AIDS) patients, who predominantly develop oropharyngeal candidiasis, which can lead to malnutrition and interfere with the absorption of medication.

C. albicans is the predominant cause of candidiasis [78], with an incidence ranging from 11.5% in Turkey to 32% in Mexico and Taiwan, and over 60% in Austria and Sweden. Currently the incidence of *C. albicans* has decreased, while the incidence of infections due to *Candida* non-*albicans* species (*C. parapsilosis*, *C. tropicalis*, *C. glabrata,* and *C. krusei*) is increasing [79]. A series of studies have shown that the incidence of *C. albicans* has declined from 70% to 50% [80]. This has been reported both in North America and in European countries, which have gradually decreased the number of infections caused by *C. albicans* and have been replaced it by *C. glabrata* infections; whereas the incidence of *C. parapsilosis* or *C. tropicalis* infections has increased in other regions [79]. This change in epidemiology could be associated with severe immunosuppression or illness, prematurity,

exposure to broad-spectrum antibiotics and older patients [78], as well as the use of fluconazole in the treatment of candidiasis and the increase in the use of venous catheters [3, 81, 82].

C. parapsilosis is considered by some authors as the second or third most common cause of candidiasis, after *C. albicans*, depending on the geographic distribution. Early reports of *C. parapsilosis* described the organism as a relatively nonpathogenic yeast belonging to the normal biota of healthy individuals, having modest clinical significance [83]; however, important factors such as catheter use and parental nutrition [84] have contributed to the increased incidence of *C. parapsilosis*, which has been mainly isolated from patients with cancer, young adults and neonates [58]. Therefore *C. parapsilosis* has emerged as a significant nosocomial pathogen with clinical manifestations that include endophthalmitis, endocarditis, septic arthritis, peritonitis and fungemia, usually associated with invasive procedures or prosthetic devices [85]. The percentage of *C. parapsilosis* isolates in candidiasis varies considerably depending on the region [86, 87]. This species is predominant in candidemia reported in Australia, Latin America, the Mediterranean area, and in countries of Africa, Asia, and Europe [59, 85]. In studies conducted by Wingar (1995), in a period from 1952–1992, *C. parapsilosis* covers only 7% of cases of candidemia in patients with cancer [88]. In 1999, Kao et al. conducted a prospective study of candidemia in two cities in the United States, Atlanta and San Francisco, from 1992 to 1993. *C. parapsilosis* was found in 21% of the isolates of patients from these populations [89]. A study from 1992 to 2001, recorded the distribution of Bloodstream Infection Isolates (BSI) of different *Candida* species. *C. parapsilosis* accounted for 13% and was the third most common *Candida* species isolated from BSI. The overall incidence in six different studies showed a prevalence between 7 and 21% of *C. parapsilosis* [3]. More recently, a retrospective study from 1991 to 2008 in the Clinic Hospital of Barcelona found *C. parapsilosis* as the second cause of candidemia after *C. albicans*, with an incidence between 14 and 20% [90]. This species presents many interesting biological characteristics that are supposed to be directly related to its virulence, such as its selective adhesion to prosthetic materials, the formation of biofilms on plastic surfaces [91], and the secretion of extracellular proteases [92, 93]. *C. parapsilosis* is usually susceptible to most antifungal agents, but there are reports of clinical isolates with decreased susceptibility to azoles and echinocandins.

C. tropicalis is one of the three most commonly isolated *Candida* non-*albicans* species [61–63, 77]. Its relative importance seems to be even greater in cases reported from Southern Europe and Latin America. *C. tropicalis* has been isolated from patients with solid tumors or hematologic diseases, and reported as the second etiological agent of invasive candidiasis in Asia and some parts of Latin America (Colombia and Brazil). In a recent epidemiological survey conducted in 12 Brazilian medical centers, *C. tropicalis* was the second most frequently recovered *Candida* species, accounting for 33–48% of all candidemia cases [63, 94]; however, a retrospective study from 1991 to 2008 at the Clinic Hospital in Barcelona found *C. tropicalis* as the third cause of candidemia after *C. albicans*, with an incidence between 13 and 14% [90]. Moreover, a review by Hobson in 2003 places *C. tropicalis* as the

fourth cause of candidiasis infections worldwide as well [95]. *C. tropicalis* is commonly associated with patients with neutropenia and malignancy [63]. Additionally, it is often found in patients admitted to intensive care units, especially in patients requiring prolonged catheterization, receiving broad-spectrum antibiotics or with cancer [96–98]. Furthermore, *C. tropicalis* appears to display a higher potential to disseminate in neutropenic individuals, when compared with *C. albicans* and other non-*albicans* species [63]. This propensity of *C. tropicalis* for dissemination, and the associated high mortality, may be related to virulence factors exhibited by this species such as biofilm formation, proteinase secretion and dimorphism [99].

Several authors reported *C. glabrata* as the third or fourth common species (18.1%) isolated from candidiasis cases. In a prospective systematic epidemiological study carried out in Intensive Care Unit (ICU) at 27 centers of India, from all cases of acquired candidemia, *C. glabrata* was the fourth common species (7.1%) in adult patients [100]; however, in a retrospective study carried out in Taiwan from July 2009 to July 2012, the proportion of *C. glabrata* increased greatly from 1.1% to 21.6% [101]. Another retrospective study from 1991 to 2008 at the Clinic Hospital in Barcelona found *C. glabrata* as the fourth cause of candidemia, with an incidence between 7 and 14% [90]. Candidiasis caused by *C. glabrata* also demands great care due to its high mortality rate (53.8% in 30 days), and relatively higher resistance to azoles, especially fluconazole. In 2015, Chakrabarti indicated that broad spectrum antibiotics, central venous catheter, mechanical ventilation, diabetes, and elderly patients (>65 years) as risk factors for *C. glabrata* candidemia [100]. It has also been found that *C. glabrata* tends to affect oncology patients with solid tumors [84].

3.5 Virulence Factors of *Candida* spp.

Virulence factors of *Candida* are traits that facilitate colonization and infection of the host cell by the fungus [102]. Many authors define these attributes as “all traits required to establish the disease”, “factors that directly interact with mammalian host cells” or as “a component of pathogen that damage the host” [103]. Here, some of them are briefly summarized.

Adhesion The adhesion of *Candida* to the host is essential to colonization and establishment of disease. The adhesion to surfaces of medical devices results in raised candidemia and antifungal resistance related to catheter insertion [103]. Adhesins are proteins that promote the adherence of *C. albicans* to the host or ligands. In *C. albicans*, hyphae are considered the best for adherence; nonetheless, the bulk of initial adhesion is between yeasts and epithelial cells, with hyphae formation being induced after the primary contact with a host cell surface. Once hyphae are formed, adhesins expressed solely on the hyphal cell surface are the main proteins for future adhesion processes [104]. *ALS* (agglutinin-like sequence) gene family encode the most studied adhesins, where the first, *ALS1–4*, encode adhesins specific for hyphae and the products of *ALS5–7* and *ALS9* are found on the surface of yeast cells [105]. *HWP1* encodes an outer surface mannoprotein that is implicated

in adherence and virulence, since the *hwp1*Δ/*hwp1*Δ mutant strain was greatly impaired in the ability to form stable attachments to buccal epithelial cells, and this mutant is less virulent than wild-type strain in the mouse model of systemic candidiasis [104, 106]. There are also other kinds of proteins involved in adherence in an indirect manner, as the Mp65 protein which modifies the cell wall structure, by its glucanase activity, thus facilitating the function of other adhesins [107].

Invasion Secreted aspartyl proteinases (SAPs) are proteins reported to be present in *C. albicans*, *C. parapsilosis and C. guilliermondii* [108]. The function of these proteins is to degrade many human proteins at lesion sites, such as albumin, hemoglobin, keratin, and secretory immunoglobulin, and this proteolytic activity is involved with tissue invasion by the fungus. There are nine known proteins of this type, Sap1–3 are expressed only in yeast, Sap4–6 are expressed in hyphae, Sap7 has never been detected in in vitro assays, Sap8 expressed only in yeast cells grown at 25 °C in defined medium, and Sap9 which is expressed in the later grown phase [103]. Additionally, the phospholipases are hydrolytic enzymes that contributes to invasion by degradation of phospholipids of the cell membrane, resulting in the loss of barrier functions. Four phospholipases genes were identified *PLA*, *PLB*, *PLC,* and *PLD*. Plb1 is a glycoprotein expressed in hyphae that has both, hydrolase and lysophospholipase–transacylase activity and it is probably secreted. In vivo experiments have demonstrated that Plb1 is necessary for virulence and tissue invasion [103, 108].

Phenotypic Switch The transition from yeast to hyphal form is considered the most important virulence factor [109]. Since yeast seems to be essential for the dissemination of the infection and hyphae contribute to host tissue damage, both morphologies are thought to be important for pathogenicity [110]. Phenotypic switch is directed by the expression of activators like Efg1 and Cph1 via cAMP/PKA or MAPK pathway as a consequence of environmental stimuli [111]. At high cell densities, quorum sensing molecule farnesol also promotes hypha formation [112]. This morphological switch is associated with the expression of hypha-specific proteins such as Hwp1, Als3, Sap 4–6, Ece1, and Hyr1 that are not required for hypha formation and maintenance but are important for virulence [111, 112].

3.6 Common Clinical Manifestations

The clinical manifestations range from asymptomatic infections to septic shock syndrome. Additionally, candidemia can be recurrent if the underlying conditions, as retained intravascular catheters, neutropenia, and corticosteroids remain unchanged. Regrettably, the mortality rates associated with candidemia are repeatedly over 40% [113, 114].

Mucocutaneous candidiasis (MC) includes noninvasive symptomatic infections of the skin, nails, and mucous membranes caused by *Candida* species [115]. Presence of some host factors such as autosomal-dominant hyper-IgE syndrome, gain of function STAT1 and IL-17 pathway defects may result in increased susceptibility to

mucocutaneous candidiasis and in some cases, may become a chronic infection [116]. Direct signs of MC are *Candida* plaques on the mucosa or thickened skin and nails [117]. The symptoms usually include pain, weight loss or failure to thrive, and may derive in complications such as squamous cell carcinoma, esophageal stricture or cerebral aneurysms [118–120].

Intra-abdominal candidiasis (IAC) is a very common type of deep-seated candidiasis [121], occurring in patients with a set of underlying conditions that specifically involves the gastrointestinal tract and digestive system [122]. In such type of infections, *Candida* may be mixed with bacteria, thus complicating the treatment, since in some cases antibiotic therapy and source control, without antifungal therapy, is enough to the improvement of the patients, but in some others, the presence of *Candida* greatly contributes to bad outcomes [123, 124]. Recent studies described that the most common types of IAC were, in order of occurrence, intra-abdominal abscesses, secondary peritonitis, primary peritonitis, infected pancreatic necrosis and cholecystitis/cholangitis, mainly caused by *C. albicans*, although non-albicans species were also reported. From the cases analyzed, over 60% exhibit mixed bacterial infections and 10% showed presence of two *Candida* species [122].

Septic arthritis caused by *Candida* is commonly developed as a complication of disseminated candidiasis [125–127], and frequently with the presence of underlying conditions such as surgery, solid organ transplantation, trauma, intravenous drug use, hematologic malignancies, among others [128]. An analysis of *Candida* arthritis cases from 1967 through 2014 showed that the apparent mechanism of infection mainly consisted of hematogenous dissemination and, in a minor level, of direct inoculation. The clinical manifestations include local pain, edema, limitation of function and movement, localized erythema and sometimes the disease is accompanied with a febrile process [128].

3.7 Diagnostic Strategies

The diagnosis of any pathological agent requires high sensitivity and specificity levels, being the microbiological culture the test of choice widely used over time. However, there are different tools other than the microbiological culture, which facilitate the diagnosis. These include smears, biopsies, molecular techniques, and serological tests.

3.7.1 Smear

Direct Exam. To make a smear, a sample is taken by scraping the affected area using a swab or spatula, the most commonly used specimens are urine, cerebrospinal fluid, feces vaginal discharge, and expectoration. The sample is spread on a slide, and treated with 10–20% KOH in the case of sputum samples. Finally, the sample is observed by conventional microscopy to search the characteristic structures of

Candida. We can observe individual or budding cells, but the number of cells found is more important [129].

Stains. The smear can be stained with different dyeing methods. The Gram staining is one of the most used, being *Candida* yeasts Gram positive structures. In this test, individual or budding cells (blastoconidia) can be detected with or without presence of pseudomycelium [129].

The smear is one of the most economical and quick tools for identifying a yeast from a bacterium. However, in both cases, direct exam or stain, the final diagnosis should consider the origin of the sample, since the presence of some species of *Candida* are considered normal biota in some parts of the body. Hence, the number of cells detected is of greater clinical importance.

3.7.2 Biopsies

The tissues most used to perform biopsies for the detection of *Candida* are esophagus, lungs, liver, muscle, skin, among others. These samples can be stained with different techniques such as hematoxylin-eosin, Periodic Acid-Schiff (PAS), Gram, Papanicolau, Gomori, Grocott, or Gridley. Through this technique, as with the smear, it is possible to observe single or budding yeasts, with or without the development of pseudohypha. Unlike the fresh examination, the biopsy allows to evaluate the invasion of the tissue by *Candida*, which demonstrates the presence of the infection. Thus, the detection of *Candida* in a biopsy is considered of great utility for the diagnosis of invasive candidiasis [129].

3.7.3 Culture

The microbiological culture has the disadvantage of being a delayed process that in turns delays the diagnosis of the patient and therefore the appropriate treatment. In addition, a sufficient quantity of microorganisms in the inoculum is required to obtain a positive diagnosis [130]. Even with these disadvantages, *Candida* culture is considered for many as the gold standard for the diagnosis of invasive candidiasis [129, 131]. The primary culture of *Candida* is achieved in 2–5 days, using Sabouraud Dextrose Agar, under aerobiosis conditions at room temperature. In this culture, it can be observed the growth of unique or budding colonies, forming pseudohypha in some occasions. From this culture, rapid tests such as germ-tube formation or thermotolerance, formation of chlamydoconidia in bile-agar medium or corn flour agar can be performed [132, 133]. In addition, tests can be performed for isolation and identification using chromogenic culture media, urease test, resistance to cycloheximide, fermentation of sugars, carrot-potatoes agar, among others. Currently, commercial automated systems allow the identification of yeast in a short time (<15 h), with time reduction in the diagnosis [130, 134].

It is important to note that a positive result for *Candida* culture does not always confirm the diagnosis of *Candida* infection, since this microorganism is considered normal biota in some parts of the body. To achieve a proper diagnosis of candidiasis, the patient's signs and symptoms must be taken into account, in addition to the laboratory results. If the culture comes from biopsy or is a blood culture, the positive result confirms the diagnosis of invasive candidiasis. On the other hand, a negative result for the culture of *Candida* is of greater diagnostic value since it allows to discard the infection by this yeast [131].

3.7.4 Molecular Tests

Like the aforementioned techniques, molecular tests will have diagnostic value when invasive candidiasis is suspected. These tools allow a quick diagnosis (<7 h); however, the cost is much higher. In addition, depending on the technique used, it is possible to detect the presence of *Candida* genetic material from nonviable microorganisms or contamination prior to amplification, resulting in a false positive, which represents the greatest problem for its use as a diagnostic tool. One of the advantages of molecular testing is the possibility of identifying a single or multiple species of *Candida* in a sample, which cannot be done by simple observation under a microscope. This is of clinical relevance due to the influence it has on the patient pharmacological treatment and the therapeutic implications.

Some of the most used molecular techniques are PCR-REA, PCR-LiPA, PCRsn, multiplex PCR, nested PCR, PCR-SB, PCR-rt., among others. However, in half the cases the studies have been performed on samples from blood cultures, so the advantage of speed in the emission of the result is lost. Hence, the use of PCR-rt. for the identification of *Candida* from serum and plasma samples has been tested, having better results than from whole blood [135–145].

In 2000, reverse cross blot hybridization assay was used for rapid detection of PCR-amplified DNA from *Candida* species, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* in clinical samples [146]. Five years later was developed a diagnostic microarray for the rapid and simultaneous identification of the 12 most common pathogenic *Candida* and *Aspergillus* species (*C. albicans*, *C. dubliniensis*, *C. krusei*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae*, *Aspergillus fumigatus*, *A. flavus*, *A. niger*, and *A. terreus)*. This microarray system was able detecting and clearly identifying the fungal pathogens within 4 h after DNA extraction [147]. L. Basková et al., described a real-time quantitative PCR assay developed for the detection of the most common pathogenic *Candida* and *Aspergillus* species. The single-reaction PCR assay targets a region of the 28S subunit of the fungal rDNA gene. The design of the universal primer/probe, including a pan-*Aspergillus* and pan-*Candida* (Pan-AC) hydrolysis probe, facilitated the detection of numerous *Aspergillus* and *Candida* species (e.g. *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, *C. guilliermondii*, *C. lusitaniae* and *C. dubliniensis*). The assay permits highly reproducible detection of 10 fg

fungal DNA, and facilitates accurate quantification of fungal load. Also, it provides an economic approach to the screening and monitoring of invasive candidiasis as a routine test in clinical diagnosis [148]. Schabereiter-Gurtner and colleagues developed a novel real-time PCR assays, targeting the fungal ITS2 region, for detection and differentiation of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*) [149]. In 2013, was developed a quantitative PCR assay based on fluorescence resonance energy transfer (FRET) for *C. albicans* in blood. The assay was highly specific and sufficiently sensitive to detect the fungal load for early diagnosis of invasive candidiasis [150]. Molecular phylogenetics of *C. albicans* has been analyzed by multilocus sequence typing (MLST), ABC typing, mating type-like locus (MAT) status, and antifungal susceptibility to a panel of 1391 *C. albicans* isolates [151, 152]. Afsarian et al., performed the first nationwide study on the genotypic relationships of *C. albicans* strains obtained from oropharynx and bronchoalveolar lavage samples from patients, based on MLST, and directly analyze the polymorphism within DNA sequences [153]. All these mentioned examples show the importance of molecular methods for *Candida* identification without disregarding conventional procedures.

3.7.5 Serological Tests

Serum tests represent an important alternative for the diagnosis of candidiasis due to the speed in generating the result, and therefore the timely treatment of the patient. There are tests for either the search for antigens or antibodies against *C. albicans*, although in most cases they exhibit cross reactions with other species. Among the most used are the detection of mannan and anti-mannan antibodies by ELISA; whose combined use yields more reliable results. The detection of *Candida* mannan alone has a sensitivity of 50–100% for the different trademarks used. The species that can be detected through this technique include *C. albicans*, *C. dubliniensis*, *C. famata*, *C. glabrata*, *C. guilliermondii*, *C. lusitaniae*, and *C. tropicalis*. Although some commercial tests show positive results for *Candida* in the presence of *Aspergillus* and *Cryptococcus*. In any case, the clinical signs and symptoms of the patient should be considered, in addition to the positive results for antigens or anti-mannan antibodies. In the case of using the test for antibody detection, special care should be taken in the results obtained from immunosuppressed patients, given their low or null response, which could generate a false negative [154]. Several methods have been used, including serum determination of β-D-glucan, which is a component of the cell wall of *Candida* and *Aspergillus*, with sensitivities ranging from 57 to 100% and 44 to 92% of specificity, with the possibility of obtaining results in approximately 1–2 h [155]. This makes serology a good diagnostic tool in the case of invasive candidiasis, because of the relatively high sensitivity and specificity and the prompt emission of results. This type of test has the characteristic of detecting β-D-glucans at the picogram level and does not react with other polysaccharides. However, false positives can be generated by contamination of β-glucans

from antibiotics and other drugs such as immunoglobulins, antitumorals, and sulfamines, dialysis membranes, materials used in surgeries, among others. The detection of β-D-glucans as a test for the diagnosis of *Candida* infection is widely used in the USA and Europe [155].

There are also commercial kits to determine the presence of *C. albicans* antimycelium antibodies by immunofluorescence. This test has cross reactivity with *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *C. guilliermondi,i* and *C. dubliniensis*.

Detection by indirect immunofluorescence of *C. albicans* germ-tube antibodies (CAGTA), is a useful diagnostic tool that discriminates between colonization and invasion. However, the standardization of this technique is complicated by its reliance on subjective interpretation [156].

A rapid enzymatic fluorometric assay for measuring D-arabinitol in serum was developed using recombinant D-arabinitol dehydrogenase from *C. albicans* (rArDH). NADH was fluorometrically measure when rArDH and NAD were added to serum extracts, and D-arabinitol concentrations were calculated from standard curves derived from pooled human serum containing known amounts of D-arabinitol. The method was precise and rapid and showed a significant improvement in terms of accuracy, cost, simplicity, specificity, and speed, compared with gas chromatography, mass spectrometry, and earlier enzymatic assays. The rArDH was highly specific for D-arabinitol, cross-reacting only with xylitol (4.9%); however, it has practically fallen into disuse [157].

Recent advances in the serological diagnosis of invasive candidiasis have allowed the development of a Lateral Flow Immunoassay for the detection of antibodies against enolase from *C. albicans*. In this test, a specificity of 98% and a sensitivity of 84% were achieved [134]. The serological diagnosis is relevant because of the speed of the results and the high sensitivity and specificity recorded for the different techniques, which has an impact on the timely therapy of patients with invasive candidiasis. In addition, it requires little investment for its implementation and the technical staff requires minimal training. In the case of patients with oral and vaginal *Candida* infection, laboratory results should be taken with caution and only used to confirm diagnosis when there are signs and symptoms compatible with the infectious process.

3.8 Treatment and Management

Pharmacological treatment used in handling of infections produced by different species of *Candida* is based on the anatomical situation of infection, the specific species of *Candida* responsible for the infection, the sensitivity of *Candida* species to specific antifungal agents, and the patient's immunological condition.

Broadly speaking, the pharmacological treatment comprises three antifungal groups: amphotericin B-based preparations, azoles antifungals, and echinocandins.

In the past years, management of infections caused by different species of *Candida* has undergone various changes. In 2016, the Infectious Disease Society

of America (IDSA) reference guides were updated, and the appropriate use of echinocandins (caspofungin, micafungin, and anidulafungin) was included, as well as the incorporation of expanded-spectrum azoles, and lipid formulation of amphotericin B.

Schemes of first-line treatment for infections caused by species of *Candida* under the IDSA guide, the expert panel from the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), and the consensus document of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC) as reference, are as follows:

3.8.1 Candidemia

All patients affected by candidemia, regardless of the presence or not of neutropenia, require the use of pharmacological treatment.

Candidemia in Non-neutropenic Patients Echinocandin is recommended as initial therapy. Echinocandins are broad-spectrum antifungals, which include caspofungin: 70 mg loading dose, followed by 50 mg IV (intravenous) once a day; micafungin: 100 mg IV once a day; anidulafungin: 200 mg loading dose, followed by 100 mg IV once a day. In every case, treatment must follow for at least a 2-week period after observing clinical improvement, and in obtaining a negative blood culture result.

Fluconazole is the drug of choice in most cases of candidemia. Initial treatment is a loading dose, 800 mg or 12 mg/kg PO (latin *per os*; by mouth) or IV respectively, then 400 mg or 6 mg/kg per day at least during 2 weeks after observing clinical improvement, and in obtaining a negative blood culture result. Fluconazole is an alternative to echinocandin as an initial treatment in stable patients without previous intake of azoles. An alternative in the treatment of candidemia with fluconazole is the use of voriconazole at a dose of 400 mg or 6 mg/kg, twice a day per two doses, followed by 200 mg or 3 mg/kg, twice a day.

A treatment with lipid formulation amphotericin B (LFAmB) at a dose of 3–5 mg/kg, once a day, is a good choice in patients with proven intolerance, limited availability, or tolerance (confirmed suspicion) to other antifungal agents.

The implementation of a change in the treatment scheme from echinocandin or LFAmB to fluconazole is recommended after a 5–7-day period in patients whose *Candida* species is sensitive to fluconazole, those who are clinically stable, and in cases in which negative repeated cultures are obtained during antifungal therapy [158, 159].

Candidemia in Neutropenic Patients Echinocandin is recommended in the treatment of candidemia gross patients with neutropenia (caspofungin: 70 mg loading dose, followed by a 50 mg IV once a day; micafungin: 100 mg IV once a day, and anidulafungin: 200 mg loading dose, followed by a 100 mg IV once a day). Provided its broad spectrum and effectiveness, LFAmB is an alternative in the treatment of

candidemia (3–5 mg/kg once a day) in neutropenic patients, although its potential toxicity limits the use. On the other hand, fluconazole is an alternative in stable patients, and without prior azole intake (800 mg or 12 mg/kg loading dose, PO or IV respectively, at a dose of 400 mg or 6 mg/kg, once a day). Voriconazol can be used when the antifungal spectrum is desired to be broadened, with an extended coverage to infections caused by mold. Initial treatment scheme is 400 mg or 6 mg/kg twice a day per 2 doses, followed 200–300 mg or 3–4 mg/kg dose, twice a day. It is recommended, in every case, that antifungal treatment must be continued in neutropenic patients until neutropenia is controlled, since it represents a major risk for treatment failure [158, 159].

3.8.2 Candidiasis

Mucocutaneous Candidiasis Patients with oropharyngeal candidiasis can be treated either with antifungal topical agents (e.g.: nystatin, clotrimazole, amphotericin B oral suspension) or with systemic azoles (fluconazole, itrazonazole, or pozaconazole). For mild cases, administration of clotrimazole is recommended in 10 mg, five times a day; or miconazole, 50 mg oral mucoadhesive tablet placed on the surface of the mucosa in the canine fossa, once a day during a 7–14-day period. An alternative to the treatment is the administration of 4–6 mL (100,000 U/mL) nystatin suspension, four times a day, or nystatin (200,000 U/tablet), 1–2 tablets, four times a day during a 7–14-day period. For patients with moderate to severe oropharyngeal candidiasis, the most suited treatment is fluconazole 100–200 mg PO once a day, during a 7–14-day period. In refractory patients to fluconazole, a solution of itraconazole (200 mg, once a day) is indicated, or a posaconazole suspension (start with 400 mg, twice a day during 3 days, then 400 mg, once a day for 28 days) [158, 160]. Other treatment options include voriconazole (200 mg, twice a day), AmB deoxycholate oral suspension (100 mg/mL, four times a day), AmB deoxycholate intravenous (0.3 mg/kg, once a day) or echinocandin intravenous (caspofungin: 70 mg loading dose, then 50 mg on a daily basis; micafungin: 100 mg, once a day; or anidulafungin: 200 mg loading dose, followed by 100 mg on a daily basis) [159–161].

The treatment of esophageal candidiasis always requires systemic therapy with fluconazole (200–300 mg orally, once a day) during a 14–21-day period. Intravenous administration is indicated in those patients with oral therapy intolerance, in which cases the recommendation is fluconazole dose at 6 mg/kg once a day, or caspofungin: 70 mg loading dose, then 50 mg on a daily basis; micafungin: 150 mg once a day; or anidulafungin: 200 mg daily. In refractory patients to fluconazole, itraconazole solution is indicated (200 mg once a day) or voriconazole (200 mg twice a day via oral or IV), during a 14–21-day period. Other treatment options in refractory patients to fluconazole, include: AmB deoxycholate (0.3 mg/kg once a day) during 21 days or the administration of one of the echinocandins in accordance to the previously described treatment Scheme [159, 160].

Genitourinary Tract Candidiasis Vaginal candidiasis can be classified in two groups: noncomplex and complex. 90% of patients report noncomplex vaginitis, and response to treatment is quick to short period, topical, or oral schemes. The indicated treatment is fluconazole 150 mg single dose. In contrast, complex vaginitis requires fluconazole, 150 mg, 2–3 doses, every 72 h. Treatment with azoles for infections caused by non-*albicans* species is not indicated. Infections by *C. glabrata*, *C. krusei*, and other non-*albicans* species frequently respond to topical treatment with boric acid (600 mg per day) or flucytosine at 17%, during a 14-day period. It is recommended that patients with recurring vaginal candidiasis be treated orally or topically under induction with fluconazole during a 2-week period, followed by a 6-month maintenance regimen [161, 162].

Most of the hospitalized patients affected by candiduria are asymptomatic, where the predisposing factor is the presence of a urinary catheter. For these patients, the removal of the urinary catheter represents the first treatment strategy, since removal is equivalent to the administration of antifungal treatment. It is important to determine if presence of candiduria is not a reflection of a disseminated infection by *Candida*, especially in newborns and immunocompromised patients (particularly neutropenic); if so, they must be treated with recommended scheme for candidemia. Therefore, pharmacological treatment with antifungals is only indicated for patients that will undergo urologic manipulation and that have asymptomatic candiduria. Treatment scheme includes administration of 400 mg (6 mg/kg) fluconazole, once a day, or AmB deoxycolate, 0.3–1 mg/kg per day during a 1–7-day period, taking into account the pre- and post-procedure [159].

Symptomatic patients with cystitis and pyelonephritis must be treated with fluconazole during a 2-week period when they have had no prior treatment with azole antifungals, or the isolate is fluconazole susceptible. If there is evidence of fluconazole-resistant isolates, the recommended treatment is AmB deoxycholate with or without flucytosine. The treatment scheme for cystitis in fluconazole-susceptible isolates is fluconazole 200 mg orally, once a day for 14 days. For fluconazole-resistant isolates (e.g., *C. glabrata* infections), AmB deoxycholate 0.3–1 mg/kg must be administered daily during 1–7 days, or flucytosine 25 mg/kg, every 6 h for 7–10 days. For infections caused by *C. krusei*, the previously described AmB deoxycholate treatment is recommended. It is strongly advised that in patients with a urinary catheter, this should be removed as first step in treatment [159, 162].

Treatment for patients with pyelonephritis implies the use of antifungal systemic therapy with fluconazole (200–400 mg daily) PO or IV during a 2-week period minimum, or AmB deoxycholate, 0.5–0.7 mg/kg daily IV can be administered with or without flucytosine at a dose of 25 mg/kg, every 6 h. Infections caused by fluconazole-resistant *C. glabrata* can be treated with flucytosine or AmB deoxycholate monotherapy, or the combination of both in the previously mentioned doses [159, 160].

Endocarditis Treatment for native valve endocarditis is indicated with LFAmB 3–5 mg/kg on a daily basis, and treatment can be combined with 25 mg/kg flucytosine

every 6 h, or high doses of echinocandins (caspofungin: 150 mg, micafungin: 150 mg, or anidulafungin: 200 mg); in every case, indicated dosage is on a daily basis. After initial treatment, it is recommended that treatment be changed to fluconazole (400–800 mg, on a daily basis) in patients with fluconazole-susceptible *Candida* strains, who are clinically stable and in who repeat cultures on antifungal therapy are negative. In fluconazole-resistant *Candida* species, voriconazole can be used (200–300 mg, twice a day), or posaconazole (300 mg, on a daily basis) as an alternative. Patients with contraindications for surgery must receive suppressive, prolonged antifungal treatment on fluconazole 400–800 mg during 6 weeks in endocarditis or pacemaker electrode cable infection and a 4-week period in case of bag or generator infection, always after surgery [158, 159].

Central Nervous System Candidiasis (Meningitis) Central nervous system (CNS) candidiasis can result as a consequence of neurosurgical procedures, being an expression of disseminated candidiasis, or an isolated chronic infection. Meningitis treatment requires the use of drugs that penetrate the blood-brain barrier. Treatment is based on monotherapy with liposomal AmB (5 mg/kg/day), or AmB deoxycholate (0.7–1 mg/kg/day), or combined with flucytosine (25 mg/kg, every 6 h). Fluconazole (400–800 mg, once a day) also obtains excellent levels in the cerebrospinal fluid, and is a useful treatment after AmB [159].

Chronic Disseminated Candidiasis (Hepatosplenic Candidiasis) The recommended treatment for this clinical case (in stable patients) is fluconazole (6 mg/kg per day), AmB deoxycholate (0.6–0.7 mg/kg/day), or liposomal AmB (3–5 mg/kg/kg). Patients in critical condition, with a more extended disease, or refractory to fluconazole must be treated with AmB (deoxycholate or lipidic formulation). Treatment must be extended until lesions calcify or heal (especially in patients being treated with continuous chemotherapy or immunosuppressed), which can happen within weeks or months after initial treatment [159].

Neonatal Candidiasis Neonatal candidiasis syndrome is characterized by the presence of an extended dermatitis in newborns infected with *Candida*, as a consequence of contamination of amniotic fluid. Premature and underweight newborns, or infants with prolonged membrane ruptures, and with clinical findings associated to disseminated newborn cutaneous candidiasis must be considered for systemic treatment. AmB deoxycholate (0.5–1 mg/kg/day) is usually the first-line treatment. Fluconazole is considered as a second-line treatment (12 mg/kg PO or IV), mainly in patients with no fluconazole prophylactic treatment. Recommended time of treatment for candidemia, with or without metastasis, is 2 weeks after observing negative results in blood cultures [158, 159, 161].

Note In 2013, the US Food and Drug Administration (FDA) emitted an advisory to not prescribe ketoconazole tablets as an elected treatment for any fungal disease (including infections by *Candida* and dermatophytes) due to extreme risk of liver damage, adrenal failure, and interaction with other drugs. In the same year, the European Union withdrew it from the market.

3.9 Basic Features of the Immune Response to *Candida* Infection

The onset of the host immune response to *Candida* species is the recognition of the conserved fungal pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PPRs), including C-type lectin receptors (CLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). These PAMPs mainly belong to cell wall components and its recognition by PPRs is always a cooperative and interactive process which induces the release of pro- and anti-inflammatory cytokines in order to mediate the immune response, thus resulting in opsonization, induction of antimicrobial peptides, inflammasome activation, pyroptosis, and phagocytosis, among others [163]. It also has been demonstrated that innate immune cells may suffer epigenetic reprogramming resulting in a phenomenon referred as "trained immunity" [164]. This comprises an enhancement on cytokine release by monocytes and macrophages in an T-cell independent manner, prior stimulation with attenuated *Candida* strains, broadening the conception of what we used to call "immune memory" [165]. Interestingly, *Candida* species have developed evasion mechanisms that can circumvent the host immune response ranging from the most elaborated, i.e., as inducing macrophages to switch from a more inflammatory M1 phenotype to a less inflammatory M2 phenotype, to a simpler shielding of PAMPs, in detriment of the elicitation of the immune response [166, 167].

3.10 Conclusion

Fungal infections caused by *Candida* species have increased their morbidity and mortality rates during the past decades, especially in nosocomial environments. These infections vary from superficial mucocutaneous candidiasis to systemic candidemias, depending on the existence of underlying conditions and the host immune competence. Mention aside, *Candida* species has also developed a set of virulence factors that enable them to switch from a friendly commensal to an invasive pathogen, along with evasive mechanisms oriented to outwit the protective host immune response. In favor of primarily limit and further eradicate the infection, the accurate identification of *Candida* is of great relevance. This is usually achieved or confirmed by laboratory methods that allow to identify the fungi from different specimen by means going from microscopic observation to biochemical or molecular tests. Finally, all patients affected by infections caused by species of *Candida*, regardless of the presence or not of neutropenia, require the use of pharmacological treatment.

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Chapter 4
When *Aspergillus fumigatus* Meets the Man

Sarah Sze Wah Wong and Jean-Paul Latgé

Abstract *Aspergillus fumigatus* is one of the most ubiquitous opportunistic fungal pathogen, which can cause life-threatening invasive pulmonary infections in immunocompromised populations. Upon the inhalation of the *A. fumigatus* conidia, the encounter between the fungus and the host presents a complex interplay. This chapter will summarize the host innate immunity against *A. fumigatus*, and emphasize on the host immune evasion mechanisms of *A. fumigatus*.

4.1 Introduction

Aspergillus species are saprotrophic thermophilic fungi living in decaying material in the soil. When the fungus is starved, it produces millions of aerial conidia which are responsible for the propagation of the fungus (Fig. 4.1). Some of the *Aspergillus* species cause clinical manifestations ranging from chronic to invasive pulmonary infections, following the inhalation of the conidia (hundreds per day in normal environments) [1, 2]. *Aspergillus fumigatus* is the most prevalent etiologic agent of aspergillosis, followed by *Aspergillus flavus*, *A. niger*, *A. nidulans*, *A. terreus* [3]. In immunocompetent individuals, the inhaled conidia rarely cause infections since the host innate immunity is efficient in the clearance of the fungal pathogen [4–9] (Fig. 4.2). In the populations with pre-existing pulmonary cavities who are otherwise immunocompetent, colonization of *Aspergillus* species will only lead to chronic pulmonary aspergillosis (CPA) [10–12]. Unlike CPA, invasive pulmonary aspergillosis (IPA) predominantly affects immunocompromised individuals [13]. Individuals with primary or secondary immunodeficiency, such as chronic granulomatous disease, hematologic malignancy, hematopoietic stem cells transplantation, solid organ transplantation, and neutropenia consecutive to cancer chemotherapy or immunosuppressive treatment are typically at risk for IPA (Fig. 4.2) [2, 14]. The mortality rates of IPA vary among groups of host with different underlying risk factors, but it is generally high [2, 14]. *A. fumigatus* benefits from immune deficiencies, and it also possesses various well-established and specific strategies which helps the

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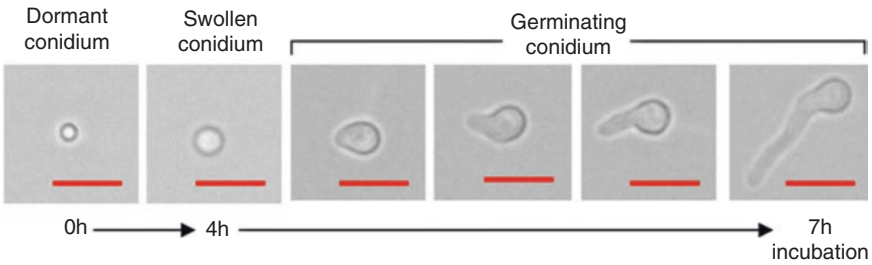

Fig. 4.1 Light microscopy (40×, scale bar = 10 μm) of the morphological change of *A. fumigatus* under incubation at 37 °C in Sabouraud medium

fungus to evade from the host debilitated immune system and colonize the lung parenchyma [15–20].

This chapter will dissect both the host innate immunity and the pathogen anti-immune strategies on the early stages of the fungal infection. Since contact between membrane-bound and soluble pattern recognition receptors (PRRs) of the host and surface pathogen-associated molecular patterns of *A. fumigatus* is the first event leading to the reciprocal recognition of the host and the pathogen (Fig. 4.3). It will especially focus on the host and pathogen molecules favoring their respective recognition, as well as the role of the innate immune cells involved in the anti-*A. fumigatus* defense. A thorough understanding of this complex "tug of war" between the host and the pathogen is fundamental in providing new insights in developing prophylactic strategies of IPA.

4.2 Molecules Responsible for the "Hide-and-Seek" Between Host and *Aspergillus fumigatus*

The fungal cell is surrounded by a cell wall with a specific composition which is very different from the phospholipid bilayer of the host cell plasma membrane. The *A. fumigatus* cell wall which surrounds the fungal cell is mainly composed of polysaccharides which are interlinked alkali insoluble β-1,3-glucans, chitin, and galactomannan and, alkali soluble α-1,3-glucans [21, 22]. These fungal structural polysaccharides are absent in mammalian cells and are therefore pathogen-associated molecular patterns (PAMPs), which are recognized by various membrane-bound and soluble PRRs of the host as foreign objects [21, 23–26].

In resting conidia, this polysaccharide core which is of similar composition both in the conidium and hyphal cell wall, is covered by a bilayered outer layer composed of hydrophobins (the most external) and melanin. The external hydrophobin rodlet layer, which is responsible for the hydrophobicity of the conidia is exclusively composed of the amyloid hydrophobic RodA proteins [27–29]. One way for *A. fumigatus* to become a pathogen is its capacity to "hide" from the host defensive

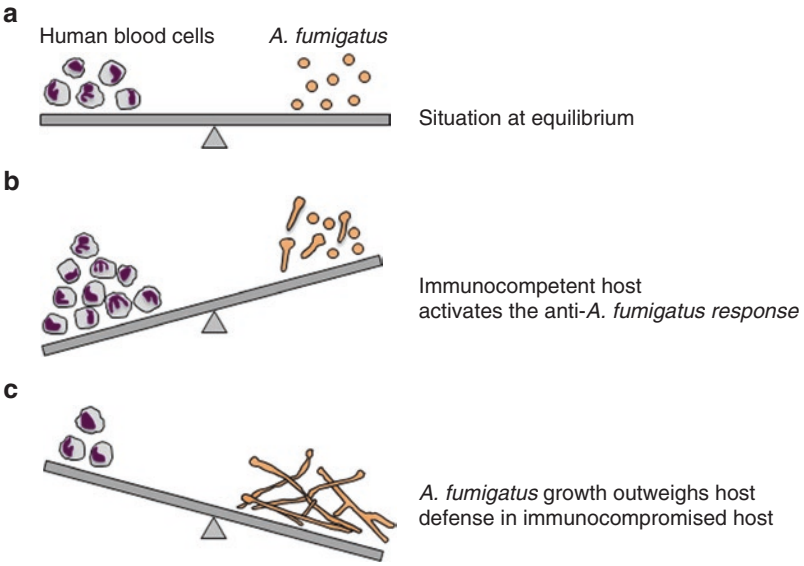

Fig. 4.2 Immune system–*Aspergillus fumigatus* interplay. The fungal disease is only established in the immunodeficient host, where the fungus can resist and escape the immune surveillance and establish the infection

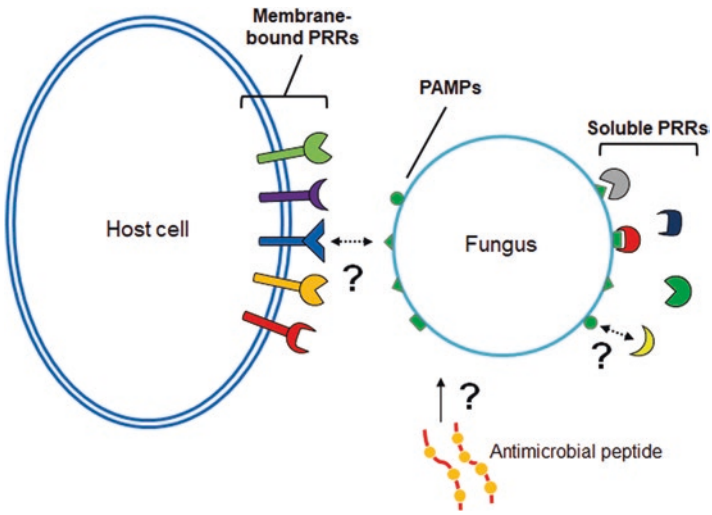

Fig. 4.3 Interaction between various membrane-bound and soluble PRRs which have been shown to bind to the PAMPs on the conidial surface. However, the corresponding PAMPs for each of the PRRs have not been elucidated (as indicated with *question mark*)

response immediately after inhalation and to go undetected after entering the respiratory tract [30–32]. This is due to the presence of the rodlet and melanin outer layers of the dormant conidia which hide the immunogenic cell wall polysaccharides. By doing so, an immediate strong inflammatory response, which would be detrimental for the fungus, is avoided. Moreover, by delaying the immune response, the initial survival of the fungus is prolonged in the host.

The loss of the rodlet and melanin layer during germination leads to an entire modification of the surface layer, which leads to the apparition of immunogenic polysaccharides on the hyphal surface. These surface molecules are recognized by PRRs that are mostly lectin receptors (carbohydrate-recognizing) involved in the initiation of the antifungal response. Indeed, conidial germination can be considered by the fungus as a form suicide. However, instead of being covered by melanin and RodA protein, the cell wall of the mycelium is covered by a specific hyphal galactosaminogalactan, which is immunosuppressive and favors the vegetative fungal growth. All these strategies for the host to seek for the fungal alien and for the fungus to counteract the host immune response after the immediate contact will be discussed below.

4.2.1 Membrane-Bound Pattern Recognition Receptors

4.2.1.1 Toll-Like Receptors

Toll-like receptors (TLRs) are a family of membrane-bound and soluble receptors on sentinel cells involved in the recognition of specific PAMPs [33]. Although the role of TLR2 and TLR4 in the immune defense against fungal pathogens have been extensively studied in the past decades [34]; so far, their precise functions have not yet been clearly elucidated. Various in vitro studies have demonstrated that TLR2 is involved in recognizing *A. fumigatus* [35, 36]. Blocking TLR2 led to a reduced phagocytic rate of *A. fumigatus* conidia, but not of control beads, suggesting that the phagocytic machinery is not impaired by TLR2 blocking [37]. Neutrophil-depleted TLR2-deficient mice had however a lower survival and produced less TNF-α upon stimulation with *A. fumigatus* [38]. The recruitment of neutrophils was severely attenuated in non-immunosuppressed mice deficient in both TLR2 and TLR4, when compared to that in mice with single deficiency [39]. Furthermore, the production of different cytokines in response to *A. fumigatus* is individually mediated by TLR2 and TLR4. For instance, the production of IL-12 and IL-6 from monocytes-derived dendritic cells are mediated by TLR2 and TLR4 respectively [40]. TLR4-mediated pro-inflammatory signals were lost during phenotypic switch from conidia to hyphae, contributing to the escape of the pathogens from the host immune defense [36, 41]. The presence of an optimal innate immune response required both TLR2 and TLR4. Although a direct binding has not been shown, polysaccharides of the cell wall seem to be recognized by TLR2 and TLR4. The IL-6 production via TLR2 and TLR4

stimulation in PBMCs (incubated with TLR2 and TLR4 ligands respectively) was attenuated by pre-incubation with α-glucan and galactomannan, while pre-incubation with β-glucan attenuated the IL-6 production via only TLR4 stimulation [42]. However, TLR2- and TLR4-knockout immunocompetent mice were not more susceptible to challenge of invasive aspergillosis, suggesting that these two receptors are not essential in preventing *Aspergillus* infection in an immunocompetent status [43].

Unlike TLR2 and TLR4, TLR9 is located intracellularly in the endosome compartment of the immune system cells [44]. TLR9 was originally thought to be activated by unmethylated CpG sequences in DNA of bacterial and viral origins only [45]. It was later found that TLR9 can also be activated by unmethylated CpG sequences in fungal DNA [46]. The *A. fumigatus* DNA contains unmethylated CpG sequences, and is therefore capable of activating TLR9 and induce the production of pro-inflammatory cytokines [47]. Since TLR9 is an intracellular receptor, it was suggested that the activation would follow the release of DNA content after fungal lysis in the phagosome. TLR9 is recruited to the phagosomes that contain internalized *A. fumigatus* conidia [48]. However, TLR9 is already recruited to the phagosome merely 1h after phagocytosis of resting conidia [48]. At this stage, the internalized conidia should still be dormant and intact, and the fungal DNA is not exposed. This leaves the underlying recognition mechanism of the fungal DNA and the activation of TLR9 unresolved. Paradoxically, in vivo, TLR9-deficient mice, immunosuppressed by cyclophosphamide, had lower fungal burden than the wild-type mice upon intranasal challenge by *A. fumigatus* [49]. Consistently, in another in vivo study, the neutrophil-depleted TLR9-deficient mice was less susceptible to challenge of dormant or swollen *A. fumigatus* conidia by showing delayed mortality [50]. The expression of dectin-1 was also significantly lowered in the bone marrow-derived dendritic cells from TLR9-deficient mice 14 days post-infection following challenge of swollen conidia [50].

Taken together, the role of TLR2, 4 and 9 in host defense against *A. fumigatus* remains to be further explored.

4.2.1.2 C-Type Lectin Receptors

C-type (Ca^{2+}-dependent) lectin receptors (CLRs), including dectin-1, dectin-2, mannose receptor, Mincle, and DC-SIGN, are important in recognizing the major carbohydrate moieties in the fungal cell wall [51].

Dectin-1 is a major transmembrane receptor for β-1,3-glucan which is found mainly on myeloid cells [52–54]. Since β-1,3-glucan is the major constituent of the fungal cell wall, it was expected that dectin-1 plays a crucial role in the control of fungal infection and especially favors recognition and phagocytosis of fungal particles [37, 55]. In the case of *A. fumigatus*, dectin-1 preferentially recognizes swollen and germinating conidia, as the β-1,3-glucan is exposed on the conidial surface [56–58]. Dectin-1 contains an extracellular lectin-like carbohydrate recognition domain and a cytoplasmic tail, which is phosphorylated at the tyrosine, upon binding

with β-1,3-glucan. The phosphorylated cytoplasmic tail then can interact with spleen tyrosine kinase (SYK), which induces cellular responses, such as respiratory burst, phagocytosis and production of pro- and anti-inflammatory cytokines [54, 58, 59]. Dectin-1 induces different cytokine responses independent or in conjunction with TLR: for example, dectin-1 alone induces the production of IL-10, but requires the adaptor MyD88 for the induction of IL-8 and IL-12 [54, 56, 60].

The ligands for the other membrane-bound CLRs remained to be fully determined. Another member of the dectin family, dectin-2, has been shown recently to recognize *A. fumigatus* galactomannan, but its role in the innate response has not been really evaluated [61–63]. Mannose receptor, Mincle and DC-SIGN recognize the N-linked mannan and N-acetylglucosamine (GlcNAc) in the fungal cell wall [51, 64]. Being a polymer of GlcNAc, chitin could also be recognized by mannose receptor [65]. However, it was recently found that chitin is mainly recognized by the Fcγ receptor [66]. Further studies should be warranted to better investigate the respective roles of these polysaccharides tested alone or in association, in the immune response.

4.2.1.3 Crosstalk Between TLRs and CLRs

Some PRRs collaborate to produce a synergetic induction of immune response [67]. For instance, dectin-1, which recognizes β-1,3-glucan, interact with TLR2, which recognizes zymosan, but not β-1,3-glucan [68]. Interestingly, crosstalk between PRRs does not always lead to enhanced immune response. The CLR Mincle suppresses the antifungal immune response mediated by dectin-1 and Mincle [69, 70]. This has also been suggested to be an evasion mechanism of fungal pathogen from the host immune defense [70]. However, this has not been studied in *A. fumigatus*.

4.2.2 Humoral Pattern Recognition Receptors

4.2.2.1 Complement Components

The complement system is often considered an irrelevant immunological weapon against *A. fumigatus* for the following reasons. First, owing to the presence of the thick fungal cell wall, the membrane attack complex (MAC) of the complement system is ineffective on *A. fumigatus* [71, 72]. Second, complement deficiency in human was not found to increase the risk of aspergillosis. However, all three of the complement pathways, classical, alternative and the lectin pathway, are indeed involved in the host defense against *A. fumigatus*, through opsonization and thus, facilitation of phagocytosis [73]. Complement component C3 can directly, or through other components, such as mannose-binding lectin or immunoglobulin, deposit on the surface of the conidia and mycelia [73–78]. The exact chemical nature of the ligand(s) of the complement components on the surface

of the conidia or mycelia remain unknown. Furthermore, *A. fumigatus* can evade from the complement attack by scavenging complement regulators, Factor H and C4b binding protein [79, 80], and secreting proteases that degrades complement proteins [81]. Alp1p, a major protease secreted by *A. fumigatus*, can degrade complement proteins C3, C4 and C5 [81].

4.2.2.2 Collectins and Ficolins

Collectins (Collagen + lectin) are a group of soluble pattern recognition receptors characterized by a collagen-domain and carbohydrate recognition domain (CRD), which is responsible in the binding to the carbohydrate moiety in a Ca^{2+}-dependent manner. Collectins in the lung bind to *A. fumigatus* conidial surface as opsonins, and facilitate phagocytosis [51, 82, 83].

Surfactant Proteins

There are four surfactant proteins (SP) in human, SP-A, SP-B, SP-C, and SP-D, that are secreted by alveolar type II cells into the alveolar space [84–86]. Both SP-B and SP-C are small hydrophobic proteins that are mainly responsible in reducing surface tension at the air–liquid interface in the lungs, and their role during *Aspergillus* infection have not been studied [87]. It was only recently found that SP-C binds to bacterial lipopolysaccharides [85, 88]. Apart from that, the immunomodulatory role of SP-C is still uncertain. Meanwhile, SP-A and SP-D, which are hydrophilic proteins, are primarily responsible for the host defense against pulmonary pathogens by facilitating phagocytosis after opsonization [84, 86, 89–91]. SP-A and SP-D are not involved in the lectin complement pathway [92].

Human SP-A and SP-D bind directly to surface of dormant conidia, swelling conidia and hyphae of *A. fumigatus* [83, 93], and the responsible ligand(s) are under investigation by our group. When bound to microorganisms via its CRD region, SP-D binds to the calreticulin/CD91 complex on the surface of macrophages, via its collagenous region, which then mediates the uptake of SP-D opsonized microorganisms by phagocytes and, stimulates inflammatory response by activating NF-κB [94, 95]. Although it was found that the mortality of SP-D-deficient mice was similar to that of the wild-type mice following intranasal challenge of *A. fumigatus* conidia, under immunosuppression of corticosteroids, shorter survival duration, higher hyphal density and more tissue damage were observed in the SP-D deficient mice in comparison to the wild-type [96, 97]. This observation suggested that SP-D plays a role in immune recognition and killing, which have been however insufficiently investigated.

Compared to SP-D, the role of SP-A in the immune defense against *A. fumigatus* is less significant. Although human SP-A bind to conidial surface, the binding is reduced in the presence of extracellular alveolar lipids, while that of SP-D remained unchanged [93]. Immunosuppressed SP-A deficient mice are resistance to lethal

IPA challenge when compared to wild-type mice [97], which could be explained by the increased level of SP-D in SP-A-deficient mice [98].

At present, no evasion mechanism from the opsonization of collectins has yet been observed in *A. fumigatus*.

Mannose-Binding Lectin and Ficolins

Mannose-binding lectin (MBL) binds to the conidial surface of *A. fumigatus* [99]. Mannose-binding lectin by binding to the MBL-associated serine proteases (MASP1, MASP2, and MASP3), activate the lectin complement pathway [100]. Non-immunosuppressed MBL-knockout mice were less susceptible to systemic invasive aspergillosis [101]. It was suggested that the lack of MBL reduced the recruitment of neutrophils, which in turn leads to less tissue damage from inflammation. In contrast, in human, a deficiency in MBL is associated with chronic and invasive aspergillosis [102, 103]. The role of MBL should be revisited.

Ficolins (fibrinogen + collagen + lectin) are a family of lectins that consist of a collagen-like domain and a fibrinogen-like domain described as recognizing specifically N-acetylglucosamine (GlcNAc) [104]. There are three types of ficolins in human, ficolin-1, 2, and 3, which are secreted into the human plasma and binding to ficolin activate the lectin complement pathway [104, 105]. Ficolin-2 binds to *A. fumigatus* [74, 106, 107]. The in vitro binding of ficolin-2 to *A. fumigatus* is inhibited by the presence of GlcNAc and Curdlan (β-1,3-glucan) [107], suggesting that chitin and β-1,3-glucan are the responsible ligands. Previously, ficolin-1 and ficolin-3 were not found to bind to *A. fumigatus*. However, recently, the role of ficolin-3 in lung immune defense was discovered. Ficolin-3 indeed binds to *A. fumigatus* resting or swollen conidia in a calcium-independent manner [74, 82], suggesting that the binding does not involve the carbohydrate recognition domain. Unlike ficolin-2, which is produced in the liver, ficolin-3 is produced by type II alveolar cells and secreted into the alveolar space. Moreover, ficolin-A (the orthologue of ficolin-2 in mouse) increases the release of IL-8 (a proinflammatory cytokine and chemokine for neutrophils) [74]. Upon binding to the carbohydrate ligand on the pathogen surface, ficolins facilitate opsonization, phagocytosis and the activation of the lectin complement pathway. However, complement activation was not impaired in ficolin-knockout mice [74, 108]. This could be explained by the similar role of MBL and ficolins, that both trigger the lectin complement pathway.

Although many elements of the immune system in the anti-*A. fumigatus* response have been discovered or rediscovered in recent years, a comprehensive picture of their roles as well as the ranking of their importance in the immunocompetent, as well as immunocompromised host remains to be elucidated.

4.3 The Cell Actors

4.3.1 Airway Epithelial Cells

Airway epithelial cells (AECs) are the first type of cells to enter in contact with the inhaled conidia [109, 110]. AECs, being nonprofessional phagocytes, are capable of internalizing and killing *A. fumigatus* conidia [111, 112]. Comparing with murine macrophage cell line (J774), the internalized conidia survive longer in the human airway epithelial cell line (A549) than in the alveolar macrophages [113]. Accordingly, a small portion of the internalized conidia are not killed by the AECs, which then germinate and break the epithelial barrier [113]. The inefficient killing of the internalized conidia by the AECs is probably due to inefficient respiratory burst. In addition, the adherence of *A. fumigatus* conidia to the A549 cell line inhibits the release of IL-6, IL-8, and TNF-α, which thus inhibits the recruitment of immune cells and apoptosis of the epithelial cells [114, 115].

AECs produce antimicrobial peptides such as human β-defensins, human lactoferrin (hLF), and histatin 5, which possess some antifungal activity against *A. fumigatus* [4, 6, 109, 110, 116, 117]. The gene expression of human β-defensins hBD2 and hBD9 were found to be induced in A549 cells that are exposed to swollen conidia [116]. hBD2 displayed direct, but low antifungal activity in vitro against *A. fumigatus* [118] and does not enhance the antifungal killing activity of neutrophils [118]. However, hBD could act as chemoattractant for immune cells and activation of professional antigen-presenting cells [119].

4.3.2 Alveolar Macrophages

The resident alveolar macrophages (AMs), which are responsible of the recognition and phagocytosis of the *A. fumigatus* conidia, serve as the major immune cells in the defense of *A. fumigatus*. Upon internalization of *A. fumigatus* conidia, phagolysosomes were not acidified [18]. Dihydroxynaphthalene-melanin (DHN-melanin) on the outer layer of dormant *A. fumigatus* conidia was shown to inhibit the acidification of phagolysosome, and thus, prevent intracellular killing [120–122]. The mutant of polyketide synthase (Δ*pksP*), the enzyme responsible for the initial step in DHN-melanin formation is devoid of DHN-melanin and avirulent [123]. Interestingly, the melanin of *A. niger*, which is different from the melanin in *A. fumigatus* [124, 125], does not inhibit phagolysosome acidification [18]. The intracellular killing of *A. fumigatus* conidia in AMs is triggered by the swelling of conidia. The phagolysosome acidification and the increase in the production of reactive oxygen species (ROS) following phagocytosis is essential for conidial killing [126, 127]. Upon swelling, the outer layer of hydrophobins and melanin is shed. In the absence of inhibition by melanin, the phagolysosome acidifies. On the other hand, the absence of the outer layer of rodlet and melanin unmasks the PAMPs in the inner layer. The exposure of PAMPs

recruits PRRs to the phagosome, which triggers production of ROS, cytokines and chemokines and the recruitment of autophagy proteins, LC3 II, Atg5 and Atg7 to the phagolysosome [57, 128, 129]. Pyomelanin, another type of melanin that *A. fumigatus* produces, can also protects the fungus from ROS attack [13, 120, 130]. However, pyomelanin-minus mutant are as pathogenic as their parental strain. In addition, DHN-melanin inhibits LC3-associated phagocytosis (LAP) [123, 131, 132]. Taken together, pyomelanin and DHN-melanin permits the fungus to escape host immune defense by inhibiting phagolysosome acidification, quenching ROS and inhibiting LC3-associated phagocytosis.

4.3.3 Neutrophils

Neutrophils act as a strong second line of innate immune defense against *A. fumigatus* [133]. Upon recruitment to the alveolar space, neutrophils constitute the majority of phagocytes to eliminate the conidia [4, 134]. Even though AMs by themselves are able to eradicate resting conidia in a mouse deprived of neutrophils, neutrophils are more important than AMs in the pathogen clearance [127, 135]. Indeed, low number of neutrophils in the host (neutropenia) renders patients at great risk of IPA [2]. Moreover, molecules of *A. fumigatus* which impairs the neutrophil action are true virulence factors. Galactosaminogalactan is one of them. This polysaccharide, which is secreted by *A. fumigatus* hyphae, suppresses the recruitment of neutrophils, which then favors the fungal survival in the host [136, 137]. In addition, it induces anti-inflammatory response by the induction of interleukin-1 receptor antagonist (IL-1Ra) and reduces neutrophil recruitment [136, 138].

An important function unique to neutrophils is its capacity of attacking *A. fumigatus* hyphae, which are too large to be phagocytosed by immune cells. Neutrophils attack the fungal hyphae extracellularly by the secretion of enzymes, which degrade and permeabilize the cell wall and makes the fungus more sensitive to the neutrophil granular toxic molecules. Recently, neutrophil extracellular traps (NETs) which are mainly composed of nuclear DNA and antimicrobial proteins, have been mentioned as playing a major role against microbial pathogens [139]. Although the production of NETs is stimulated by *Aspergillus* hyphae [140], the hyphae are reported to be only slightly susceptible to the killing by NETs [141]. It is suggested that NETs only has a fungistatic effect on the hyphae and prevent further spreading of the disease [141]. This finding is in contrast to the one regarding *Candida albicans*, which is completely susceptible to the killing by NETs [142]. The fungistatic activity of NETs towards *A. fumigatus* hyphae is attributed by calprotectin, a Zn^{2+} chelator, [143]. Interestingly, it was shown recently that *A. fumigatus* hyphae generate hyphal branching upon interaction with neutrophils, which allows lower host immune interference and increases invasive growth [144].

Moreover, *A. fumigatus* seems to have other mechanisms to evade the neutrophil antagonistic action since *A. fumigatus* is less sensitive than *A. niger* for which the germination and hyphal length are more significantly reduced by neutrophils [18].

For example, fumagillin, secreted by the *A. fumigatus* hyphae, inhibits the antifungal function of neutrophils by inhibiting the formation of NADPH oxidase and reduces the degranulation [145]. Neutrophils exposed to fumagillin in vitro also demonstrated reduced rate of phagocytosis of *A. fumigatus* conidia [145]. An analysis of the differences between *A. fumigatus* and other non- or poorly pathogenic *Aspergillus* species, such as melanin structure and cell wall composition, should definitely reveal more information of the success of *A. fumigatus* as pathogen.

4.4 Perspectives

The current arsenal of antifungal agents available for the treatment of IA is mainly limited to azoles, echinocandins, and polyenes [146]. However, there are major drawbacks regarding the drug resistance, efficiency, and toxicity of these current agents [147, 148]. Furthermore, the pipeline of antifungal development has not been introducing any new classes of antifungal agents, especially since there is not a market for drugs for rare diseases such as aspergillosis. Can immunotherapy lead to the development of specific drugs or antibodies that are able to block virulence factors that allow the evasion of *A. fumigatus* from the host immune system? The past decade has witnessed a shift in paradigm of antifungal development, in which the virulence factors are targeted for drug candidates instead of essential genes [149–151]. Analysis of the transcriptomic and proteomic changes occurring after internalization of the pathogen and especially in the phagolysosome, where the fungus is inhibited in the immunocompetent host but not in the immunocompromised host, may lead to the identification of essential virulence factors in vivo. Such approach focused on the inhibition of virulence factors would exert less selective pressure on the pathogens, and thus, the chance of resistance development is lower [152]. However, such immunotherapeutic approach has not been yet undertaken with *A. fumigatus* but the situation may be difficult in the case of pulmonary aspergillosis, which occurs mainly among immunocompromised patients. The identification of monoclonal antibodies able to block hyphal emergence could be also possible. Such strategy has been proposed a few years ago [153, 154], however it has not been pursued yet. Although a lot of progresses have been obtained in recent years, the overall picture to fully understand the innate immune defense against *A. fumigatus* is missing. For example, the respective role of the different PRRs and the lack of definition of all the ligands binding to complement proteins, collectins and other PRRs are essential gaps to fill. The reasons for the ineffectiveness of NETs to kill *A. fumigatus* hyphae should also be investigated. Comparative characterization of major *Aspergillus* species may also provide valuable understanding of how *A. fumigatus* took the "throne" and became the most predominant and successful pathogen among the *Aspergillus* species. Finally, it has been reported that many cytokines and chemokines plays a major role in the defense reactions (not discussed here but see for review [155, 156]. However, the interweaving network between all these chemokines, cytokines and the defense against

A. fumigatus remains obscure. In conclusion, the early events "when *A. fumigatus* meets the man" remain enigmatic and this chapter paves the way for the direction of future exploration.

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Chapter 5
Histoplasma capsulatum and Histoplasmosis

Chelsea Bueter, George S. Deepe, and Chad A. Rappleye

Abstract The fungal pathogen *Histoplasma capsulatum* infects tens to hundreds of thousands of individuals every year. Ensuing disease severity is largely dependent on the host immune response with activation of cell-mediated immunity essential for eventual pathogen control. During the early stages of infection normally fungicidal phagocytes provide a permissive intracellular host niche for Histoplasma yeasts. These fungal elements avoid destruction by phagocytes by minimizing exposure of immunostimulatory molecules on their surface and by detoxifying antimicrobial reactive compounds produced by phagocytes. Within the phaogcyte, yeasts overcome nutrient limitations including organic sulfur, iron, and zinc. Activation of T-cells and the ensuing production of pro-inflammatory Th1 and Th17 cytokines (especially TNFα, IFNγ, and GM-CSF) stimulate phagocyte reactive oxygen and nitric oxide production, thereby changing the host–pathogen balance to favor phagocyte clearance of intracellular Histoplasma yeasts. Clinical management of histoplasmosis involves identification of susceptible individuals with functional or therapeutic reduction of factors mediating activation of cell mediated immunity and augmentation of fungal control through administration of azole- and polyene-class antifungals.

5.1 Introduction

Histoplasma capsulatum is the causative agent of the respiratory and systemic disease histoplasmosis. Areas of endemicity as defined by clinical case prevalence and skin reactivity to fungal antigens include the Midwest and Eastern parts of the United States (especially around the Mississippi and Ohio River valleys) and many parts of Latin America (particularly Colombia, Venezuela, Brazil, and Argentina)

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[1–4]. *Histoplasma* spp. are also found in Africa and reports are emerging of cases in China and India indicating *Histoplasma* species are more globally distributed than originally thought [5–8]. Multiple phylogenetic groups of strains have been defined from both clinical and animal isolates which correlate with these different geographic regions and likely represent distinct subspecies of *Histoplasma* [9, 10]. The major phylogenetic groups defined include two North American clades (NAm1 and NAm2), two Latin American clades (LAmA and LamB) and the African clade. Consequently, we will refer to them collectively using the genus name *Histoplasma* without reference to species or subspecies. Although the annual incidence is likely under reported, estimates of hundreds of thousands of infections occur each year [11, 12]. Fortunately, the majority of infections are self-limited and do not require clinical intervention.

Histoplasma infects both immunocompromised and immunocompetent individuals. *Histoplasma* is initially acquired through inhalation of conidia released from an environmental site. Upon germination, the conidia establish a respiratory infection. Histoplasmosis manifests as a gradient of disease which is ultimately dependent on the balance of *Histoplasma* virulence factors versus the efficiency of immune defense mechanisms. Clinical disease typically manifests in individuals lacking a critical attribute of immune defenses (e.g., CD4 deficiency in AIDS patients or cytokine inhibition therapies) or individuals receiving a large inoculum of conidia/spores [12, 13]. One study of hospitalizations in the United States for histoplasmosis indicated less than half were associated with immunocompromising comorbidities indicating the existence of some, as yet undetermined, additional susceptibility factors [2]. Control and ultimate resolution of *Histoplasma* infection requires cell-mediated immunity and the consequent activation of phagocytes. This indicates that the innate immune system, by itself, is insufficient and that *Histoplasma* cells (yeasts and conidia) can efficiently escape innate immune defenses. This conveniently divides the course of infection into two phases in otherwise normal individuals: an initial phase during which *Histoplasma* conquers host macrophages and a later phase during which activation of macrophages defeats the intracellular yeasts. In this chapter, we discuss factors at the host–pathogen interface that facilitate these two phases of infection: (1) how *Histoplasma* survives and parasitizes host innate immune cells before activation of cell-mediated immunity and (2) how onset of adaptive immunity reverses this situation, leading to control and elimination of *Histoplasma*.

5.1.1 Thermal Dimorphism

The pathogenesis of *Histoplasma* is linked to thermal dimorphism. In the environment, *Histoplasma* grows as a saprobic mold (Fig. 5.1a) in environments with decaying organic matter. The mycelia produce conidia (Fig. 1a, inset) which facilitate dispersal of *Histoplasma* to new environments where they germinate into mycelia again. *N*-acetylglucosamine, a widespread component of microbes, appears to

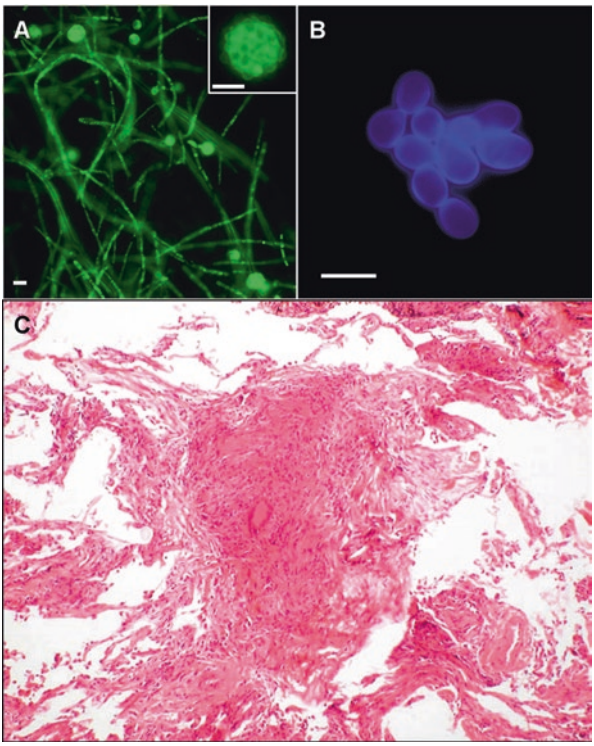

Fig. 5.1 Histoplasma dimorphism. (**a**) At lower temperatures, *Histoplasma* grows as filamentous hyphae that produce microconidia and tuberculated macroconidia (**a**, inset), which function for dispersal. (**b**) At mammalian body temperatures, Histoplasma grows as a budding yeast form. (**c**) Within host tissues, the immune response organizes into granulomas in an attempt to contain the yeasts which have invaded phagocytes. *Scale bars* in each panel indicate 5 μm

provide one cue signaling the soil environment since this monosaccharide enhances the differentiation into filamentous growth [14]. However, upon inhalation into a mammal, the conidia germinate into a yeast form (Fig. 5.1b) and express factors that enable pathogenesis. The principal cue inducing differentiation into the yeast form is the elevated temperature of the mammalian host (i.e., 37 °C). Genetic studies have identified an orphan sensor-kinase that is required for the temperature-dependent switch to the yeast phase as well as a set of transcription factors (Ryp = required for yeast phase) that control expression of the yeast-phase regulon [15–18]. In the absence of these factors, or through pharmacological inhibition of the transition, *Histoplasma* cells remain as mycelia at 37 °C and are unable to establish infections in mammals [16, 19], demonstrating the differentiation into yeasts is necessary for *Histoplasma* pathogenesis.

5.1.2 Symptoms and Diagnosis

Patients with acute *Histoplasma* infection present with fever, chills, headache, respiratory symptoms, cough, and chest discomfort. Histoplasmosis is not often suspected until patients fail to recover after a standard antibiotic therapy. Diagnosis

can be confirmed by the presence of yeast cells in tissue samples stained with methenamine silver, or by culturing blood or tissue [20]. Histoplasmosis is a granulomatous disease (Fig. 5.1c). As histoplasmosis can result in respiratory failure or death, rapid diagnosis is vital. Diagnostic tests including: enzyme immunoassay (EIA), complement fixation (CF), immunodiffusion (ID) and PCR assays, all of which deliver quick determinations. EIA detects antigen in urine or serum with a sensitivity of over 80–90% [21–23]. However, relying on just one sample may result in failure to detect histoplasmosis, and consequently testing both blood and serum is recommended [23]. Antigen has also been discovered in the BAL of pulmonary histoplasmosis patients with over 93% accuracy [24]. Aside from its utility in diagnosis, the antigen assay is an effective tool for monitoring treatment responses. One caveat of the EIA method is that there is a 90% cross-reactivity with blastomycosis that may obscure results. CF identifies host antibodies to *Histoplasma*. Persistence of antibodies years after exposure makes this assay less useful; however, a single CF titer of 1:32 may indicate a more recent infection [20, 25]. The ID assay detects the M and H precipitin bands; the M band will persist for years, making it less useful for diagnosis, but the H band is typically only found during active infection [20, 24]. Finally, a PCR assay is in development and has been successful in culture isolates and clinical isolates, though as of yet there is no commercially available test [26].

5.2 Innate Immunity Phase: Success of *Histoplasma*

5.2.1 Infection of Macrophages

Within the lung, *Histoplasma* cells overcome challenges to survival by successfully invading phagocytic cells. The small size of *Histoplasma* microconidia enable them to penetrate into the alveoli of the lung. Within alveolar spaces, *Histoplasma* cells encounter surfactant, a lipid-rich fluid that also contains the collectins SP-A and SP-D [27]. At physiological concentrations, these two surfactant proteins can permeabilize yeast cells [28], and this antifungal activity represents a significant barrier to *Histoplasma* colonization within the lumen of the alveoli. To overcome this, *Histoplasma* cells find an intracellular refuge by infecting alveolar macrophages, thereby sheltering itself from the activities of SP-A and SP-D.

Resident alveolar macrophages encounter *Histoplasma* first and are the primary host cell. By day 3 post-infection, yeasts are found inside these cells and are also within inflammatory macrophages. By day 7, yeasts reside in inflammatory macrophages and neutrophils [29]. To become intracellular, *Histoplasma* conidia and yeast rely on non-opsonic phagocytosis by macrophages [30, 31]. To accomplish this, *Histoplasma* conidia and yeast bind to β-integrin family complement receptors [e.g., complement receptor 3; CR3 (CD18/CD11b)] on phagocytes, which triggers phagocytic uptake [31, 32]. On the fungal side, this adhesion is mediated by heat shock protein 60 (Hsp60) molecules present on the yeast cell surface [33, 34]. Yeast

binding to CR3 is temperature sensitive and requires divalent cations (e.g., Ca2+ and Mg2+) indicating internalization occurs through structural dynamics of the integrins [32]. Vaccination with Hsp60 provides protection to the host, possibly by blocking the critical Hsp60-CR3 ligand-receptor interaction and subsequent phagocytosis or by redirecting phagocytosis through FcγR [35–37].

5.2.2 Avoidance of Detection

Although intracellular residence protects *Histoplasma* from extracellular immune defenses, infection of phagocytic cells carries risks of its own. Upon interaction with fungal cells, phagocytes are normally antifungal. However, CR3-mediated phagocytosis, in the absence of other activating signals, is generally noninflammatory. Thus, to promote uptake into a permissive intracellular environment, *Histoplasma* yeast must bind to phagocytic receptors but avoid stimulating signaling receptors that would activate phagocyte defenses. Signaling pattern recognition receptors (PRRs) that detect fungal pathogen-associated molecular patterns (PAMPs) commonly have a carbohydrate recognition domain that binds to molecules of the glycan/glycoprotein-rich fungal cell wall [38].

5.2.2.1 β-Glucan Concealment

Chief among these PRRs for detecting fungal pathogens is Dectin-1, a C-type lectin receptor that recognizes β-glucans that are ubiquitous constituents of fungal cell walls [39]. Recognition of fungal β-glucans by Dectin-1 receptors on macrophages and neutrophils triggers inflammatory responses that include production of reactive oxygen species (ROS) and the secretion of pro-inflammatory cytokines (e.g., TNFα and IL-6) [40–44]. Mouse studies show that Dectin-1 contributes relatively little to the control of *Histoplasma* yeasts [45, 46]. The cell walls of *Histoplasma* yeasts contain abundant β-glucans and therefore the potential to be recognized by Dectin-1. However, *Histoplasma* yeast minimize this detection through two separate mechanisms. The yeasts cells of most strains of *Histoplasma* also produce an α-linked glucan polysaccharide and this α-glucan constitutes the outermost layer of the cell wall, effectively concealing the underlying immunostimulatory β-glucans from detection [47, 48]. The α-glucan polysaccharide is synthesized by α-glucan synthase (Ags1) which is thought to extend short glucan oligomers formed by an α-amylase-like protein (Amy1) [48, 49]. The second mechanism for minimization of β-glucan detection is through enzymatic removal of β-glucan fragments. *Histoplasma* yeasts secrete an endo-β-glucanase (Eng1) which acts on the fungal cell wall and hydrozlyzes surface-exposed β-glucans [50]. These two mechanisms, shielding of β-glucans and pruning away exposed β-glucans effectively prevent detection of *Histoplasma* yeasts by Dectin-1, thereby preventing macrophage

responses. Consistent with their pathogenesis-enabling role, avirulent mycelia do not produce α-glucan or Eng1 [51, 52].

5.2.2.2 Toll-Like Receptors

Histoplasma yeasts can be detected by Toll-like receptors (TLRs) as well as Dectin-2. Immune cells expressing Dectin-2, but not Mincle, respond to *Histoplasma* yeasts suggesting a mannan-type PAMP is present on yeasts [46]. The involvement of TLRs in yeast detection comes from studies in mice lacking the MyD88 adaptor protein which is required for TLR2 and TLR4 signaling. In the absence of MyD88, mice show a modest defect in the control of *Histoplasma* infections beginning at 7 days post-infection which coincides with the activation of cell mediated immunity [45]. Consistent with this, mice lacking MyD88 have lower pro-inflammatory cytokines, notably IL-12 which is required for generation of the Th1 response necessary for efficient control of *Histoplasma* (see section below on the adaptive immunity phase). A yeast cell wall extract can stimulate TLR2 but cytokine production by macrophages interacting with whole yeast cells does not depend on TLR2 [30] suggesting, like β-glucan exposure discussed above, the spatial organization of the yeast cells minimizes detection of PAMPs by TLR2.

Additional fungal molecules have been localized to the yeast cell wall. Surface exposed proteins include the Hsp60 adhesin mentioned above, an extracellular catalase (CatB/M-antigen), histone 2B, and Hsp70 [53–56]. In addition, yeast cells of some, but not all, *Histoplasma* lineages secrete Yps3, a protein that associates with the yeast cell surface via interactions with cell wall chitin [57–59]. Yps3 contributes to extrapulmonary dissemination through an unknown mechanism but is dispensable for pulmonary infection [60].

Minimizing detection by macrophages sets *Histoplasma* apart from opportunistic fungal pathogens which are readily detected by multiple PRRs. The ability to conceal itself from macrophages provides at least a partial explanation for why innate immune cells are unable to fully control *Histoplasma* yeasts.

5.2.3 Neutralization of Macrophage Defenses

5.2.3.1 Reactive Oxygen

Phagocytic cells are armed with multiple factors to either kill or restrict the growth of microbes. Production of antifungal reactive oxygen species (ROS) is initiated by formation of superoxide by the phagocyte NADPH oxidase complex. Superoxide and other reactive oxygen molecules derived from it (i.e., peroxides and hydroxyl radicals) directly damage microbes. Superoxide dismutases are antioxidant enzymes produced by aerobic organisms; however, in most microbes, superoxide dismutases are intracellular to elminate damage from superoxide produced as a by-product of

respiration. However, for intramacrophage pathogens, antimicrobial superoxide produced by the phagocyte is extracellular to the microbial cell. Thus, for cells interacting with macrophages or neutrophils, intracellular superoxide dismutases provide little protection.

Histoplasma yeasts secrete two factors that combat phagocyte-derived reactive oxygen and enable its survival in phagocytic cells. *Histoplasma* yeasts produce an extracellular superoxide dismutase (Sod3) as well as an extracellular catalase (CatB) which dismute superoxide and eliminate peroxide ROS, respectively [51, 61, 62]. Both enzymes can associate with the yeast cell wall in addition to being soluble. Importantly, the extracellular localization positions Sod3 and CatB to act on ROS produced by the phagocyte against *Histoplasma*. Without Sod3 or CatB, *Histoplasma* yeasts are readily killed by macrophages and neutrophils [61, 62]. Consistent with its secretion, Sod3 does not act on, nor does it protect against, metabolically-derived ROS within the yeast cells [62]. Thus, *Histoplasma* yeasts release an efficient collaboration of antioxidant enzymes that negate phagocyte-derived ROS. Consequently, *Histoplasma* yeasts survive levels of ROS which are lethal to the opportunistic pathogen *Candida albicans*. The rapid destruction of ROS mediated by these enzymes also explains why earlier studies failed to detect ROS production by phagocytes in response to *Histoplasma* (i.e., the ROS was destroyed by Sod3 and CatB before it could be measured) [63–65]. Interestingly, the expression of extracellular superoxide dismutases is common for bacterial pathogens that infect macrophages suggesting that elimination of superoxide is a necessary defense for survival in phagocytes. As with α-glucan, expression of Sod3 and CatB are restricted to the pathogenic yeast consistent with their function specifically in enabling the pathogenic lifestyle of yeasts [51, 66].

5.2.3.2 Reactive Nitrogen

In addition to ROS, *Histoplasma* yeast are confronted with reactive nitrogen species (RNS) which is fungistatic to *Histoplasma*. Yeast cells respond to RNS by upregulating multiple stress-response genes including a nitric oxide reductase, although its functional role has not been defined [67]. Inhibition of RNS production decreases the ability of macrophages to control *Histoplasma* [68, 69]. RNS production against *Histoplasma* is regulated by pro-inflammatory cytokines. This underscores the necessity of *Histoplasma* yeasts to avoid detection and minimize inflammatory cytokine production.

5.2.3.3 Phagosome Acidification

Within the macrophage, *Histoplasma*-containing phagosomes can fuse with lysosomes exposing *Histoplasma* yeast to hydrolytic enzymes. Regardless of phagolysosomal fusion, *Histoplasma* yeasts prevent complete acidification of the *Histoplasma*-containing phagosome, maintaining a pH around 6 [70–73]. This partial acidification

is thought to permit release of one of the iron molecules from transferrin (see below) but prevent activity of acidic hydrolases of the lysosome. Yeast modulation of pH in the phagosome is an active process as killed yeast cells are found in acidified compartments [70]. At least part of this mechanism is exclusion of proton ATPases from the phagosomal membrane that mediate transport of H^+ ions into the phagosome [73]. Yeasts that artificially acidify the phagosome environment are killed, indicating the pathogenesis requirement to maintain a more neutral pH [74].

5.2.4 Overcoming Nutrient Limitations

5.2.4.1 Pyrimidines and Essential Cofactors

Once established in the phagosome, *Histoplasma* yeast scavenge available nutrients in order to proliferate. The *Histoplasma*-containing phagosome lacks available nucleic acids and essential vitamins for *Histoplasma* utilization. Consequently, *Histoplasma* yeast synthesize these compounds from simpler organic molecules derived from central metabolism. For example, growth in macrophages requires the pyrimidine biosynthetic pathway to overcome the paucity of uracil in the phagosome [75]. Similarly, biosynthesis of riboflavin and pantothenate cofactors is necessary for intramacrophage growth of *Histoplasma* yeasts [76]. Without these pathways, *Histoplasma* yeasts can survive macrophage defenses, but are unable to proliferate within macrophages and have severely attenuated virulence in murine models of histoplasmosis. Biotin synthesis is not required, indicating either the availability of biotin within the phagosome or effective scavenging mechanisms [76].

5.2.4.2 Organic Sulfur

Histoplasma yeast, unlike mycelia, are auxotrophic for organic sulfur [77]. This is due to the lack of expression of sulfite reductase by yeast cells, preventing the incorporation of inorganic sulfate into cysteine. Interestingly, the lack of sulfite reductase activity is dependent on temperature with the elevated temperature of mammals correlating with lack of activity [77–79]. Although growth of yeasts in culture requires an organic sulfur source, they proliferate well in macrophages [80]. Together these findings suggest that yeasts have adapted to an abundant supply of organic sulfur within the macrophage phagosome.

5.2.4.3 Iron and Zinc

Sequestration within the phagosome imposes challenges for acquisition of trace metals such as iron and zinc. Indeed, reducing the availability of metals is a defense strategy used by macrophages to restrict microbial pathogens [81]. *Histoplasma*

yeast have multiple strategies to acquire iron. Iron in the host is bound to transport molecules such as transferrin and these must be brought to the phagosome for iron to become available to yeasts within the macrophage. Although *Histoplasma* yeast prevent acidification of the phagosome, a drop in pH is necessary to liberate iron from transferrin [82]. Thus, *Histoplasma* maintains a lumenal pH around 6.0 at which it is acidic enough to release one of the two iron atoms bound to transferrin but sufficiently neutral to prevent acidic hydrolase activity. Addition of the weak base chloroquine to infected macrophages prevents *Histoplasma* proliferation by raising the phagosomal pH and reducing available iron [82].

In addition to phagosome pH modulation, *Histoplasma* yeast steal iron by secretion of iron chelating hydroxamate siderophores and iron reduction systems. Restriction of iron causes upregulation of siderophore biosynthesis genes through an iron-responsive GATA family tanscription factor (Sre1) [83, 84]. Prevention of siderophore synthesis reduces proliferation of *Histoplasma* yeasts in macrophages [85, 86]. In addition to siderophore synthesis, some strains of *Histoplasma* express genes encoding a high-affinity iron reduction/transport system (Fet3 and Ftr1) as well as a glutathione-dependent iron reduction system catalyzed by a secreted γ-glutamyl transferase (Ggt1) [87, 88]. Loss of Ggt1 function blocks the ability of yeast to proliferate inside macrophages confirming yeasts must overcome iron limitations [88]. In murine models of disease, the operation of various iron acquisition mechanisms causes differences in *Histoplasma* pathogenesis. For example, strains that lack siderophore production are attenuated early in infection if they also lack the Fet3/Ftr1 iron reduction system whereas strains that express Fet3/Ftr1 do not exhibit decreased virulence until 2 weeks post infection, a time point at which adaptive immunity and the production of IFNγ has activated macrophages to further restrict iron availability [85, 86].

As with iron, zinc availability can also be modulated by macrophages imposing an additional nutritional limitation on intramacrophage yeasts. *Histoplasma* yeasts express a high affinity zinc transporter (Zrt2) when faced with low zinc concentrations [89]. The Zrt2 transporter facilitates zinc import into *Histoplasma* yeasts and facilitates growth in macrophages. However in a murine disease model, Zrt2-dependent zinc transport was not required until after 5 days of infection suggesting that zinc levels in resting macrophages are sufficient for *Histoplasma* yeast proliferation but that activation of macrophages restricts zinc availability. Consistent with this, treatment of *Histoplasma*-infected macrophages with GM-CSF reduces levels of intramacrophage iron and zinc impairs growth of yeasts [90]. This cytokine upregulates zinc-binding metallothioneins and macrophage zinc exporters, the end result of which is to reduce the zinc available to *Histoplasma* yeasts within the phagosome [91]. Together these findings show that *Histoplasma* expresses a zinc transporter to overcome the cytokine-induced zinc limitation in macrophages. Preliminary work indicates a third essential metal, copper, is similarly restricted after activation of macrophages and that *Histoplasma* yeasts express a high-affinity copper transporter to overcome copper limitation (Shen Q and Rappleye CA, personal communication).

5.2.4.4 Cbp1

Within the phagosome, *Histoplasma* yeasts secrete a small calcium-binding protein [92, 93]. Although the protein has nanomolar affinity for calcium, whether Cbp1 influences calcium homeostasis of yeasts is unknown. As with the α-glucan, Eng1, Sod3, and CatB virulence factors, Cbp1 production is restricted to yeasts and absent from mycelia arguing for a role in pathogenesis [94, 95]. Cbp1 has multiple intramolecular disulfide bonds which makes Cbp1 resistant to proteases, a feature consistent with Cbp1 functioning in the macrophage phagosome [96]. Without Cbp1, yeast proliferation in macrophages is impaired confirming Cbp1's importance to the intramacrophage growth of yeasts [97, 98].

Unlike nonpathogenic and opportunistic fungi which are readily controlled by innate immune cells, *Histoplasma* yeast survive their encounter with phagocytes. The mechanisms that distinguish *Histoplasma* from other fungi include the ability to promote their phagocytosis while minimizing detection by host phagocytes and the production of factors to defend the yeasts against phagocyte-produced ROS (Fig. 5.2). *Histoplasma* yeasts not only survive, but they also use the macrophage as their primary host cell. Within the *Histoplasma*-containing phagosome, yeasts successfully combat nutrient limitations or synthesize essential compounds de novo that are unavailable. In this way, yeasts convert the macrophage phagosome into a host cell permissive for yeast proliferation. However, upon activation of cell-mediated immunity, these mechanisms prove insufficient and the macrophage gains the advantage.

5.3 Cell-Mediated Immunity: Host Prevails

5.3.1 Kinetics

Macrophages and DCs produce cytokines to stimulate the type 1 helper T cell (Th1) response and present antigens to T cells, and by day 7 of infection, the adaptive response has been activated. In mice, T cells produce interferon γ (IFNγ) that induces macrophages to kill or inhibit the growth of yeast cells. Human macrophages induce fungistasis independent of IFNγ, at least in vitro, but are dependent on interleukin-3 (IL-3), granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) and collagen adherence [99, 100].

Histoplasma infection results in the formation of granulomas, a spatial organization of immune cells formed to wall off the pathogen it cannot eliminate (Fig. 5.1c). In hosts with intact cell-mediated immune responses, these granulomas consist of macrophages and lymphocytes with *Histoplasma* contained in the center [101]. T cell-depleted mice are still able to form granulomas, but they are much less capable of containing the infection and have elevated fungal burdens [102, 103]. Both humans and mice form granulomas, though the latter tend to not caseate [104]. Granulomas can be seen as early as day 7 post intraperitoneal infection in mice [101]. The timing of granuloma

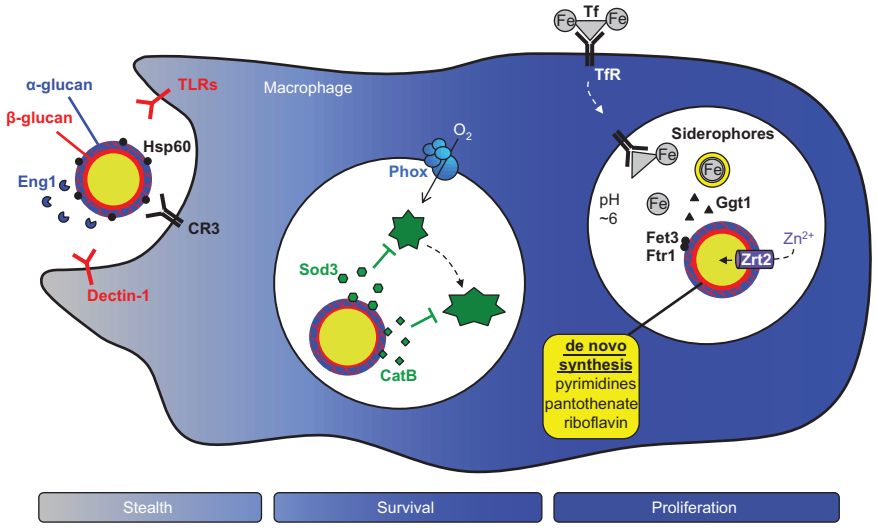

Fig. 5.2 Mechanisms of *Histoplasma* pathogenesis of macrophages. Schematic showing three aspects of macrophage infection: stealth, survival of defenses, and intramacrophage proliferation. *Stealth interaction*: *Histoplasma* yeasts utilize Hsp60 binding to phagocytic receptors (*black*; CR3) to induce uptake while simultaneously reducing detection by signaling receptors (*red*; Toll-like receptors (TLRs) and Dectin-1). *Histoplasma* minimizes β-glucan detection by Dectin-1 by production of α-glucan to conceal cell wall β-glucans and secretion of the endo-β-glucanase (Eng1) which prunes away any remaining exposed β-glucans. *Survival of defenses*: *Histoplasma* produces extracellular superoxide dismutase (Sod3) and extracellular catalase (CatB) to destroy reactive oxygen species generated by the phagocyte NADPH-oxidase (Phox) and ensure survival of yeasts. *Proliferation*: *Histoplasma* prevents complete phagosome acidification and acquires iron from host transferrin (Tf) by production of siderophores (*yellow*), a gamma-glutamyl transferase (Ggt1), and the Fet3/Ftr1 iron reductase. Within the phagosome, a high affinity zinc transporter (Zrt2) aids in zinc acquisition and de novo biosynthesis provides intramacrophage yeasts with pyrimidines and essential vitamin cofactors (riboflavin and pantothenate) to meet metabolic needs

formation in humans is a bit more difficult to determine; specific immune responses are assumed to be initiated between 7 and 18 days post exposure and once activated, formed granulomas begin to resolve often resulting in the lesions being encapsulated and then calcifying [105]. In an immunosuppressed host undergoing granuloma dissolution, previously trapped fungi may be released and start replicating again.

5.3.2 Innate-Adaptive Immunity Connections

5.3.2.1 Monocytes/Macrophages

Monocytes/macrophages, along with DCs, assist in the initiation of the adaptive response. Macrophages may promote a Th1 response through cytokine release and presentation of antigen to CD4+ T cells, to act in concert with the stimuli provided

by the DCs [99, 106, 107]. Although macrophages are initially permissive to *Histoplasma*, ultimately, macrophages are responsible for clearance of *Histoplasma* infection. Once the adaptive response has been initiated, T cells produce Th1 cytokines such as IFNγ, which impel macrophages to become the final effector cells, enabling them to kill or inhibit the growth of the *Histoplasma* [99]. IFNγ is likely acting by stimulating NO release, which inhibits *Histoplasma* growth through the chelation of iron, the effects of which can be reversed by adding iron to in vitro cultures [99, 108, 109].

Human and mouse macrophages differ in their responses upon ingestion of *Histoplasma*. Human macrophages produce a vigorous ROS response but no phagolysosomal fusion, whereas murine macrophages exhibit phagolysosomal fusion [99]. While most studies have demonstrated little effect of IFNγ on human macrophages in vitro [72, 99], Brummer et al. found in human monocyte-derived macrophages, short-term growth inhibition may be enhanced by the addition of IFNγ [110]. Aside from proinflammatory cytokines, human macrophages do exhibit enhanced phagolysosomal fusion when they are able to adhere to collagen [72]. The mechanisms of activation may differ between human and mouse macrophages, but ultimately both are able to induce fungistasis.

5.3.2.2 Dendritic Cells

DCs recognize *Histoplasma* surface-exposed cyclophilin A via very late antigen 5 (VLA-5) [111, 112]. Unlike macrophages, human DCs are able to directly kill *Histoplasma* without activation by the adaptive immune response. Upon phagocytosis, human DCs kill *Histoplasma* yeasts via lysosomal hydrolases after phagolysosomal fusion occurs [113]. The killed yeasts are degraded and antigenic peptides are generated that can activate T cells while the DCs are also making IL-12 prompting Th1 differentiation. Both MyD88 and TLR 7/9 are essential for infected resident DCs to facilitate proper T cell activation and IFNγ production [45, 114]. Additionally, DCs have been reported to ingest *Histoplasma* infected macrophages and produce antigenic epitopes that are cross-presented to $CD8^+$ T cells [115]. DCs stimulate both $CD4^+$ and $CD8^+$ T cells, making them central in the defense against *Histoplasma*.

There are many described DC subsets. Two of these have important roles during *Histoplasma* infection. $CD8^+$ DCs are potent initiators of a proinflammatory Th1 response in response to *Histoplasma*. They are able to prime both $CD4^+$ and $CD8^+$ T cells and produce IL-12 thereby activating a strong Th1 adaptive response [116]. Additionally, these DCs are able to cross-present antigen: phagocytosing extracellular particles, that would normally be processed through the MHCII pathway, and utilizing the MHCI pathway to present the antigens on their surface to CD8+ T cells [116].

The CD103+ DC subset has also been shown to have a significant role in *Histoplasma* infection. They are the major producer of type I IFN in response to *Histoplasma* infection of the lungs, and Van Prooyen et al. demonstrated type I IFN is required for successful restriction of fungal growth by DCs. Similar to the facilitation of the IFNγ response by DCs, production of type I IFN TLR 7/9 [114].

Contradictory data have been published that type I interferons may actually be deleterious to host resistance since type I IFN receptor deleted animals restricted growth [117].

5.3.3 Important Cytokines and Their Effects

Cytokines produced by innate and adaptive cells that encounter *Histoplasma* are crucial for controlling fungal infection. The proinflammatory cytokines TNFα, IFNγ, GM-CSF and IL-17/23 are all vital for host defense, enabling clearance of the infection. IL-4, IL-10, and IL-33 on the other hand impede an effective host response resulting in enhanced fungal growth. Deficiencies and the interplay of these cytokines determine the outcome of infection (Fig. 5.3).

5.3.3.1 TNFα

TNFα is one of the earliest cytokines induced by *Histoplasma* and it is fundamental in primary *Histoplasma* infection [118]. TNFα is required in primary and secondary infection in mice for successful clearance and neutralization results in accelerated mortality in mice [119, 120]. TNFα signaling is necessary for successful defense as TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice manifest a high mortality rate, with the former much more susceptible to this fungal pathogen than the latter [121]. The importance of TNFα in human *Histoplasma* infections has been highlighted with the advent of TNFα blocking drugs and the resulting rise in disseminated *Histoplasma* infections (discussed further below).

TNFα combats *Histoplasma* infection in two ways. Neutralization of TNFα is associated with a decrement in CD8+ T cells in the lungs, suggesting it is involved in the recruitment and/or expansion of CD8+ T cells [122, 123]. An absence of TNFα also diminishes NO production in primary infection [119, 120]. In secondary infection, TNFα deficiency impairs fungal clearance by skewing the response towards Th2, and the negative effects can be rescued by also neutralizing IL-4 and IL-10 [119].

5.3.3.2 IFNγ

IFNγ is the necessary defensive cytokine against *Histoplasma* infection. It is produced by T cells and acts on macrophages to induce fungistasis. IFNγ production depends principally on IL-12, which is upregulated within 3 days of infection. After initiating the IFNγ response during this time period, IL-12 is dispensable for host defense [65, 108, 124]. Neutralization of IL-12 at the start of infection leads to accelerated mortality, as its absence results in no induction of IFNγ and therefore no protective effect by this cytokine [125]. While IFNγ is vital for survival of a primary

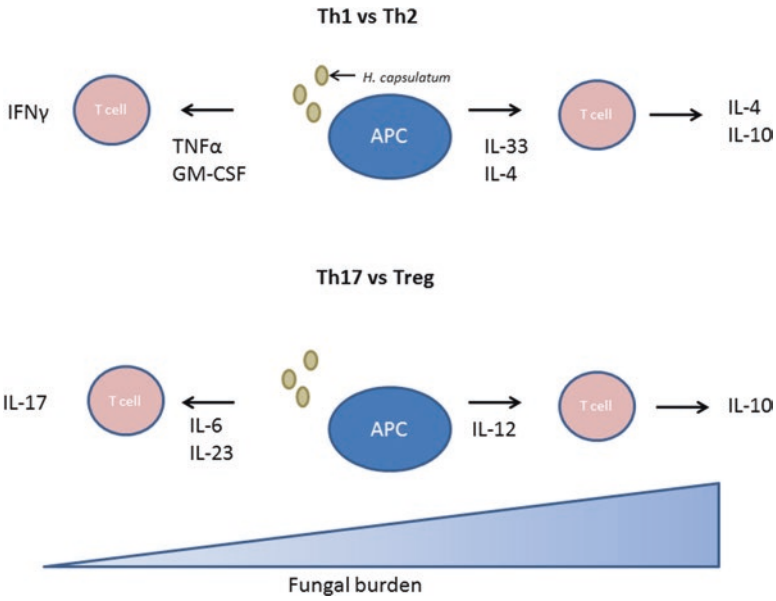

Fig. 5.3 Balancing the T cell response: the good vs. the bad. Different T cell subsets have varied effects on a *Histoplasma* infection. The antigen-presenting cells (APCs) that encounter the yeasts produce cytokines that polarize naïve T cells. Immunocompetent hosts will generate Th1 and Th17 T cells, producing IFNγ and IL-17 respectively, that enable the host to clear the infection. Conversely, hosts skewed towards a Th2 or Treg response have elevated levels of IL-4 and/or IL-10 that impairs their ability to clear the infection

infection in a mouse model, the animal is able to survive a secondary infection without IFNγ, at least in part due to increased TNFα levels [120], suggesting there is some overlap in efficacy with other proinflammatory cytokines able to substitute for the lack of IFNγ.

IFNγ is crucial for defense against *Histoplasma* in both the mouse model and human infections. Most in vitro studies have shown IFNγ does not activate human monocytes/macrophages to inhibit intracellular growth of *Histoplasma*, yet there is evidence that IFNγ is still involved in clearing human *Histoplasma* infections. Human deficiency in the IFNγ receptor is a risk factor for refractory and recurrent disseminated histoplasmosis [126]. Furthermore, Sampaio et al. described patients with dominant STAT1 gain-of-function mutations and disseminated histoplasmosis. Stimulation of IFNγ receptors leads to phosphorylation of STAT1, which homodimerizes and heterodimerizes before translocating to the nucleus and activating interferon-induced genes. These patients with delayed dephosphorylation of STAT1 likely have decreased ability to respond to IFNγ [127]. While IFNγ may not be required for the final macrophage activation step in humans, it is involved in the initiation of the Th1 response and a lack of IFNγ may allow for a stronger Th2 response and therefore impaired fungal clearance.

5.3.3.3 GM-CSF

GM-CSF is another critical cytokine for control of *Histoplasma* infections. Neutralization of it during primary infection increases mortality in association with fewer CD4+ and CD8+ T cells in the lungs, a decrement in TNFα, IFNγ and NO, and an elevation in IL-4 and IL-10 [128]. Similar to IFNγ, neutralization during secondary infection exacerbates infection but does not increase mortality [128]. This result indicates TNFα is the primary cytokine during secondary infection. As discussed earlier, GM-CSF induces nutritional immunity against *Histoplasma* by lowering the levels of intracellular iron and zinc in macrophages and thereby inducing fungistasis [90]. GM-CSF sequesters zinc by upregulating zinc exporters in macrophages and enhancing binding of free zinc to metallothioneins thereby preventing its use by *Histoplasma* [91]. GM-CSF neutralization, and the resulting skew towards a Th2 response, results in more free zinc and an increase in fungal growth.

5.3.3.4 IL-17/23

IL-17 has a role in optimal clearance of *Histoplasma*. Neutralization of IL-17A impedes efficient fungal clearance, however does not lead to progressive infection [129]. In the absence of IL-12, and therefore IFNγ, IL-23 extends survival through an IL-17-dependent mechanism [129]. Furthermore, Galectin-3 negatively regulates IL-17A production; when mice are deficient in Galectin-3, they have enhanced fungal clearance as increased IL-23 production by DCs skews the response towards a Th17 response and away from a Th1 response [130]. There is an optimal balance between Th1 and Th17 responses for clearance of infection; augmenting the Th17 response enhances clearance and determining how to boost IL-17 may aid in the development of treatments for this infection.

5.3.4 Impaired Immunity

IL-4, IL-10, and IL-33 are all detrimental to resolving *Histoplasma* infection. Most individuals with an intact immune system will respond with the appropriate cytokines to infection; however, perturbations to the immune response may skew towards a detrimental Th2 response.

5.3.4.1 IL-4 and IL-33

Infection by *Histoplasma* induces a Th1 response; yet when a Th2 response develops concurrently there is poor control of fungal burden. IL-4 is a Th2 inducing cytokine produced by leukocytes such as eosinophils, basophils and group 2 innate lymphoid cells (ILC2) [131]. CCR2−/− mice exhibit defective inflammatory

monocyte recruitment and an influx of eosinophils producing IL-4, skewing their response towards Th2 and resulting in elevated fungal burdens and increased mortality [132]. The CCR2 ligands, CCL2 and CCL7, diminish the Th2 response by constraining IL-4 generation, likely through reducing eosinophil influx [116]. One mechanism of action exerted by IL-4 to negatively affect the outcome of *Histoplasma* infection is by enhancing zinc transport into macrophages [90]. IL-4 also acts to suppress GM-CSF and IFNγ signaling, thereby subduing the antifungal response of the host [128, 133, 134].

Traditionally IL-33 is a precursor to IL-4 [135]. In *Histoplasma* infection the opposite occurs, with IL-4 appearing first and driving IL-33 production in the $CCR2^{-/-}$ model mouse [136]. These mice have elevated fungal burdens that are resolved by neutralizing IL-33 [136]. This suggests that while IL-4 has multiple known roles for subverting an effective Th1 response to *Histoplasma*, in the CCR2−/− model, IL-33 is responsible for the exacerbated infection.

5.3.4.2 IL-10

IL-10 inhibits the antimicrobial effect of phagocytes and dampens T cell activation [137]. GM-CSF in primary *Histoplasma* infection and TNFα in secondary infection suppress IL-10, enabling the Th1 response to effectively control the infection [119, 128]. An absence of IL-10 aids clearance as IL-10−/− mice resolve both primary and secondary *Histoplasma* infections more quickly than wild-type mice [138]. This effect is independent of increases in protective cytokines during the adaptive phase; nonetheless, endogenous IFNγ or TNFα is required for clearance in these animals in a primary infection whereas TNFα is sufficient in a secondary infection [138].

It has recently been demonstrated that HIF-1α is a negative regulator of IL-10 expression in macrophages. A myeloid-specific $HIF\text{-}1\alpha^{-/-}$ mouse model exhibits increased IL-10 levels and elevated fungal burdens leading to increased mortality [139]. IL-10, like IL-4, impedes successful resolution of *Histoplasma* infection, but suppression by Th1 cytokines and transcriptional regulation prevent this cytokine from being produced in a normal response.

5.3.5 Primary Immunity

5.3.5.1 Th1 cells

$CD4^+$ T cells are required for defense against *Histoplasma*. Without them mice die within 2 weeks from a sublethal challenge [123]. These cells are the main IFNγ producers and a lack of $CD4^+$ T cells results in a dramatic decrease in IFNγ levels and therefore no activation of macrophages and no control of *Histoplasma* growth [122, 140]. Antigens are recognized through the T cell receptor (TCR) and the variable element of the β chain (Vβ) allows for many different epitopes to be recognized.

In a primary infection $CD4^+$ $V\beta4^+$ T cells are required to defend against *Histoplasma*, they expand during the peak and early resolution phases of infection and depletion of this subset hinders optimal clearance [141–143]. This suggests a dominant *Histoplasma* antigen is responsible for driving expansion of a single T cell subset.

$CD8^+$ T cells are necessary for optimal clearance of *Histoplasma*, but unlike $CD4^+$ T cells, they are not required. While loss of $CD8^+$ T cells is associated with increased CFUs and depletion of this subset delays clearance, mice survive and eventually recover [123]. Like $CD4^+$ T cells, $CD8^+$ T cells produce IFNγ, but they additionally generate granzyme to kill infected cells as an additional mode of defense [116]. $CD8^+$ T cells recognize antigens presented by DCs on their cell surface from ingested macrophages infected with *Histoplasma* and also act directly against infected macrophages through perforin-mediated killing, thereby destroying a site of replication for the fungus [115].

5.3.5.2 Th17 Cells vs. Regulatory T Cells

The Th17–Treg balance is tightly regulated. While Th17 cells facilitate elimination of *Histoplasma*, an excess of Tregs is likely to dampen the inflammatory response, preventing the host from recruiting cells to properly fight the infection. Nevertheless, Tregs are essential for preventing uncontrolled inflammation and are a key cell type for dampening the inflammatory response once the infection has been cleared. Th17 cells and Tregs exist in a balance; while both are promoted by TGF-β, the Th17 inducing cytokine IL-6 restrains Treg development and Tregs themselves suppress Th17 cells [144]. Reduced numbers of Tregs, such as found in $CCR5^{-/-}$, results in an increase in Th17 cells and enhanced *Histoplasma* clearance [145]. Although Th17 cells are dispensable when it comes to defense against *Histoplasma* in a normal mouse, they augment clearance in mice where the balance of Tregs and Th17 cells is skewed towards Th17.

While fewer Tregs have a positive outcome on infection resolution, more Tregs do not necessarily mean impaired resolution. Animals supplemented with zinc exhibit significant skewing towards Tregs and away from Th17 cells yet have no change in fungal burden when compared to animals that were not supplemented with zinc [146]. These animals have fewer Th17 cells, but their IFNγ producing Th1 cells were unaffected and still able to combat infection efficiently [146]. Tregs induced by zinc supplementation do not impair immunity to *Histoplasma* infection, yet in animals where TNFα has been neutralized there is a population of Tregs that dampens antigen-specific immunity to *Histoplasma* and results in elevated fungal burden [147]. Different subsets of Tregs have different roles during *Histoplasma* infection and dysregulation of these subsets leads to very different outcomes.

5.3.6 Secondary Immunity

5.3.6.1 Memory T Cells

As in the primary response, secondary infection resistance is dominated by T cells. In the latter, protective immunity is hindered only when both $CD4^+$ and $CD8^+$ T cells are depleted, elimination of a single subset does not enhance mortality [122, 148]. The $V\beta4^+$ T cell subset remains important, but there is also a significant role for $V\beta6^+$ T cells. Simultaneous depletion of $V\beta4^+$ and $V\beta6^+$ T cells impairs fungal clearance in secondary infection [142].

Vaccine based immunity to *Histoplasma* requires the generation of memory T cells. The potent *Histoplasma* antigen, Hsp60, has been successfully used to vaccinate mice; with recombinant Hsp60 (rHsp60) conferring protection against lethal challenge [36, 149]. There are variations in the subset of T cells that expand depending on if mice are immunized with a fragment or the whole Hsp60 protein. $CD4^+$ $V\beta6^+$ T cells make up approximately 90% of the T cell clones in mice vaccinated with the immunoactive fragment 3 (F3) of Hsp60, their presence is accompanied with elevated levels of IFNγ, TNFα and GM-CSF, and depletion of these cells abrogated protection [148]. Alternatively, immunization with rHSP60 results in $V\beta8.1/8.2^+$ T cells preferentially expanding and protects from a subsequent challenge [143] In the absence of $CD4^+$ T cells, $CD8^+$ T cells are able to confer vaccine protection, through MHC I and TNFα production [150]. Alternatively the vaccine-based immune response to *Histoplasma* is dependent upon Th17 while Th1 immunity was dispensable [151]. Being able to induce a vaccine response from $CD8^+$ T cells and/or Th17 T cells is imperative as some of the most vulnerable to this fungus are immunocompromised and lack a $CD4^+$ response.

5.3.6.2 Role of B Cells and Antibodies

While DCs, T cells, and IFNγ-activated macrophages are fundamental in protection against *Histoplasma*, the importance of B cells during an in vivo infection is more difficult to determine. Since humoral immunity works in conjunction with cell-mediated immunity, experimental models to determine the efficacy of serum or a specific antibody independently are convoluted [152]. B cell deficient animals do not manifest a difference in fungal burdens compared to wild-type animals [122], but this does not necessarily mean that there are no protective antibodies conferring protection. Specific monoclonal antibodies can be used to augment protective immunity; phagocytosis and phagosomal maturation are enhanced when yeasts are opsonized by antibody and they interfere with the ability of *Histoplasma* to control the phagosomal pH, thereby facilitating killing of the fungus and prolonging survival in a mouse model [37, 56, 153]. The antibodies produced in vivo will not be monoclonal, they will be responsive to a wide variety of epitopes, some may be protective but in small numbers while others may be nonprotective and able to interfere with the

protective antibodies [152]. Furthermore, the isotype is also very important, with IgG1 and IgG2a being protective against *Histoplasma*, but not IgG2b [37]. Cell-mediated immunity is undoubtedly vital, and while the role of B cells and antibodies is still undetermined, there is evidence suggesting a protective role.

5.3.7 Perturbations to the Immune Response

Most individuals infected with *Histoplasma* resolve the infection. Less than 1% of those infected are even aware they have been infected [12]. Immunocompromised patients, which are unable to mount an effective cell-mediated immune response to *Histoplasma*, have a difficult time resolving an infection upon exposure and frequently develop disseminated disease. Patients with AIDS, solid organ transplant, Hodgkin's disease, chronic and acute lymphocytic leukemia, and those receiving TNFα blockers or corticosteroids are all at increased risk for developing disease [12]. Immunocompromised hosts develop disease in multiple organs through a primary infection or through reactivation of a previous infection [154]. Older adults are also at risk for chronic progressive disseminated histoplasmosis. This is generally slowly progressive and an often fatal infection that occurs in older adults who are not overtly immunocompromised [155–157]. Understanding why these conditions increases the risk for contracting histoplasmosis allows insight into the immune response to this fungus.

5.3.7.1 HIV/AIDS

The hallmark of AIDS is a low $CD4^+$ T cell count. As these cells are required for successful clearance of *Histoplasma*, AIDS patients infected with *Histoplasma* are more likely to develop disease. In fact, the diagnosis of progressive disseminated histoplasmosis was one of the early definitions of AIDS. With the advent of successful antiretroviral therapy, there has been a significant decrease in the number of disseminated histoplasmosis cases in the United States, nevertheless HIV-associated histoplasmosis hospitalizations remain significant [12, 158]. Areas with poor access to antiretroviral drugs, such as Central America, have a considerable problem with disseminated histoplasmosis in AIDS patients [159]. Those patients with $CD4^+$ T cell counts of less than 150 cells/μL are most at risk [12]. When correctly diagnosed, treatment with antifungals resolves infection in these patients [160].

5.3.7.2 Solid Organ Transplants/Immunosuppression/Corticosteroids

Patients on immunosuppressive drugs and/or corticosteroids after receiving a solid organ transplant (SOT) are at increased risk of developing disseminated histoplasmosis. The first year after transplant is the period of highest risk, with the median

time from transplant to diagnosis around 27 months [161, 162]. A year of antifungal treatment successfully resolves the infection, with rare relapse [161]. SOT patients with human leukocyte antigen HLA-B37 may be at slightly higher risk for disseminated histoplasmosis, though [163] did not find statistical significance in their small sample size [163]. SOT patients on immunosuppressant drugs that live in endemic areas may develop a new infection or if they had previously infection that was contained in granulomas, the fungi may escape due to the drugs causing reactivation.

5.3.7.3 TNFα Blockers

While the development of TNFα blockers has been salutary for many with conditions like rheumatoid arthritis and plaque psoriasis, they elevate the risk of developing histoplasmosis. *Histoplasma* complications have been found to be more common in patients taking anti-TNFα monoclonal antibodies than in those taking a soluble TNFα receptor [164]. The monoclonal antibody infliximab neutralizes TNFα, but may also disrupt granulomas, resulting in previously sequestered *Histoplasma* being released and preventing the necessary TNFα response to resist infection [165]. Antifungal therapy for 12 months while stopping TNFα blocker therapy resolves infection in most cases. Resumption of TNFα blocker therapy is safe following successful antifungal treatment, as long as patients are closely clinically monitored [166]. An alternate method of action is enhanced activity and increased number of regulatory T cells upon treatment with TNFα blockers, thereby suppressing inflammation and enabling the *Histoplasma* to escape confinement [167]. Blocking TNFα increases susceptibility for developing histoplasmosis and yet only a small percentage of patients on these drugs in endemic regions develop infections, suggesting some patients are less able to compensate for the loss of TNFα than others. They may be unable to increase IFNγ or GM-CSF levels to counteract the lack of TNFα or have an underlying additional immunodeficiency.

5.4 Clinical Management of Histoplasmosis

5.4.1 Treatment

Most *Histoplasma* infections resolve without treatment. For the more vulnerable immunocompromised patients and the rare immunocompetent patients that develop histoplasmosis, the current Infectious Diseases Society of America (IDSA) guidelines are to treat with a combination of liposomal amphotericin B and itraconazole [168]. Other azoles, including: fluconazole, posaconazole, isavuconazole, and voriconazole, exhibit activity against *Histoplasma* and provide additional options for patients intolerant of itraconazole [168]. However, fluconazole is much less effective than other azoles, and clinical studies with isavuconazole are limited. The echinocandins class of antifungals, which includes caspofungin, are not recommended for treatment as they have minimal effect on Histoplasma yeasts [169, 170].

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Chapter 6
Cryptococcus and Cryptococcosis

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Abstract Cryptococcosis is a fungal disease caused by *Cryptococcus neoformans* and *C. gattii*. Lung disease and meningocephalitis are the most common clinical outcomes of cryptococcosis, especially in immunosuppressed patients. Current estimates suggest 278,000 cases of human cryptococcal meningitis annually. This syndrome is fatal if untreated. Treatment of cryptococcosis is expensive and poorly effective. This complex scenario makes clear the need for innovation in the field of *Cryptococcus* and cryptococcosis.

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Cryptococcosis is an infectious disease with a worldwide distribution that is commonly caused by two pathogenic encapsulated fungal species: *Cryptococcus neoformans* and *Cryptococcus gattii*. Historically, the *Cryptococcus* genus has been classified into three varieties and five serotypes based on the antigenic determinants of the capsular polysaccharide: *C. neoformans* var *grubii* (serotype A), *C. neoformans* var *neoformans* (serotype D), *C. neoformans* (hybrid AD), and *C. gattii* (serotypes B and C) [1]. To date, molecular methodologies have established at least eight different molecular types for the *C. neoformans*/*C. gattii* complex, and these molecular types correlate with specific serotypes. For *C. neoformans,* these classifications include VNI/AFLP1, serotype A; VNII/AFLP1A, serotype A; VNIII/AFLP3, serotype AD; and VNIV/AFLP2, serotype D. The molecular types of *C. gattii* (VG1/AFLP4; VGII/AFLP6; VGIII/AFLP5; and VGIV/AFLP7) are all associated with both serotype B and serotype C [2].

The first clinical case describing a *Cryptococcus* species as the etiological agent of a fatal disease dates from the late nineteenth century [3]. Nearly one million cases of cryptococcal meningitis occurred in 2006, resulting in approximately 625,000 deaths within 3 months after infection [4]. Estimates of the global incidence of AIDS-related cryptococcal meningitis have been decreasing based on early diagnosis and improvements in antifungal treatment in some countries [5]. However, cryptococcosis remains the most fatal mycosis among AIDS patients worldwide [4].

Cryptococcal infection occurs primarily via the inhalation of fungal particles, which reach the lungs and can disseminate to the brain in immunocompromised hosts. Although an immunosuppressive condition is often associated with cryptococcosis, healthy hosts may also develop the disease, primarily through *C. gattii* infection [6].

Both *C. neoformans* and *C. gattii* display several virulence factors that modulate their interactions with their hosts. The most studied among these virulence factors are the ability to survive at mammalian body temperature, enzyme secretion, the addition of melanin to the fungal cell wall, and the abundant secretion of polysaccharides that may surround the cell to form a capsular structure [1]. All of these virulence factors appear to interact with the human host in a deleterious manner, subverting the immune response or protecting the fungus from immune effector molecules.

Clinical presentations may vary from self-limiting cutaneous infections to fatal meningoencephalitis. Amphotericin B, flucytosine, and fluconazole are recommended for treatment, although the high costs, wide array of side effects, and drug resistance that are associated with these drugs represent problems in health policy implementation [7]. Efforts to identify new fungal therapeutic targets and improve existing antifungal treatments are necessary.

Central aspects related to *Cryptococcus* spp. and cryptococcal infection will be discussed in more detail below.

6.1 Recent Data Related to the Epidemiology of Cryptococcosis

6.1.1 Clinical Epidemiology

In the early twentieth century, the occurrence of cryptococcosis was sporadic, with less than 300 cases reported before the 1950s [8]. In the 1970s, the number of cases increased due to the association of the infection with organ transplantation, immunosuppressive treatments, and malignancy [9]. The emergence of cryptococcosis as an important systemic mycosis worldwide came with the emergence of acquired immunodeficiency syndrome (AIDS) in the 1980s. In fact, the major risk factors for cryptococcosis are HIV infection, immunosuppressive therapies, solid organ transplantation, hematologic malignancy, sarcoidosis, treatment with some monoclonal antibodies or anti-granulocyte-macrophage colony stimulating factor (GM-CSF) antibodies, systemic lupus, rheumatoid arthritis, CD4+ lymphopenia, decompensated chronic liver disease, renal failure, peritoneal dialysis, hyper IgM or IgE syndrome, and tuberculosis [10].

Epidemiologic differences are observed between the agents of cryptococcosis. Most patients infected with *C. neoformans* are immunocompromised, with a few cases reported in individuals without an immunodeficiency-related condition. In contrast, *C. gattii* affects primarily immunocompetent individuals. Another important difference between these two species is that *C. neoformans* infections occur worldwide, while *C. gattii* shows some endemic areas of occurrence [8]. Epidemiological studies have demonstrated a lower incidence for *C. gattii* infections than *C. neoformans* infections. In 1996, only 3 of 248 cases of cryptococcosis were attributed to *C. gattii* (serotype B) in HIV-negative individuals in the US [1]. *C. gattii* isolates have a restricted global distribution, with infections occurring in tropical and subtropical areas. However, some studies have attributed some *C. gattii* outbreaks in uncommon areas, such as Canada and areas in the US that are outside of California, to climate changes [3]. To illustrate this point, in 2006, British Columbia reported an average annual incidence of 5.8 per million persons, while endemic areas in Australia confirmed 0.61 *C. gattii* cases per million. This figure represented the highest incidence of *C. gattii* infection in the world [11].

Infections caused by *C. neoformans* are largely associated with HIV infection [9]. In 2006, Park and colleagues estimated that nearly one million cases of cryptococcal meningitis occur annually in this group of patients, with a mortality rate higher than 60%. Thus, cryptococcal meningitis is one of the leading causes of death in HIV/AIDS patients in Africa, accounting for more deaths than tuberculosis [4]. Current estimates cite 278,000 cases of cryptococcal meningitis annually, and this infection is associated with 100% mortality in untreated AIDS patients [5]. The total death counts were recently 233,000. This reduction can be explained by improved treatment for established cases, with the WHO recommending therapy with amphotericin and flucytosine [3], as well as the detection of asymptomatic antigenemia and oral treatment with fluconazole after excluding meningitis [5].

The majority of these affected patients are from sub-Saharan Africa, which is a region with several difficulties in terms of the diagnosis and treatment of this disease [12]. Primary cutaneous cryptococcosis can occur in both immunocompromised and immunocompetent individuals living in poverty [13]. Pulmonary cryptococcosis is usually seen in immunocompetent individuals but can also occur in immunosuppressed patients, with further dissemination to the central nervous system [14]. The real burden of cryptococcosis caused by *C. neoformans* in at-risk patients without HIV is not well-recognized [12].

The lack of *C. neoformans/C. gattii* differentiation by most clinical laboratories worldwide makes the knowledge of cryptococcosis caused by *C. gattii* inaccurate [15]. In general, most affected individuals are immunocompetent, including a significant number of children [16]. The major endemic areas of this pathogen are Australia, Botswana, Brazil, Canada, Papua New Guinea, South Africa, the United States of America, and Vietnam [15]. The major manifestations of cryptococcosis caused by *C. gattii* in humans are meningoencephalitis and pulmonary disease [17]. Most authors report the central nervous system as the major site of infection by *C. gattii*. However, in North American outbreaks, pulmonary disease predominates [15].

6.1.2 Ecoepidemiology

Current knowledge of *C. neoformans* and *C. gattii* ecology shows that these species do not have specific habitats and instead inhabit diverse niches and substrates in the regions where they can be found [18]. Although both species are ubiquitous yeasts, their main ecological niches have some differences. For instance, *C. neoformans* is usually isolated from pigeon droppings, while *C. gattii* is more frequently isolated from decaying wood and soil [19]. However, both species can be isolated from household dust [20].

The occurrence of *C. neoformans* in soil and pigeon droppings has been widely studied. Poultry manure is considered to be a natural substrate for *C. neoformans*. Several birds, especially *Columba livia* pigeons, can bear this fungus on their beaks, legs, and feathers, as well as present colonization by *Cryptococcus* on the crop. These birds act as fungal dispersers in the environment, generating a source of infection for humans and other mammals [21]. Other animals, such as ants (*Odontomachus bauri*), can also harbor *C. neoformans* and contribute to its dispersal in the environment [22].

One or both species of *Cryptococcus* have been found in different trees from around the world. Some species from which *C. neoformans* has been isolated include *Tipuana tipu*, *Tabebuia avellanedae*, *Thuja* sp., *Peltophorum dubium*, *Cassia grandis* and *Eucalyptus* trees [23]. *C. gattii* was first isolated from *Eucalyptus* trees in Australia, and for several years, these trees were thought to be the habitat of this species. However, at the end of the last century, *C. gattii* began to be isolated from several other trees around the world, and attempts to isolate this species from *Eucalyptus* trees in other countries were unsuccessful. Some species that have been reported to harbor *C. gattii* include *Acacia visco*, *Cassia grandis*, *Guettarda acreana*, *Ficus soatensis*, and *Pinus* spp. [24].

6.1.3 Molecular Epidemiology

With the advance of molecular methodologies and their use in medical mycology, isolates of *C. neoformans* and *C. gattii* were separated into eight different molecular types: for *C. neoformans,* VNI-VNIV, and for *C. gattii,* VGI-VGIV. This improvement in *Cryptococcus* classification clarified several epidemiological aspects of these species, since they exhibit differences in habitats, antifungal susceptibility, clinical manifestations, virulence, antifungal susceptibility and epidemiology [18]. Despite the importance of the molecular typing of *Cryptococcus* species, molecular data are unavailable in several countries in Africa, Asia, and Eastern Europe, as well as the United Kingdom, Ireland, Norway, and Finland [25].

C. neoformans VNI is the most frequent molecular type worldwide, except in Australia and Papua, New Guinea, where *C. gattii* VGI predominates. VNII is less frequent, but has been isolated on all continents. The molecular type VNB was thought to be exclusive to Africa, but this type has been also found in Brazil and Colombia. The frequencies of VNIII and VNIV are very low on all continents, except for Europe, where they account for 18.5% and 18.3% of the *Cryptococcus* strains [25].

As reviewed by Chen [15], *C. gattii* VGII is the most frequently isolated molecular type from environmental samples, followed by the molecular types VGI, VGIII, and VGIV, which is rarely isolated. In clinical samples obtained from humans or animals, however, the frequencies of the molecular types VGI and VGII are very similar; together, these types account for 77% of all *C. gattii* isolates. The frequencies of the molecular types VGIII and VGIV in clinical samples are lower but very similar to each other (13% and 10%, respectively). The geographic distribution of these molecular types is also heterogeneous; the VGII molecular type predominates in the Americas, VGI is most prevalent in Europe, Asia, and Oceania, and VGIV predominates in Africa.

The relevance of *C. gattii* VGII as an emerging pathogen was demonstrated during the Vancouver Island outbreak [2]. The initial molecular analysis of this highly virulent molecular type revealed that these fungi originated in Australia or South America [26]. Further studies confirmed South America as the origin of *C. gattii* VGII dispersal. It was initially proposed that the Amazon rainforest was the original source of *C. gattii* VGII [27], but recent studies changed the origin of this molecular type to the semi-arid desert region in Northeast Brazil [28].

A novel study of *C. gattii* VGIII that used multi-locus sequence typing and whole-genome sequencing revealed that this molecular type is highly diverse genetically, with minor differences between geographic origins, sources of isolation, serotypes, and mating types. This molecular type has two subpopulations, which comprise serotypes B and C of *C. gattii*. The origin of the serotype B VGIII population, which is more virulent, is very likely to be Mexico and the USA, while serotype C VGIII originated in Colombia [29].

6.2 Clinical Aspects of Cryptococcosis

Cryptococcal infection occurs via the inhalation of environmental infectious propagules, resulting in initial lung colonization. In general, the presence of yeast cells in the lung elicits an immune response that results in a lymph node complex [30]. An effective immune response is able to eradicate the fungi, leading to the clearance of lung infection [9]. Asymptomatic cryptococcal infection is prevalent in certain geographic regions, as indicated by serologic analysis [31]. However, depending on the inoculum, the immune status of the host, or the virulence of the strain, the infection may progress to acute disease or become latent [32]. In latent infections, the individual is asymptomatic, although *Cryptococcus* remains alive inside macrophages in the thoracic lymph node, where the fungus can persist for years. When local immunity is suppressed, the dormant yeasts begin to grow, multiply, and disseminate outside of the pulmonary-lymph node complex, leading to disease. Immunosuppressive conditions, such as HIV coinfection and organ transplantation, represent potential risks for the development of acute cryptococcosis from latent infection [9]. The reactivation of latent infections upon immunosuppression is observed more frequently in *C. neoformans*/HIV coinfection, while *C. gattii* is primarily responsible for acute infection in immunocompetent individuals [1].

After cryptococcal cells have reached the alveolar space and come into contact with the immune system, the fungi reach the bloodstream, cross the blood-brain barrier and disseminate into the central nervous system (CNS), causing a meningoencephalitis that is life-threatening if left untreated. Some clinical features of cryptococcosis are dependent on the host immune status and the fungal species. Pulmonary cryptococcal disease is more frequent in immunocompetent hosts (60%) and associated with *C. gattii* infection, while meningitis is common in AIDS patients (86%) [33]. In *C. gattii*-associated pulmonary disease, a progressive inflammatory response and cryptococcoma formation are typical, as is concurrent lung and brain infection [11]. The presence of single mass lesions in the lungs and brain has been mistaken for neoplastic disease or pyogenic abscesses [34], and these lesions commonly produce neurologic sequelae that require surgery or prolonged antifungal therapy [35]. In contrast, HIV-associated cryptococcosis presents with outstanding CNS and extrapulmonary involvement, poor inflammatory reactions and a high burden of *C. neoformans,* which is frequently cultured from the blood, urine and skin lesions [11]. Although *Cryptococcus* species have diverse infection profiles, some clinical features, such as fever and pulmonary or neurological symptoms, are similar [11].

Although cryptococcosis most commonly affects the lung and brain, many sites in the body may be involved, presenting a wide array of clinical presentations. The skin, prostate, eyes, urinary tract and bone are some organs that are infected by these fungi, primarily in HIV-associated cryptococcosis [36].

6.2.1 Pulmonary Cryptococcosis

Pulmonary cryptococcosis ranges from asymptomatic infection of the airways to severe pneumonia with respiratory failure. Typically, symptoms include coughing, pleuritic chest pain, fever, dyspnea, weight loss, malaise, and more rarely, hemoptysis [37]. As these symptoms are similar to those of other pulmonary infections, cryptococcosis may have a delayed diagnosis, which can lead to rapid progression and disease dissemination in some cases [38]. The host immune status influences the clinical presentation of pulmonary cryptococcosis. In immunocompetent individuals, infection may be asymptomatic and can occur in approximately one-third of healthy hosts [39]. Occasionally, these hosts can progress from isolated pulmonary infection to disseminated disease, as occurred in the Vancouver Island *C. gattii* outbreak [40]. In contrast, immunocompromised hosts develop symptomatic cryptococcal pneumonia with the presence of interstitial infiltrates or pleural effusions. These conditions can progress rapidly to acute respiratory distress syndrome (ARDS) [41]. Primarily in AIDS patients, CNS dissemination is a natural consequence after pulmonary involvement and is fatal if untreated. Between 10 and 55% of patients with cryptococcal meningoencephalitis develop pulmonary infection [42]. Although immunosuppressive hosts are considered to be at greatest risk of developing cryptococcosis complications, fatal cases and CNS dissemination have been reported in non-immunocompromised groups [43].

In HIV-positive patients, chest radiograph abnormalities include the presence of interstitial infiltrates or pleural effusions, which are often associated with disseminated disease [38]. In immunocompetent individuals, the presence of single or multiple pulmonary nodules or cryptococcomas in the lung is typical of *C. gattii* cryptococcosis, occurring in 77% of cases [11] (Fig. 6.1).

6.2.2 Meningeal Cryptococcosis

Although both *Cryptococcus* species are able to invade the brain and cause life-threatening disease, *C. neoformans* is more frequently associated with CNS cryptococcosis [44]. *Cryptococcus* infects the meninges and the brain parenchyma, leading to an inflammatory process that is responsible for meningoencephalitis. In addition to the typical meningoencephalitis, many other syndromes have been reported in *C. neoformans*-infected patients, including meningitis, encephalitis, ventriculitis, increased intracranial pressure (ICP) and space-occupying lesions (such as cerebral abscesses, cysts and cryptococcomas) [45]. Headache accompanied by neck stiffness and photophobia, fever, memory loss, lethargy, altered consciousness and personality changes are common symptoms in patients with neurological cryptococcosis [45]. Secondary infections of the lungs, skin, eyes and prostate are less frequent [45]. Depending on the host immune status, the signs and symptoms of disease may be chronic and last for months or occur within days. HIV patients present a high

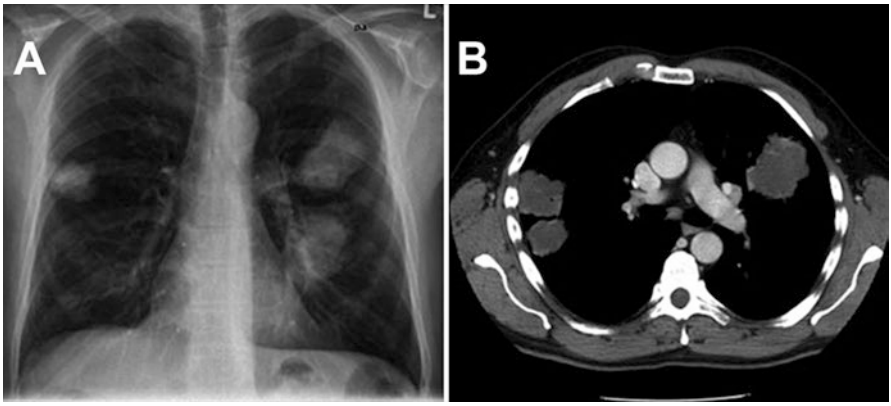

Fig. 6.1 *C. gattii* lung infection. Chest radiograph (**a**) and computed tomographic scan of chest and abdomen (**b**) showing nodular infiltrates near the pleura. Extracted from: Lindberg, J, Hagen, F, Laursen, A, Stenderup, J, Boekhout, T. (2007). *Cryptococcus gattii* Risk for Tourists Visiting Vancouver Island, Canada. Emerging Infectious Disease 13(1): 178–179 DOI: 10.3201/eid1301.060945. Available from https://wwwnc.cdc.gov/eid/article/13/1/06-0945_article

fungal burden, high polysaccharide titers in the CSF (cerebrospinal fluid), elevated ICP, and slow CSF sterilization, even after antifungal treatment initiation. Consequently, these immunosuppressed individuals have a shorter onset of signs and symptoms that may extend for months, leading to death [46]. In contrast, immunocompetent individuals may present subacute or chronic complaints of headaches or altered mental status. Lower rates of fungemia (10%) and mortality (25%) and an intense inflammatory response are common in these hosts [47].

Elevated ICP (>25 cm water) is typical in meningitis caused by both *C. gattii* (50–60% of cases) and *C. neoformans* (30–75% of cases) [44]. This increased ICP is related to the accumulation of cryptococcal polysaccharides, mannitol and/or encapsulated fungal cells, which impairs CSF resorption [48]. The common signs and symptoms of altered ICP include severe headache, hearing loss, seizures, vomiting, altered mental consciousness and lethargy [48]. HIV patients may present with ophthalmologic complications derived from ICP due to underlying meningitis or optical nerve infiltration by yeast cells [49]. In both cases, visual loss occurs, which may be either rapid with few effective treatments during widespread disseminated disease or gradual and reversible with medical management to decrease ICP [9]. In addition, photophobia, diplopia and ocular motility deficits have been reported as common ophthalmologic complaints [49].

Ophthalmoplegia, papilledema, neuroretinitis with or without vitritis and optic atrophy are the ophthalmic complications associated with this infection, and most of these complications are caused by CNS infection [50]. ICP diagnosis is associated with a poor prognosis and neurological sequelae [51].

In general, *C. gattii* has a predisposition to infect the brain parenchyma rather than the meninges and to develop cryptococcomas, which must be considered in the differential diagnostic (Fig. 6.2). These large lesions in the brain may cause symp-

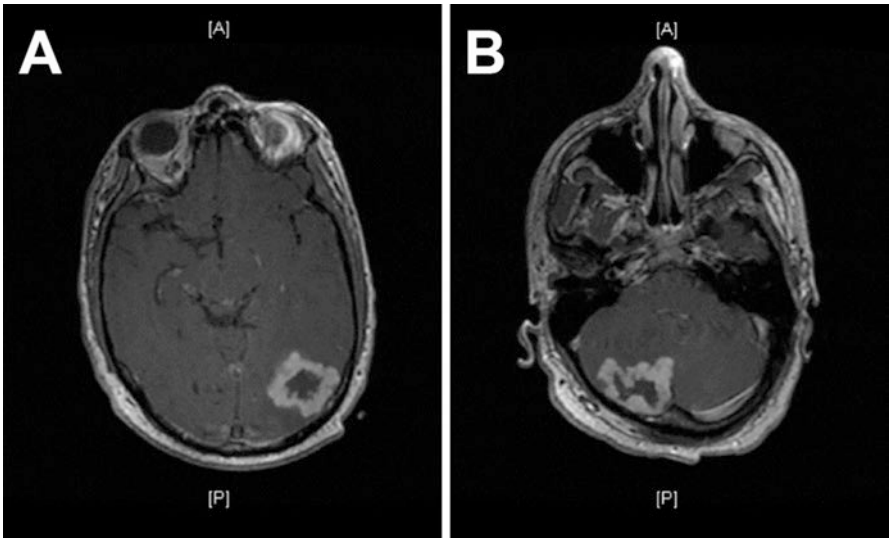

Fig. 6.2 *C. gattii* CNS infection. Magnetic resonance imaging of brain cryptococcomas from a patient with a *C. gattii* infection showing a large mass in left parietal lobe (**a**) and in the right cerebellar hemisphere (**b**). Extracted from: Byrnes EJ III, Li W, Lewit Y, Perfect JR, Carter DA, et al. (2009) First Reported Case of *Cryptococcus gattii* in the Southeastern USA: Implications for Travel Associated Acquisition of an Emerging Pathogen. PLoS One 4(6): e5851. doi:10.1371/journal.pone.0005851

toms that range from headaches to increased ICP or acute obstructive hydrocephalus [44]. Patients who present with this condition typically respond poorly to antifungal therapy, requiring neurosurgical intervention to control pressure or improve treatment efficacy [34].

6.2.3 Cutaneous Cryptococcosis

Cutaneous cryptococcal infection is the third most common clinical manifestation of cryptococcosis, accounting for approximately 10–15% of cases [52]. Skin lesions are found in 5% of patients with cryptococcal meningitis [53]. Local inoculation and dissemination from distant sites of infection represent two ways to establish the disease. Primary cutaneous cryptococcosis is rare and is associated with direct inoculation through a skin injury, which is the main portal of fungal entry and the site at which the lesion will develop. Most cases are observed in older individuals and rural workers who have no underlying disease [54]. A single skin lesion, such as a whitlow or phlegmon, is typically observed and occurs in a sun-exposed area. The absence of fungal dissemination is common in this type of infection. In most cases, the skin lesions are described as ulcers, cellulitis and abscesses that may be located at a site of trauma anywhere on the body [55]. Primary cutaneous infection is caused

by *C. neoformans* serotype D [56], although *C. gattii* has been isolated in cutaneous infections, primarily in tropical and subtropical areas with eucalypt reservoirs [57]. Secondary cutaneous cryptococcosis occurs consecutive to hematogenous dissemination, and immunosuppressive conditions are considered to be risk factors. Skin disease presents with multiple skin lesions, which are distributed in clothed and unclothed body areas and frequently present as papules or macropapules with a central ulceration [58]. Other cutaneous lesions include purpura, vesicles, nodules, cellulitis, abscesses, ulcers, pyoderma gangrenosum-like lesions, molluscum-like lesions and herpes-like plaques, among others [59].

6.2.4 Nonmeningeal, Nonpulmonary Cryptococcosis

Cryptococcus species can cause disease in any organ, primarily in immunocompromised individuals, particularly in those with severely impaired cellular immunity, such as patients with AIDS, lymphoreticular malignancy or immunosuppression after corticosteroid therapy or organ transplantation.

Cryptococcal eye infection is rare and a consequence of disseminated disease. However, conjunctivitis, iritis, choroditis, chorioretinitis, vitritis, endophthalmitis and intraocular cryptococcomas have been reported [60]. HIV patients are the patients most frequently affected by ophthalmologic complications, although some cases involving immunocompetent individuals have been reported [61].

Prostatic infection by *C. neoformans* is also rare and is associated with systemic cryptococcosis in immunocompromised individuals [62]. This infection is usually asymptomatic and may result from fungal dissemination from the CNS and/or the lungs or occur due to descending urinary spread [63]. Although primary prostatitis has been reported infrequently, a latent infection of the prostate may develop into disseminated cryptococcosis after urologic surgery on the prostate or other genitourinary manipulation, as reported previously [64]. Thus, the prostate is considered to be a reservoir of infection from which systemic relapse may occur [65]. Even while undergoing appropriate antifungal therapy, AIDS patients have positive cultures from urine and seminal fluid, suggesting that the fungus persists at this site [66]. Patients with prostatic cryptococcosis present high levels of prostate-specific antigen (PAS), which represents an important inflammatory process and may help track therapeutic responses [63]. In addition to the prostate, other organs that belong to the genitourinary tract, such as the kidney, epididymis and testis, have been involved in cryptococcal infections in individuals with severe immunosuppression [67]. Patients typically present with symptoms that include urgency, dysuria, suprapubic pain, hematuria, and bladder outlet obstruction [68].

Active cryptococcal infection in unusual body sites is rare, although some case reports have been described, such as cryptococcal peritonitis [69], arthritis [70], myositis [71], olecranon bursitis [72] and hepatitis [73]. Infections of the parathyroid [74] and parotid glands [75], infections of the biliary tract and adrenal involve-

ment [76] are infrequent but have been observed. Bone infection is associated with osteolytic lesions in any bone of the body, and cryptococcal osteomyelitis has been associated with underlying sarcoidosis [77]. Rare cases of fungal colonization in the colon have been reported, although peritonitis has been described in patients with cirrhosis [69]. Cryptococcuria and cryptococcemia were reported in several case series [36]. In some conditions, even after initiating antifungal therapy, cryptococcal infection may persist in these uncommon sites, suggesting that these sites may be a source for future recurrence and dissemination [74].

6.2.5 Cryptococcal Immune Reconstitution Inflammatory Syndrome

Immune reconstitution inflammatory syndrome (IRIS) is considered to be a clinical deterioration that is associated with new or worsening clinical manifestations of cryptococcal disease caused by the recovery of *Cryptococcus*-specific immune responses. In this syndrome, a dysregulated reversal of the anti-inflammatory response into a strong proinflammatory immune response occurs [78]. Typically, IRIS affects HIV-infected patients with CNS infection and solid organ transplant (SOT) recipients [36]. However, this syndrome has been observed in healthy hosts, such as pregnant women [46]. In immunosuppressed patients, IRIS development is associated with the withdrawal or reduction of immunosuppression and/or the restoration of immune functions [79].

When observed upon the initiation of highly active antiretroviral therapy (HAART), cryptococcal IRIS can be defined as paradoxical or unmasking. Paradoxical IRIS occurs in up to one-third of patients with pre-HAART cryptococcosis and presents as a worsening or recurrence of previously treated cryptococcal disease in the same or new anatomical sites. In the unmasking form of IRIS, patients with or without a prior diagnosis may develop acute symptoms of cryptococcosis, such as meningitis or necrotizing lymphadenopathy [80].

Epidemiologic data demonstrated that 5–11% of SOT recipients with cryptococcosis develop cryptococcal IRIS 4–6 weeks after the initiation of antifungal therapy [81]. For HIV patients, the incidence of IRIS ranges from 8% to 50% after HAART initiation [82].

IRIS is associated with significant morbidity and mortality and is responsible for diverse sequelae, including neurological deficits, which may be associated with inflammatory mass lesions, hydrocephalus, meningeal enhancement and increased CSF inflammation [44]. Patients are rarely asymptomatic, and most IRIS cases present with CNS involvement and clinical features that are similar to an active cryptococcal infection, although pulmonary manifestations are uncommon [9]. The signs and symptoms in these cases may range from headaches and drowsiness to new or recurrent neurological manifestations, such as ataxia, deafness, blindness, and seizures [83].

6.3 Therapy and Management of Cryptococcosis

The gold standard treatment for cryptococcosis that is recommended by the World Health Organization (WHO) and the Infectious Diseases Society of America (IDSA) guidelines includes three off-patent drugs: amphotericin B (AmB), flucytosine, and fluconazole [84].

AmB belongs to the polyenes class and binds ergosterol, which is present in fungal cell membranes. AmB can form pores in the plasma membrane, disrupting cell integrity, and is also known to induce oxidative stress in the cytoplasmic environment [85]. AmB is the fastest drug for *Cryptococcus* spp. clearance based on different clinical trials [86]. Because of its low bioavailability, AmB cannot be administrated orally using the existing formulations and thus requires hospital admission for intravenous administration [1]. Furthermore, prolonged treatment with AmB is inadvisable due to substantial side effects, such as anemia, nephrotoxicity and local infusion reactions. Thus, AmB usage is limited in regions without a satisfactory medical infrastructure [87].

AmB displays a very low solubility in water and is therefore administrated as a deoxycholate salt (AmBd) [88]. Newer formulations of AmB, such as liposomal AmB, an AmB-lipid complex and an AmB colloidal dispersion, have fewer toxic side effects and are better tolerated, allowing the administration of higher doses [3]. However, the high cost of lipid AmB is a barrier to the implementation of this drug, as its use is economically unfeasible in low- and middle-income countries [89].

Flucytosine or 5-fluorocytosine (5-FC) is a pyrimidine analogue that is converted into 5-fluorouracil (5-FU) inside fungal cells by the enzyme cytosine deaminase [90], which is not present in mammalian cells [91]. The accumulation of 5-FU inside fungal cells inhibits RNA and DNA synthesis, causing an antifungal effect, although the mechanism of action is not fully elucidated [92]. Mammals are unable to produce 5-FU from 5-FC; however, bacteria in the human microflora can perform this conversion, causing some side effects, such as hepatotoxicity, hematological toxicity and gastrointestinal toxicity [93].

Fluconazole inhibits the final step of ergosterol synthesis, leading to the accumulation of toxic steroidal components in cellular membranes [94]. This drug is largely used in the initial phase of treatment and is also used during the maintenance phase to avoid relapses. Fluconazole has excellent bioavailability and easily reaches the central nervous system (CNS) [95]. However, the development of resistance to fluconazole is a critical problem in cryptococcosis therapy, with resistance reported in more than 60% of culture-positive individuals in South Africa [51].

Despite the guideline recommendations and the inclusion of these three drugs in the 19th WHO Model List of Essential Medicines [81], AmBd and 5-FC are still unavailable in most low- and middle-income countries, where cryptococcosis is a leading cause of death among HIV-infected patients, with an alarming mortality rate of 70% for cryptococcosis patients in sub-Saharan Africa [4].

This section will discuss cryptococcosis management and therapy in three groups of patients: HIV-infected individuals, solid organ transplant (SOT) patients and non-HIV/non-SOT individuals. A summary of the first line of therapy proposed by the IDSA for these three groups is presented in Table 6.1.

Table 6.1 First-line treatment for cryptococcal meningitis according to the IDSA guidelines

	HIV-positive patients	SOT recipients	Non-HIV/Non-SOT patients
Induction phase	At least 2 weeks: AmBd (0.7–1.0 mg/kg per day iv) 5-FC (100 mg/kg per day orally in 4 doses)	At least 2 weeks: ABLC (5 mg/kg per day orally in 4 doses)	At least 4 weeks: AmBd (0.7–1.0 mg/kg per day iv) 5-FC (100 mg/kg per day orally in 4 doses)
Consolidation phase	At least 8 weeks: Fluconazole 400 mg (6 mg/kg per day orally)	At least 8 weeks: Fluconazole 400 mg (6–12 mg/kg per day orally)	At least 8 weeks: Fluconazole 400 mg (6–12 mg/kg per day orally)
CSF sterilization			
Maintenance or suppressive phase	At least 12 months: Fluconazole 200 mg (3 mg/kg per day orally)	6–12 months: Fluconazole 200 mg (3–6 mg/kg per day orally)	6–12 months: Fluconazole 200 mg (3–6 mg/kg per day orally)

The treatment is divided into the induction, consolidation and maintenance phases. Complete fungal clearance in the CSF should be reached at the end of the consolidation phase in order to continue to the maintenance phase. Otherwise, the patients should repeat the induction phase. The three groups of patients affected by cryptococcal meningitis (HIV-positive, SOT, and non-HIV/non-SOT) are treated differently

6.3.1 Cryptococcosis Treatment in the HIV-Infected Population

HIV infection is considered to be the leading predisposing condition to the development of cryptococcal meningitis [96], with 80–90% of cases in HIV-positive patients [97]. Therefore, the extensive use of highly active anti-retroviral therapy (HAART) starting from the mid-1990s resulted in a remarkable decrease in the cryptococcal meningitis incidence in developed countries [98]. However, in low- and middle-income countries, access to HAART is limited and is initiated during late phases of HIV infection [89]. In fact, the failure to continue monitoring patients after hospitalization is the first barrier to positive outcomes in HIV-associated cryptococcal meningitis treatment in South Africa [99]. Indeed, according to IDSA guidelines [100], patients with disseminated cryptococcosis or cryptococcal meningoencephalitis should be immediately tested for HIV infection.

The first line of therapy recommended by the IDSA guidelines includes an induction phase that is characterized by at least 2 weeks of treatment with intravenous AmBd (0.7–1.0 mg/kg per day) plus 5-FC (100 mg/kg per day orally). This strategy is associated with rapid *Cryptococcus* clearance and is highly effective based on data from randomized clinical trials [86]. Ideally, sterilization of the CSF should be achieved after 14 days of primary therapy, which is associated with a positive outcome [101]. In places where 5-FC is not registered or not available at the supplier level, such as several countries in Africa and Asia [89], alternative regimens should be considered as primary therapies [102]. For instance, AmBd (1 mg/kg per day, intravenously) plus fluconazole (800 mg/day, orally) for at least 2 weeks is the stan-

dard primary therapy recommended by the Southern African HIV Clinicians Society guidelines [103]. However, the absence of 5-FC in induction therapy is associated with treatment failure in 3 months [104]. In situations where AmBd is not available or cannot be given safely, fluconazole monotherapy at higher doses (1200 mg/day orally) is largely used, although this regimen is associated with poor outcomes, such as higher mortality rates, a prolonged CSF sterilization time and a risk of relapse [105]. Bicanc and colleagues observed 32 relapse episodes among 27 patients who received fluconazole monotherapy during the induction phase [106].

After 2 or more weeks of induction therapy, the consolidation phase is established, with at least 8 weeks of fluconazole 400 mg (6 mg/Kg) per day orally. In a clinical trial with 306 patients, 72% of patients presented negative CSF cultures after fluconazole treatment, while 60% of patients had negative CSF cultures after itraconazole treatment, supporting the preferential use of fluconazole during the consolidation phase [107].

After fungal clearance, the maintenance or suppressive phase starts. During this phase, patients should take 200 mg fluconazole per day, orally, for a period of 12 months or more. A clinical trial demonstrated that fluconazole maintenance therapy reduced the chance of relapse to zero, while the placebo group displayed a relapse rate of 15% in patients who completed successful induction and consolidation therapies [108]. As an alternative maintenance therapy, the use of itraconazole (400 mg/day orally) or AmBd (1 mg/kg per week intravenously) is suggested for azole-intolerant individuals or patients with fluconazole therapy failure [100]. Both alternative drugs require patients to be monitored due to toxicity-related side effects. The results of two different trials highlighted fluconazole as the most effective drug in the maintenance phase. Patients undergoing fluconazole treatment showed significantly lower relapse rates than patients undergoing itraconazole or AmBd regimens [109]. In the HIV coinfected population, the suppression phase should be maintained for at least 1 year and should be discontinued only after the CD4 cell count is restored and higher than 100 cells/μL and the HIV RNA serum level is undetectable for at least 3 consecutive months [110]. If the CD4 cell count decreases to less than 100 cells/μL or if cryptococcal antigenemia increases, the guidelines recommend the restitution of maintenance therapy [111]. In the case of asymptomatic antigenemia, lumbar puncture and blood cultures are highly recommended [100]. If the culture results are positive, the patients should repeat the induction phase [100].

To date, the most effective strategy for preventing and reducing cryptococcosis mortality and morbidity remains early HIV diagnosis and adhesion to HAART, especially before the CD4 count falls below 350 cells/μL [84]. On the other hand, in the presence of a high fungal burden, HAART initiation can be harmful due to the risk of immune reconstitution inflammatory syndrome (IRIS) development [112]. In a randomized clinical trial, which was designed to address the optimal HAART timing, a cohort was initiated 72 h after antifungal therapy, and this strategy resulted in a high mortality rate [113]. The restoration of the immune status upon HAART initiation may induce an exacerbated inflammatory response, which contributes to tissue damage and increases the severity of cryptococcosis, particularly in the CNS

[114]. However, later HAART initiation and prolonged immunosuppression may pose risks of developing complications associated with HIV infection and the worsening of cryptococcal disease [115].

The optimal starting time for HAART remains to be elucidated, and new trials with larger cohorts are necessary [116]. The Southern Africa HIV Clinicians Society guideline currently recommends a HAART initiation window between 4 and 6 weeks [103]. Management of major IRIS complications has not yet been studied extensively in clinical trials. However, the IDSA guideline considers the use of anti-inflammatory drugs, such as corticosteroids, in patients with CNS inflammation and increased intracranial pressure. Corticosteroid therapy should be monitored carefully and administered concomitant with antifungal therapy [117].

6.3.2 Cryptococcosis in Solid Organ Transplant (SOT) Recipients

Opportunistic *Cryptococcus* infection affects approximately 2.8% of the SOT population [118]. The mortality rate reaches 15% 90 days after cryptococcosis diagnosis and 27% 12 months after cryptococcosis diagnosis [119].

As in other patient groups, cryptococcosis in SOT individuals manifests primarily as meningoencephalitis or pulmonary disease. Between 48 and 89% of the *Cryptococcus*-infected SOT population has CNS involvement [120], and between 25 and 64% of SOT recipients have pulmonary disease, with the disease limited to the lungs in 6–33% of these patients [121]. It is noteworthy that in patients with disseminated disease, several sites can be infected by *Cryptococcus* simultaneously, such as the soft tissues, joints, bones, eyes, and the thyroid and adrenal glands [74].

After a SOT procedure, the use of immunosuppressive drugs is necessary to avoid organ rejection. However, this therapy increases not only the risk of acquiring cryptococcosis but also the severity of this disease [122]. Immunosuppressive regimens include calcineurin inhibitors (such as tacrolimus or cyclosporine) [123], mycophenolate mofetil, which is an inhibitor of lymphocyte de novo purine synthesis [124], and corticosteroids [118]. Cryptococcosis management in the SOT population includes the gradual reduction of immunosuppression prior to the initiation of antifungal therapy [100]. The abrupt removal of immunosuppressive therapy may result in a higher risk of organ rejection or IRIS development [125].

Approximately 5% of SOT recipients with cryptococcosis present renal dysfunction (creatinine level > 2.0 mg/dL) [100]. The use of calcineurin inhibitors and AmBd is known to be highly nephrotoxic [118]. Therefore, AmBd is not recommended as a first-line therapy in SOT patients [100]. Liposomal AmB formulations are used preferentially in these patients according to the IDSA guideline [100] because these formulations are associated with a lower incidence of nephrotoxicity [126].

The induction therapy established by the IDSA for SOT patients consists of liposomal AmB (3–4 mg/kg per day) or an AmB lipid complex (ABLC) (5 mg/kg per day) plus 5-FC (100 mg/kg per day divided into 4 doses) for at least 2 weeks. For the consolidation phase, the guidelines recommend 6–12 mg/kg per day of oral fluconazole for 8 weeks. Finally, the maintenance phase consists of 6–12 months of orally administered fluconazole at a dose of 3–6 mg/kg per day. This regimen is recommended for CNS disease and severe pulmonary or disseminated non-CNS cryptococcosis [100]. For patients with mild to moderate symptoms with no CNS involvement, no evidence of disseminated infection and nondiffused lung infiltrates, the guidelines recommend 6 mg/kg per day of fluconazole for 6–12 months [127]. Patients who received maintenance therapy for 6 months or more had a relapse rate of 1.3% [128]. In the absence of AmB lipid formulations, AmB deoxycholate should be used with extra caution. The guidelines suggest a maximum dosage of 0.7 mg/kg per day of AmBd, with frequent renal function monitoring. In areas with no access to 5-FC, induction therapy guidelines may include liposomal formulations of AmB (6 mg/kg per day) for 4–6 weeks. The absence of 5-FC during this phase is considered to be a risk factor for poor treatment outcomes [104]; however, this drug remains unavailable in most countries in Africa, Asia, and Latin America [89].

The mean CSF sterilization time is 16 days after treatment initiation [121]. After this period, another lumbar puncture is recommended, and if the CSF presents a positive fungal culture, the induction therapy must be repeated. Therapeutic lumbar punctures are recommended to relieve intracranial pressure during cryptococcal meningitis, and this management strategy is associated with decreased cryptococcal meningitis mortality in HIV-infected patients [129].

The gradual reduction of immunosuppressive therapy is highly recommended to restore cellular immunity against *Cryptococcus* in SOT patients [79]. However, the abrupt reduction of immunosuppressive therapy may induce a potent shift in the immune status to a Th1 proinflammatory response, which is related to IRIS development in SOT patients [130]. This strong proinflammatory response may result in tissue lesions in the CNS and the lungs, worsening the clinical condition of the patient [125]. Therefore, IRIS can be misdiagnosed as therapy failure [131]. This syndrome has a prevalence of 4.8% in SOT-related cryptococcosis patients and is associated with a high risk of organ rejection [125].

6.3.3 Cryptococcosis in Non-HIV and Non-SOT Patients

Concerning the treatment of cryptococcosis in virtually immunocompetent hosts, there is no consensus among specialists that is clearly expressed in the IDSA guidelines, primarily because the major controlled clinical trials performed in the non-HIV non-SOT population included individuals with an immunosuppressed status, such as cancer patients and patients undergoing steroidal drug treatment [132]. Patients with systemic lupus erythematosus are at risk of developing cryptococcosis, which reinforces the importance of the host immune status [133]. In a Taiwan

study, *C. neoformans* was responsible for 6 deaths among 15 patients with lupus erythematosus [134].

The first line of induction therapy that is recommended for cryptococcal meningitis in this group of patients is AmBd (0.7–1.0 mg/kg per day, intravenously) plus 5-FC (100 mg/kg per day, orally in 4 doses) for 4–6 weeks. The subsequent consolidation phase is characterized by 8 weeks of treatment with fluconazole 400 mg/day, orally. Maintenance therapy is continued for 6–12 months, with fluconazole 200 mg/day, orally. For pulmonary cryptococcosis in non-immunosuppressed patients, the treatment is fluconazole 400 mg per day, orally, for 6–12 months [100]. Severe pulmonary cryptococcosis should be treated as CNS disease [135]. Immunocompetent hosts are particularly susceptible to infections caused by *C. gattii* species [136]. These infections tend to form more cryptococcomas in the lungs and in the CNS than *C. neoformans* infections; therefore, the therapeutic response tends to be slower [137]. The presence of cerebral cryptococcomas may require surgical removal if the lesions are larger than 3 cm [138]. Surgery is also recommended if the cryptococcoma is compressing the optic nerve to avoid the development of blindness [139]. The management and therapy principles used for *C. gattii* infection are the same as those used for *C. neoformans* infection [44].

6.4 Laboratory Diagnosis: Classic and New Diagnosis Trends

The definitive laboratory diagnosis of cryptococcosis is based on the direct observation of the yeast through India ink staining of bodily fluids, the direct examination and culture of *Cryptococcus* from clinical specimens and immunoassays for cryptococcal antigens [9]. Other methods are also used for the diagnosis of this mycosis, including histopathology of infected tissues and molecular diagnosis methods, which are applied extensively for research but not widely used in the clinic [140].

6.4.1 Direct Examination

Direct microscopic observation is the most rapid and low-cost method used for the diagnosis of cryptococcosis, with the detection of encapsulated yeasts in a variety of samples, such as cerebrospinal fluid (CSF), sputum, bronchial lavage, pus from mucosal cutaneous lesions, urine, and macerated tissues obtained through biopsy. In direct examinations, *C. neoformans* is characterized as globular or oval-shaped cells, which may present a single bud, surrounded by a polysaccharide capsule that ranges from 5 to 20 μm in diameter. The visualization of the capsule is evident when the samples, primarily CSF and urine, are stained with India ink. India ink is a negative stain that creates a dark background, with the yeast appearing to be surrounded

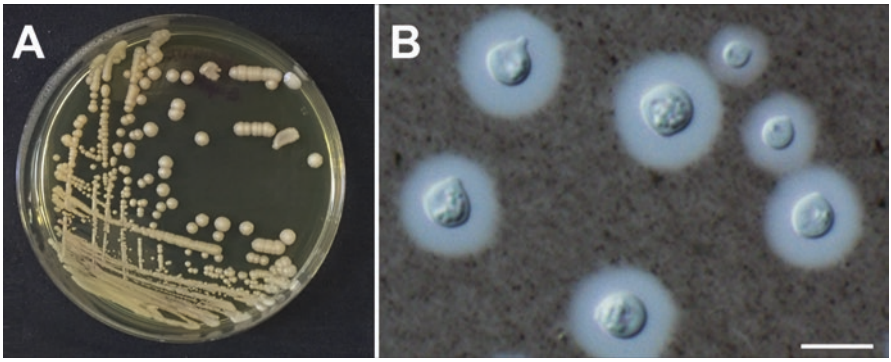

Fig. 6.3 *Cryptococcus* yeasts in culture. (**a**) Isolated colonies in agar Sabouraud. (**b**) India ink negative staining showing the capsule as an exclusion zone surrounding the cell. *Scale bar*: 10 μm

by a clear halo generated by the polysaccharide capsule, which repels the India ink staining (Fig. 6.3). Sputum and other purulent samples can be treated with a 10% potassium hydroxide solution. The purpose of this sample preparation is to disrupt cellular proteinaceous debris, cleaning the specimen for easier fungal detection. The sensitivity of microscopic observation in samples from AIDS patients reaches 80% of positive cases of cryptococcal meningitis, but in immunocompetent patients, this percentage drops to 50% [141].

Histological preparations of tissues, such as lung, skin, bone marrow, brain, and other organs, can reveal the presence of *Cryptococcus* by using special stains that label the polysaccharide capsule, such as mucicarmine, periodic acid-Schiff, and Alcian blue stains. Another reagent, the Fontana–Masson staining agent, identifies melanin deposited in the yeast cell wall. Other staining procedures are able demonstrate the presence of fungal chitin (Calcofluor White) and of the fungal cell wall (Grocott's methenamine silver) in clinical specimens [142].

6.4.2 Culture

Cryptococcus species are easily isolated and maintained in culture on Sabouraud agar, with or without antibiotics. The production of melanin is a feature that is widely used for the identification of *C. neoformans* and *C. gattii*. These yeasts produce the enzyme phenoloxidase, which oxidizes phenolic and diphenolic compounds found in some media, such as Sunflower Seed agar (*Helianthus annus*), Niger seed agar (*Phalaris canariensis*), Bird Seed agar (*Guizotia abyssinica*) and L-3,4-dihydroxyphenylalanine (L-Dopa), which is a chemically defined medium. Under these conditions, the colonies grow with a dark brown color [143]. To differentiate *C. neoformans* from *C. gattii*, L-canavanine glycine bromothymol blue agar (CGB) can be used because *C. gattii* grows in the presence of canavanine and uses glycine as a source of carbon and nitrogen; thus, the pH of the medium increases and the color of the indicator, bromothymol blue, changes to a vivid cobalt blue

color after approximately 48 h of incubation at room temperature [144]. In contrast, *C. neoformans* is sensitive to the presence of canavanine and does not use glycine as a sole source of carbon, leaving the medium's pH unaltered and its coloration unchanged [144].

6.4.3 Immunological Methods

Immunological tests that are sensitive to the cryptococcal polysaccharide capsular antigen (CrAg), which is shed during infection, are used to aid in the diagnosis of invasive cryptococcosis. Latex agglutination and immunoenzymatic techniques using serum, CSF, and urine are widely available.

The detection of the capsular antigen using the latex agglutination test has proved to be an important tool in the diagnosis of cryptococcosis, as this test is more sensitive than microscopy [145] and faster than the isolation of the fungus in culture. In addition to being highly specific, this test can be performed as a qualitative and semi-quantitative assay [145].

Although rare, false-positive results can be obtained in the latex agglutination test due to cross-reactions with polysaccharides from other microorganisms. False-negative results are also rare but may occur due to a low antigen concentration, the presence of immunocomplexes, a prozone effect, or thinly encapsulated strains [141].

The lateral flow immunochromatographic assay for the detection of cryptococcal antigen (CrAG LFA) was recently developed for the diagnosis of cryptococcal meningitis as a true point-of-care test, which is accessible in any setting without the need for a laboratory structure (Fig. 6.4). The CrAg LFA uses a combination of two monoclonal antibodies against the most abundant capsule polysaccharide, which is termed glucuronoxylomannan. The first monoclonal antibody reacts with the polysaccharide of serotypes A, B, and C; the second monoclonal antibody is highly reactive with the polysaccharide of serotypes A and D. Combined, the monoclonal antibodies are highly reactive with polysaccharides from *C. neoformans* and *C. gattii.* This test is more accurate than the CrAg-latex test or the enzyme immunoassay (EIA) and has advantages with respect to the detection range. The test can be performed for screening and preemptive treatment of subclinical cryptococcosis and to monitor serum/CSF polysaccharide titers for treatment control. This test is less time consuming than other tests ($\leq$10 min), requires little or no equipment, can be performed without electricity, is stable at room temperature and is relatively inexpensive [142].

6.4.4 Molecular Methods

Molecular methods, although not applied in routine diagnosis, are widely applied in research for several purposes; for example, these methods have high sensitivity and specificity and are important alternative tools for overcoming the limitations of conventional diagnostic tests. These methods can be employed for identification and

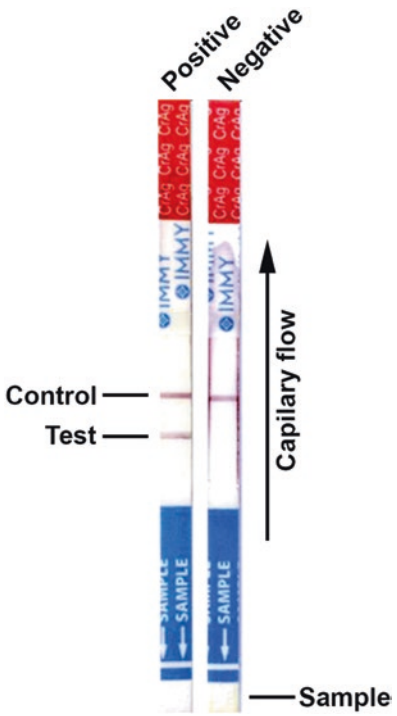

Fig. 6.4 Positive and negative strips from the lateral flow immunochromatographic assay for the detection of cryptococcal antigen (CrAG LFA)

typing and have been applied primarily for molecular epidemiology studies. Although numerous molecular techniques have been applied for typing *C. neoformans* and *C. gattii* strains, only three methods were proved to produce comparable results: PCR fingerprinting, AFLP, and MLST [25]. At present, eight major molecular types are widely recognized: VNI, VNII, VNIII, and VNIV among *C. neoformans* isolates and VGI VGII, VGIII, and VGIV among *C. gattii* isolates [25].

6.5 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is widely used in research laboratories and has high sensitivity and specificity in detecting minimal amounts of DNA from species of the *Cryptococcus* genus from clinical and environmental samples. Nested PCR, multiplex PCR, and real-time PCR are the most common variant PCR methodologies used to identify *C. neoformans* and *C. gattii*. Several target sequences are available, including URA5 (the orotidine monophosphate pyrophosphorylase gene), CAP59 (the capsule synthesis gene), M13 (minisatellite-specific core sequence), and ITS (internal transcribed spacer regions 18S, 5.8S, and 28S) [146].

6.6 Polymerase Chain Reaction: Restriction Fragment Length Polymorphism

Polymerase chain reaction—restriction fragment length polymorphism (PCR–RFLP) is similar to restriction fragment length polymorphism (RFLP), but in this case, the probe hybridization step is not needed because the genomic DNA fragments are previously subjected to PCR with specific primers and then cleaved with restriction enzymes [147]. PCR-RFLP that targets the URA5 gene during the amplification reaction has been employed in molecular epidemiology studies to determine the potential relationships among molecular types in clinical and environmental isolates of *Cryptococcus*. This approach is also recommended when it is necessary to obtain more information about a specific strain [148].

6.7 Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) is another alternative for typing *C. neoformans* complex individuals. AFLP is very useful in detecting genetic variability, since this technique reveals the presence or absence of polymorphisms at restriction sites. The AFLP genotyping technique is both more sensitive and more expensive than PCR fingerprinting and is also performed in several steps. First, the genomic DNA is cleaved with restriction enzymes, followed by the ligation of specific adapters to the sticky ends of the cleaved genomic fragments. The amplification of the generated segments is carried out via PCR with specific primers that recognize sequences in the adapter, and the separation of the subset of amplified fragments is achieved through high-resolution gel electrophoresis. This process enables the grouping of the *C. neoformans* species complex into six molecular types: AFLP1 to AFLP6 [149].

6.8 Multi-locus Sequencing Typing

The multi-locus sequencing typing (MLST) technique is highly reproducible and trustworthy. MLST is based on variations in the nucleotide sequences of multiple housekeeping genes, and the following set of genetic loci that encode capsule, urease, phospholipase, and laccase production was proposed as a consensus international standard for multi-locus sequence typing for *C. neoformans* and *C. gattii*: CAP59, GPD1, LAC1, PLB1, SOD1, URA5, and IGS1 [150]. MLST is of great value for determining the circulating *Cryptococcus* complex genotypes in different parts of the world [29]. After amplification and sequencing of the desired regions, MLST analysis is fully automated. A work-group created and supported by the International Society of Human and Animal Mycoses (ISHAM) proposed MLST as

a consensus methodology for genotyping *C. neoformans* and *C. gattii.* This recommendation was supported by all major research groups that are involved in molecular strain typing of the *C. neoformans* species complex [150].

6.9 *Cryptococcus* Virulence Factors

6.9.1 Survival at High Temperatures

The ability to survive at high temperatures is a key characteristic of fungi that are able to become invasive in mammalian hosts due to the mammalian body temperature [151]. Only two species of *Cryptococcus* can cause invasive systemic disease in mammalian hosts and exhibit optimal growth at temperatures higher than 30 °C, despite the presence of classical virulence factors, such as melanin and capsule production, in other members of this clade [152].

Mammalian hosts with increased body temperature, such as rabbits, are naturally resistant to experimental cryptococcosis [153]. However, upon immunosuppression with corticosteroidal drugs, *Cryptococcus* is able to colonize the rabbit CNS [153]. Moreover, an increase in the environmental temperature prolonged the lifespan of infected mice [154]. These findings eventually led to early attempts to treat cryptococcosis patients using hyperthermia; however, such treatments had no success [155].

Cryptococcal thermo-tolerance has been studied extensively, and the application of several molecular techniques revealed that a huge shift in gene expression occurs during yeast exposure to mammalian body temperatures. The temperature-regulated genes involve a long list of processes, including signaling pathways (*RAS1*, *CNA1*, *CNB1*, *MPK1,* and *CTS1*) [156], energetic metabolism (*VPH1*) [157], structural components of the polysaccharide capsule (*UGD1*) [158] and even amino acid metabolism (*ILV2* and *SPE3*) [159] and mitochondrial detoxification functions, such as the manganese-superoxide dismutase (*SOD2*) [160]. Conserved processes, such as sugar metabolism and ribonucleotide synthesis, are also involved in thermo-tolerance, as evidenced by the avirulent temperature-sensitive phenotype of mutants in the trehalose synthesis machinery [161] and de novo pyrimidine ribonucleotide formation [162].

6.9.2 Melanin

Melanin is a widespread pigment that is expressed in a broad range of fungi [163] and was first described in the *Cryptococcus* genus by Staib in 1962 (reviewed in [164]). Two metabolic pathways can lead to melanin synthesis in fungi: the 1,8-dihydroxynaphthalene (DHN) pathway and the L-3,4-dihydroxyphenylalanine (L-DOPA) substrate pathway, which shows closer similarity to the mammalian pathway [165]. The latter pathway is present in *C. neoformans* [166]. Melanin produced is deposited in the cell wall and can be observed commonly in a culture medium containing with D-DOPA (Fig. 6.5).

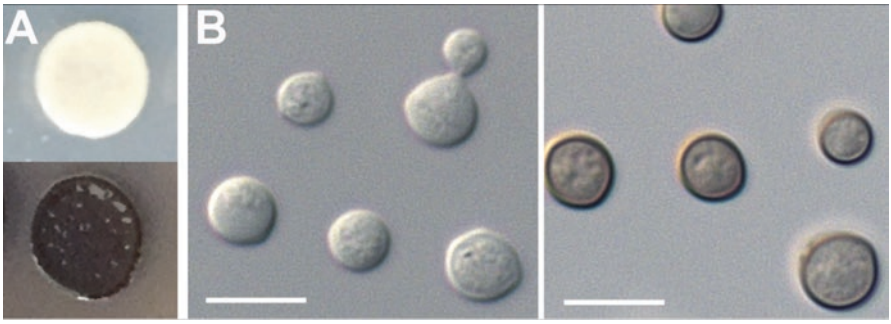

Fig. 6.5 Melanin production. *Cryptococcus* grown on medium containing 0.1% L-DOPA to demonstrate melanization (*brown pigment*). Colony morphology (**a**) and optical microscopy (**b**) in presence and absence of L-DOPA. *Scale bar*: 10 μm

Throughout this process, the enzyme laccase converts L-DOPA molecules into dopaquinone, which is subjected to the addition of amino groups to form cyclodopa. Oxidized cyclodopa is converted to dopachrome, which undergoes tautomerization to generate dihydroxyindoles that ultimately polymerize spontaneously into melanin, which is a negatively charged pigment [166]. The cryptococcal enzyme laccase is encoded by two genes: *LAC1* and *LAC2* [167]. The *LAC1* product is the main component responsible for melanin production. The association of this gene with virulence in *Cryptococcus* was observed using deletion mutants, and those studies showed that deletion mutants for this gene are less virulent [167].

The *LAC1* laccase is targeted to the cell wall in a Sec6p-dependent manner [168]. The *LAC2* laccase was demonstrated to be localized primarily throughout the cytoplasm [169]. It is possible that this enzyme reaches the cell wall and the supernatant inside the lumen of secreted vesicles, as Sec6 molecules are also related to extracellular vesicle secretion according to data that demonstrated that laccase and its activity are present in purified extracellular vesicles produced by *C. neoformans* [170].

During infection, melanin appears to confer resistance against host defenses [171]. Several studies demonstrated protection against phagocytosis in melanized fungi [172]. It is possible that the increased negative charge generated by melanin deposition is one of the factors responsible for this antiphagocytic effect [173].

Inside macrophages, the melanization process increases the ability of *Cryptococcus* to resist oxidative stress produced by host cells, such as oxygen- and nitrogen-derived oxidants [174]. This effect is achieved through melanin's natural ability to combat free radicals by transferring its electrons, as shown by electron spin resonance experiments [173].

Another characteristic of fungal melanin is to interfere with the host immune response [172]. Melanized *Cryptococcus* can reduce TNF-α cytokine secretion by alveolar macrophages and decrease lymphocyte expansion [175]. Melanization also increases the dispersion of this fungus, as it becomes more likely to survive in the spleen, liver, and brain of infected animals [171]. The fungus causes severe tissue damage in the murine central nervous system, with decreased cytokine responses in comparison with nonmelanized strains [175].

Despite its importance in cryptococcal infection, upon immunization, purified fungal melanin can trigger the host innate and adaptive immune responses. This activation is indicated by specific antibody production [176] and the activation of the complement system alternative pathway, with ultimate binding to the C3 protein [177]. However, the localization of melanin under the cryptococcal capsule appears to prevent this molecule from activating the complement system during infection [177].

In addition to interfering with the host immune system, melanin is able to sequester and protect fungi from synthetic anti-fungal agents, such as amphotericin B [178], caspofungin [179] and azoles [180], as observed in experimental murine infection assays.

6.9.3 Secreted Enzymes

Although *Cryptococcus* secretes several classes of enzymes, two of these classes have been directly implicated in fungal virulence: phospholipase B (PLB) [181] and urease [182].

Phospholipases are a heterogeneous class of enzymes [183]. Specifically, fungal PLB is expressed by several fungi (including *Cryptococcus*) and presents three distinct activities: hydrolase activity (the ability to remove *sn*-1 or *sn*-2 fatty acids from a glycerophospholipid), lysophospholipase activity (LPL), and transacylase activity (fatty acid addition) [184]. Genetic studies in *C. neoformans* demonstrated that a single gene (*PLB1*) is responsible for phospholipase activity in this yeast [184].

Known as an "invasin," PLB is important for cryptococcal tissue dissemination during traversal of the alveolar membrane, probably because the primary surfactant present in this environment, namely dipalmitoyl phosphatidylcholine, is a substrate for cryptococcal PLB [185]. This finding is in accordance with early observations by Chen [186] that the amount of PLB secreted is directly related to the fungal burden in a murine model of infection.

Another role for PLB is related to brain colonization during cryptococcosis. Cox [184] demonstrated that a *C. neoformans PLB1* knockout was less able to cross the blood-brain barrier (BBB), and an in vitro model of the BBB was less permeable to the *plb1*Δ strain than to the wild-type strain [187].

Although the absence of PLB does not appear to influence *Cryptococcus* uptake by macrophages, this mutation appears to delay fungal replication inside host phagocytic cells [184]. Once phagocytosed, the fungi inside phagolysosomes come into contact with lysosomal enzymes that are capable of inhibiting fungal growth, such as cathepsins B and L, as demonstrated in vitro [188]. It has been proposed that PLB activity against the phagolysosome membrane increases the permeability of the membrane, reducing the concentrations of these enzymes inside this compartment [184]. This finding explains the observed delay in fungal replication in the absence of PLB and the observation that cathepsin L activity decreases progressively inside the phagolysosome during the phagocytosis of live but not heat-killed *C. neoformans* cells [189].

Another characteristic of PLB is the capacity to generate bioactive eicosanoids using host arachidonic acid [190] to interfere with cellular levels of these molecules. This imbalance in eicosanoid homeostasis can shift the immune response toward a TH2 pattern, which is associated with fungal proliferation and tissue dissemination, as observed in mice infected with the wild-type strain in comparison to mice infected with *plb1*Δ, which exhibited an intense TH1 response and fungal clearance [190].

Another important enzyme for cryptococcal virulence is urease, which is a secreted nickel-dependent metalloenzyme that drives the hydrolysis of urea into ammonia and carbamate. The urease produced by *Cryptococcus* is encoded by a single gene named *URE1* [191]; however, similar to bacteria, accessory proteins are required for urease activity, namely Ure4, Ure6 and Ure7 (homologs of the UreD, UreF, and UreG proteins in bacteria, respectively) [182] plus a nickel/cobalt transporter protein named Nic1. A major difference between cryptococcal and bacterial urease activity is that the former lacks the nickel chaperone UreEp, which is present in bacteria [192]. In *Cryptococcus,* Ure7p contains histidine-rich domains that are capable of nickel binding and may overcome the absence of a UreE homolog [182].

Long studied as a virulence factor in some bacteria [193] and produced by many pathogenic fungi [194], urease activity and its role in cryptococcal virulence have only begun to be clarified.

Although this enzyme does not appear to be essential for the early stages of cryptococcal infection, published data showed a strong induction of IL-4 and IL-13 cytokines, high IgE antibody production and eosinophil infiltration into the lungs of mice infected with the wild-type strain, in contrast to mice infected with the *ure1*Δ strain [195]. Moreover, the same study reported a larger number of immature dendritic cells in the lung-associated lymph nodes of wild-type-infected animals in comparison to those infected with the urease-deficient mutant [195]. Taken together, these results point to a TH2 activation pattern that is implicated in persistent infection by *Cryptococcus* to the detriment of the host. This pattern is absent in the *URE1* knockout [195].

Although urease does not appear to be essential for cryptococcal survival in the brain [196], animals infected with the *ure1*Δ strain showed no apparent neurological damage and a decreased CFU load in organs with small capillary circulation, suggesting that the role of urease in brain colonization could be related to the crossing of the BBB [196]. To elucidate this issue, Shi and collaborators [197] used intravital microscopy to observe the initial steps of brain colonization *in vivo*. Using this approach, those authors demonstrated that *Cryptococcus* is rapidly trapped in brain capillary beds in a microembolic event [197], probably due to its size rather than a receptor-associated mechanism. The lack of urease activity does not influence this process; however, urease activity is essential to the transmigration of the BBB, as it increases transmigration sites through a mechanism that remains unknown [197].

It has also been proposed that the importance of urease in brain cryptococcosis could be related to tissue damage in the BBB caused by ammonia production and/or pH increases caused by the activity of this enzyme. In vitro tracking of a tight junction marker (ZO-1) in HBMEC cells exposed to wild-type and *ure1*Δ fungi showed that the deficient fungi are less efficient in loosening these junctions [182].

6.9.4 Polysaccharide Capsule

Since the early isolation of this fungus, the existence of an extracellular capsule surrounding cryptococcal cells was noted (Fig. 6.6) [198]. It was years before Benham [199] proposed that this structure could play an important role in pathogenesis.

From a poorly structurally and functionally described feature, the cryptococcal capsule became the most important and well-studied virulence factor related to this genus. Nevertheless, many aspects of the synthesis, structure and functions of the cryptococcal capsule remain to be elucidated. The first attempts to investigate the polysaccharide capsule were based on paper chromatography and identified traces of mannose, xylose, glucuronic acid, and galactose [200]. It was then proposed that this structure could be used to separate *Cryptococcus* species into serotypes defined by reactions with rabbit hyperimmune sera [201].

Rebers [202] shed the first light onto the structural problem by demonstrating that the polysaccharides from the cryptococcal capsule could be fractionated into two groups. Later, Batacharjee and colleagues [203] demonstrated that the largest fraction of purified polysaccharides did not contain galactose. Those authors termed this polysaccharide glucuronoxylomannan (GXM). It was only in 1982 that Cherniak [204] published the first detailed study of the minor, galactose-containing capsular polysaccharide, which was then termed galactoxylomannan (GalXM) [later renamed glucuronoxylomannogalactan (GXMGal)].

GXM is by far the most abundant of the two capsular polysaccharide components [205]. The molecular weight of GXM is approximately 1700 to 7000 kDa, comprising 90% of the overall polysaccharide composition of the capsule [206]. The current proposed structure of this molecule consists of the repetition of one or more motifs composed by a core triad of α-1,3-linked mannose with side substitutions of β-1,2-linked glucuronic acid and β-1,2- or β-1,4-linked xylose [207]. In addition, these triads undergo O-acetylation on their mannose residues due to the action of an enzyme called Cas1 [208].

The other polysaccharide component of the capsule, GXMGal, presents a molecular weight range of 101–275 kDa [209]. Recent data concerning the structure of GXMGal purified from an acapsular mutant (which does not produce GXM) showed this polymer to be organized as an α-1,6-linked galactose core with alternating β-1,3 galactose side chains α-1,4-linked to a dimer of mannose-α-1,3-mannose. These alternate side chains can receive substitutions of α-1,3- or β-1,3-linked xylose molecules. In addition, molecules of β-1,3-linked glucuronic acid may also be present [210].

The basic components of these sugars are UDP-glucuronic acid, which is synthesized by the Ugd1 UDP-glucose dehydrogenase [157], UDP-xylose, which is generated via the conversion of UDP-glucuronic acid by Uxs1 decarboxylase [211], UDP-galactose, which is generated via the conversion of UDP-glucose by Uge1 epimerase [212], and GDP-mannose, which is synthesized by a phosphomannose isomerase named Man1 and possibly other enzymes [212].

It was proposed that these sugar molecules are synthesized inside the Golgi cisternae [213], and transporters for GDP-mannose and UDP-galactose that are pres-

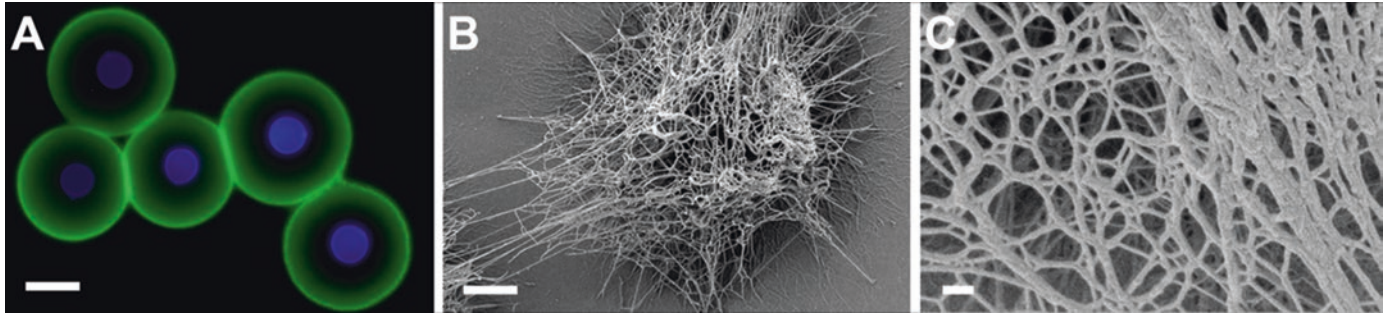

Fig. 6.6 Different views of *C. neoformans* capsular polysaccharide. Immunofluorescence microscopy using monoclonal antibody against GXM and the cell wall stained with Calcofluor White. *Scale bar*: 10 μm (**a**). Scanning electronic microscopy showing fibrillar morphology of capsule (**b** and **c**). *Scale bars*: 1 μm and 200 nm

ent in the membrane of this structure have been described to play a role in capsular polysaccharide production [214].

Once outside of the cell, the anchoring of the polysaccharide capsule appears to be dependent on several components and structures. Both α-1,3 and β-1,6 glucan influence capsule attachment [215]. The first molecule influences the attachment of the whole capsular structure [216], and the second molecule influences the capsule density [215].

Chitin and chitosan, which are also components of the fungal cell wall, are determinants in capsule formation. Mutants containing decreased chitosan levels and increased chitin levels exhibited an augmented capsule size in comparison to wild-type individuals [217]. Reinforcing that finding, chitin and chito-oligomers were shown to bind GXM in vitro [218], which may indicate a role for these molecules in capsule attachment.

Finally, among the proteins that insert into cell wall structures, two GPI-anchored proteins are likely to be related to capsule structure: MP98 and phospholipase. MP98 affects chitosan levels [217], and phospholipase binds to β1,6 glucan, affecting cell wall integrity and capsule density [219].

Once attached to the cell wall, GXM fibers are believed to aggregate with each other via interactions with divalent cations [220]. Newly secreted fibers attach to the outer portion of the preexisting capsular structure, as indicated by irreversible staining of GXM fibers with complement component C3 during capsule enlargement [221]. This finding is consistent with the observation that antigenic determinants appear first at the distal portion of the capsule in vivo [222].

The most established function of the cryptococcal capsule is the avoidance of phagocytosis, which occurs very early during lung infection [223]. This impairment of innate immunity is probably a consequence of the capsule acting as a barrier between the immunogenic epitopes in the cell wall and their receptors on phagocytes [224]. This function is profoundly important, as without internalizing and killing the fungus, these cells cannot correctly present fungal antigens to T cells. Thus, this process negatively impacts the host's ability to mount an adaptive immune response, leading to fungal persistence and dissemination [225].

Among the receptors implicated in the recognition of the fungal capsule is the mannose receptor, which was shown to influence the uptake of *Cryptococcus* by dendritic cells [226]. Fc receptors that are present in these cells also engage capsular polysaccharide, leading to opposite outcomes. When bound to FcRIIB or FcRγII, GXM can trigger phagocytosis [227] or induce inhibitory signals, respectively [228].

After being internalized, the cryptococcal capsule plays a decisive role in survival and replication inside phagocytes. Acapsular mutants are unable to replicate inside the phagolysosome and cannot escape the cell by extrusion [229]. This phenotype can be rescued by coating the acapsular yeast with exogenous GXM [229].

The observed increased survival inside phagocytes may be related to protection against reactive oxygen species, which can either be trapped by the abundant negative charge of the capsular polysaccharide's glucuronic acid residues [230] or diluted by the

expansion of the phagolysosome. Phagolysosome expansion may be driven by capsular enlargement and is believed to make this compartment leaky and less concentrated [231].

Encapsulated strains disseminate from the lungs better than acapsular strains [232], and this difference appears to be linked to the engagement of receptors in epithelial cells. GXM was demonstrated to bind and enter the intracellular environment of the pulmonary alveolar epithelium via interactions with CD14 [233].

The role of the polysaccharide capsule in *Cryptococcus* dissemination among host tissues appears to be related not only to capsule enlargement but also to capsule organization. Fungal cells modulate capsule design during spreading, as demonstrated by differential binding to specific antibodies while infecting different organs, which represents a clear modification of the exposed epitopes [222].

Capsular GXM is also shed constitutively into the host environment and can be detected in large quantities in several tissues [234]. This secreted exopolysaccharide produces several deleterious effects on the host. For example, large quantities of polysaccharide can decrease antibody production [235] and lymphocyte proliferation [236]. Purified GXM was also shown to impair T cell activation, either via dendritic cells exposed to this polysaccharide or by directly rendering T cells unresponsive to polyclonal stimuli upon contact with this polysaccharide [237]. In addition, brain microglial cells appear to be insensitive to stimuli, such as LPS, after polysaccharide contact [238].

GXM can interact with TLR4 in macrophages, inducing the expression of FasL, which can promote T cell apoptosis through the activation of caspases 8 and 9 [239]. Macrophages can also undergo apoptosis themselves through the expression of both Fas and FasL receptors.

Neutrophil migration is also impacted by GXM, as this molecule decreases surface levels of the TNFα receptor and L-selectin and engages CD18, which is part of the integrin LFA1, to impact the tight binding of those cells to the activated endothelium [240].

Cryptococcus virulence relies upon the externalization of several molecules, such as melanin, enzymes and polysaccharides. However, several virulence-related molecules do not contain any secretion targeting sequences. Nevertheless, these molecules are commonly observed in the extracellular environment. Rodrigues and colleagues [241] described the presence of extracellular vesicles in *C. neoformans* culture supernatants, and these compartments revealed a complex composition of extracellular vesicle cargo, including polysaccharides, active enzymes, nucleic acids, pigments, toxins, and lipoproteins [242].

The secretion of vesicles during infection has proven difficult to assess. However, indirect observations, such as the recognition of EV proteins by antibodies present in serum from cryptococcosis patients [243], reinforces the idea that these structures play an important role in cryptococcal infection, possibly by acting as virulence delivery bags, as proposed previously [243].

Virulence factors described above and other cryptococcocal components impacting disease progress are directly linked to immunological mechanism of host responses. The immune response against *Cryptococcus* is out of the scope of this chapter and will not be discussed here. Major mechanisms, however, are summarized in Table 6.2.

Table 6.2 Summary of major immune mechanisms against *Cryptococcus*

Cell type	Cell phenotype	Effector molecules	Protection mechanisms	Experimental host phenotype	Fungal evasion mechanisms
Macrophage	M1	TNF, IL-12, NO, ROS	Oxidative killing by ROS and NO [244–246]; cytokine-induced immune response modulation [230, 247–249]	Associated with increased survival and decreased fungal burden [244]	Apoptosis induction [250]; phagocytosis inhibition [223, 224, 251, 252]; protection against ROS and NO [230, 253–255]; M2 polarization induction [255–260]; cytokine production inhibition [227, 261]
Macrophage	M2	IL-10, TGFβ, arginase-1	–	Associated with decreased survival and increased fungal burden [256, 258, 260]	Efficient intracellular replication [262–265]; benefits from phagolysosome low pH [266]; nonlytic exocytosis [267–269]; CNS dissemination (“trojan horse”) [222, 270, 271]
Neutrophil	–	ROS, sphingomyelin synthase	Intracellular killing by ROS [272]; extracellular killing [273]; yeast removal from brain microvasculature [274, 275]	No influence in fungal burden; may increase immunopathology [195, 276, 277]	Phagocytosis inhibition; protection against ROS [230]; NETs inhibition [274]; migration inhibition [240, 278]
Innate Lymphoid cells	ILC1 (NK)	IFNγ, perforin	Extracellular killing by perforin-rich granules [279–281]; growth inhibition [282, 283]; early production of IFNγ and macrophage activation [284–286]	–	–
Innate Lymphoid cells	ILC2	IL-5, IL-13	–	Associated with M2 macrophages, increased fungal burden and increased immunopathology [287]	–

Cell type	Cell phenotype	Effector molecules	Protection mechanisms	Experimental host phenotype	Fungal evasion mechanisms
Dendritic cells	–	TNF, IL-6, IL-12, IL-23, ROS, CD40, D80, CD86	Intracellular oxidative killing [188, 288, 289]; growth inhibition [188, 290]; antigen presentation [291]	Immunization with pulsed DCs increases survival, decreases fungal burden and reduces immunopathology [292, 293]	Phagocytosis inhibition [225, 294]; inhibition of cytokine and chemokine production, antigen presentation and costimulatory molecules expression [288, 294–296]
T CD4+ Lymphocytes	Th1	TNF, IFNγ	Stimulates oxidative killing (ROS and NO) by M1 macrophages [244, 256]	Associated with M1 macrophages, increased survival, and decreased fungal burden [244, 247]	Inhibition of proliferation [236, 237]; direct induction of apoptosis [297]; indirect induction of apoptosis [359, 360]
T CD4+ Lymphocytes	Th2	IL-4, IL-5, IL-13, IL-10	–	Associated with M2 macrophages [298–301]; Associated with decreased survival, increased fungal burden and increased immunopathology [300–302]	Inhibition of proliferation [236, 237]; direct induction of apoptosis [297]; indirect induction of apoptosis [239, 250]
T CD4+ Lymphocytes	Th17	IL-17, IL-22, IL-23	Leucocyte migration; Th2 polarization inhibition [303, 304]	No influence in survival, no influence in fungal burden, limits Th2-driven inflammation [300, 303]	Inhibition of proliferation [236, 237]; direct induction of apoptosis [297]; indirect induction of apoptosis [239, 250]
T CD8+ Lymphocytes	–	Granulysin	Killing by granulysin release [305, 306]	Associated with increased survival and decreased fungal burden [307]	–
B Lymphocytes	–	Antibodies	Opsonization [308, 309]; increases killing by phagocytes [282, 283]; growth inhibition [310, 311]; inhibits fungal budding [312, 313]; inhibits capsule formation [310]; inhibits GXM release [314]; inhibits biolfim formation [315]	Associated with increased survival, decreased fungal burden and restriction of detrimental inflammation [316–321]	–

6.10 Final Considerations

Cryptococcal meningitis is considered to be a global problem, based on the high incidence and mortality estimated by the US Centers of Disease and Prevention (CDC). Although AIDS patients in poor countries are the most affected by cryptococcosis, healthy individuals were victims of important outbreaks in the US and Canada [322]. The annual deaths caused by this infectious disease could be decreased to 70,000 if rapid and accurate diagnostics and appropriate fungal treatments were available for all people, especially in high-burden countries [5]. To reach this goal, investments in research, development, and innovation are required to better understand important aspects related to *Cryptococcus* and cryptococcosis and to improve antifungal therapies and diagnostic tools.

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Chapter 7
Pneumocystis jirovecii and Pneumocystosis

Olga Matos, Ana Luisa Tomás, and Francisco Antunes

Abstract *Pneumocystis jirovecii* pneumonia (PcP or pneumocystosis) remains a major cause of respiratory illness among immunocompromised patients, especially those infected with the human immunodeficiency virus (HIV). *P. jirovecii* infection has also been detected in healthy individuals and people with chronic lung diseases, raising the possibility that the spectrum of illness caused by this pathogenic fungus may extend well beyond the immunocompromised host. In industrialized countries, while the number of acquired immunodeficiency syndrome (AIDS) patients suffering from PcP decreases, there is a rising number of non-HIV immunocompromised patients at risk for life-threatening PcP. In contrast, in developing countries a limited number of epidemiological studies have evaluated PcP prevalence; however, recent reports have described an increased rate of the disease in AIDS patients in Africa, Asia and South America.

After nearly three decades of intensive research, a significant progress has been made in the understanding of PcP; however, this disease continues to be a challenge for clinicians and microbiologists; in particular, the processes of detection and interpretation of the PcP testing, because even the most current diagnostic methods cannot adequately differentiate between colonized (asymptomatic) and symptomatic patients.

This chapter reviews the basic biology of *P. jirovecii* and the epidemiology of the caused infection, including genetic diversity. It also discusses pathogenesis and host defences against *P. jirovecii*, and the clinical presentation in HIV-infected and non-HIV-infected immunocompromised patients. Attention will be devoted to classic and modern approaches for diagnosis, as well as to treatment and prophylaxis of

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PcP. This chapter is designed to present a balanced, comprehensive, and concise examination of the latest advances in the field of *P. jirovecii* pneumonia, and to contain the most up-to-date references.

7.1 Introduction

Pneumocystis jirovecii (formerly *Pneumocystis carinii* f. sp. *hominis*) is an atypical fungus causing pneumonia in humans. This microorganism was first reported in Brazil, in 1909 and 1910, by Carlos Chagas and Antonio Carini, respectively, who misidentified the organism found in murine lungs with stages in the life cycle of *Trypanosoma cruzi* [1, 2]. Antonio Carini, doubting that the cysts were part of the life cycle of *T. cruzi*, sent his specimens to the Pasteur Institute in Paris, where in 1912, the researcher duo of Pierre and Marie Delanoë described the organism identified as a new biological entity, and suggested the name *Pneumocystis carinii* because cysts, their characteristic morphological form, were found only in the lungs of hosts and as a tribute to Carini [3].

For several decades this organism was considered an enigmatic lung pathogen, and in the beginning of the 1980s of the twentieth century it became one of the leading causes of death in the pandemic of acquired immunodeficiency syndrome (AIDS) [4].

Pneumocystis was classified for many years as a protozoan, because of the histological characteristics of its life cycle forms, trophozoite and cyst, inability to maintain in vitro cultures of the organism, successful response to treatment with anti-parasitic drugs and by contrast not responding to a broad spectrum of antifungal agents, such as amphotericin B, and the number of copies of ribosomal ribonucleic acid (rRNA) genes, having only one nuclear gene unlike other fungi studied, which have hundreds of copies for this gene [5]. Later, based on DNA studies it was reclassified as an atypical fungus of the *phylum* Ascomycota [6–9]. *Pneumocystis* infects a variety of mammalian hosts, and the human form because of its host specificity was renamed *Pneumocystis jirovecii* in honor of Otto Jirovec, who linked it to epidemics of interstitial plasma cell pneumonia in neonates in Europe [7], while *P. carinii* is now reserved for the species affecting rats.

The life cycle of *Pneumocystis*, which develops extracellularly in the alveolar cavities of mammals, is not completely defined, containing an asexual mode of replication via binary fission of the trophic form (formerly called 'trophozoite') and a sexual mode of replication that gives rise to a cystic form or ascus containing eight spores [10]. The asexual mode of replication was observed in lung tissues of infected rats and humans by ultrastructural transmission electron microscopy studies [11, 12]. Nevertheless, the sexual replication is still putative, based on the presence of a pheromone receptor protein on the surface of some trophic forms, suggesting mating of two haploid trophic forms, giving rise to a diploid zygote that progresses through meiosis to produce four haploid nuclei [10, 13]. Meiosis is followed by an

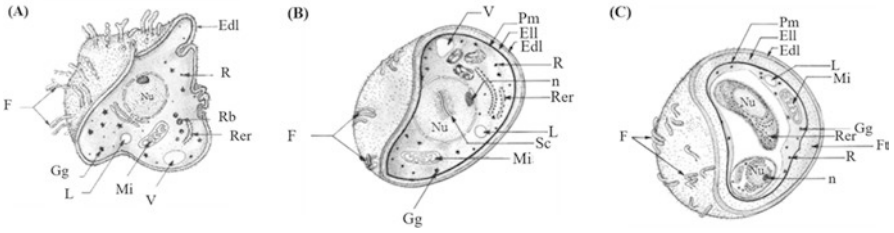

Fig. 7.1 Structure of the developmental stages of *Pneumocystis*. (**a**) Structure of the trophic form of *Pneumocystis*; (**b**) Structure of the sporocyte of *Pneumocystis*; (**c**) Structure of the cystic form of *Pneumocystis*. *F* filopodia, *Gg* glycogen granules, *L* lipid bodies, *Mi* mitochondria, *V* vacuoles, *Rer* rough endoplasmic reticulum, *Rb* round bodies, *R* ribosome, *Nu* nucleus, *Sc* synaptonemal complex, *n* nucleolus, *Pm* plasma membrane, *Ell* electron-lucent layer, *Edl* electron-dense layer, *Ft* focal thickening of the electron-lucent layer (adapted from Matos [14])

additional mitosis to produce eight nuclei that will turn into spores by invagination of the cyst cell membranes to produce eight double-membrane spores, and finally excystment occurs with the release of the spores to the alveolar cavity.

Figure 7.1 presents the structure of the developmental stages of *Pneumocystis*. The trophic forms appear as mononuclear, 2–8 μm in diameter, mostly haploid eukaryotic cells, amoeboid in shape, presenting a thin cell wall consisting of an electron-dense single layer (Fig. 7.1a). They attach specifically to type-1 epithelial alveolar cells, emitting cytoplasmic expansions or filopodia that may penetrate deeply into the cytoplasm of the host cell. However, no disruption of host cell membrane results from either attachment or filopodial activity [15, 16]. The intermediate sporocyte, probably diploid, measures 4–5 μm in diameter and has initially an oval form (Fig. 7.1b). As the cell matures it becomes spherical and an electron-lucent layer appears between the electron-dense layer and the plasma membrane [12]. It further thickens at the late sporocyte stage to finally produce a thick-walled mature cyst that measures 4–7 μm in diameter (Fig. 7.1c) [12, 15]. Trophic forms are the most abundant of all *Pneumocystis* lifecycle stages, representing 90–95% of the total population in the lungs of hosts with PcP [15].

Pneumocystis jirovecii is usually found to the lungs, although extrapulmonary sites have been reported [17].

7.2 Epidemiology of *Pneumocystis jirovecii* Infection

7.2.1 Preponderance of PcP

P. jirovecii is often found in the lungs of healthy people; however, PcP is rare in persons with an intact immune system [18]. As such, prior to the human immunodeficiency virus (HIV)/AIDS pandemic, PcP was an uncommon disease. In the 1980s, with the onset of that pandemic, there were significant changes in the incidence of

Table 7.1 Summary of risk factors for *Pneumocystis jirovecii* pneumonia in different underlying conditions

Underlying condition	Risk factor	References
HIV infection/AIDS	CD4+ T count <200 cells/μl	[22, 23]
	CD4+ T cell percentage < 14%	[23, 24]
	History of oral thrush	[22–24]
	History of previous PcP	[23, 24]
	Viral replication not controlled	[22–24]
	Recurrent bacterial Pneumonia	[23, 24]
	Unintentional weight loss	[23, 24]
	Tobacco	[25]
Haematological malignancies	CD4+ T count <200 cells/μl	[26]
	Lymphocytopenia	[26, 27]
	Steroid and cytotoxic therapy	[26–28]
	Administration of purine analogues	[26, 29]
	Chemotherapy with R-CHOP14 or high-dose methotrexate	[27, 30]
	Administration of Monoclonal antibodies	[26, 30, 31]
Solid organ transplantation	CD4+ T count <200 cells/μl	[32, 33]
	Immunosuppressive therapies	[33–35]
	CMV disease	[33, 36, 37]
	Co-morbidity (tuberculosis, bacterial pneumonia, hepatitis C)	[38]
	Allograft rejection	[33, 35, 38]
	Neutropenia	[33]
	Exposure to cases of PcP	[33, 39]
Solid tumors	Corticosteroid therapy	[40, 41]
	Chemotherapy	[42]
	Radiotherapy	[43]
Inflammatory and collagen-vascular disease	Steroid and cytotoxic therapy	[44]
	Administration of monoclonal antibodies	[45, 46]

PcP, as it turned from a rare infection into a common pneumonia [19–21]. In this period, before the widespread use of PcP prophylaxis and combination antiretroviral therapy (cART), PcP occurred in 70–80% of patients with AIDS [22] and the disease was associated with a mortality rate of 20–40% of these patients. Approximately, 90% of PcP cases occurred in patients with CD4+ T cells count <200 cells/mm^3, or with a percentage < 14%. Other risk factors associated with a higher risk of PcP included, oral thrush, previous episodes of PcP, recurrent bacterial pneumonia, unintentional weight loss, higher plasma HIV RNA levels and tobacco use. Table 7.1 summarizes the risk factors for PcP in HIV-infected and in non-HIV-infected persons. However, with the introduction of PcP prophylaxis since late 1980s, and the development and availability of cART since mid-1990s, there was a decline in PcP cases in the United States and Western Europe [21]. The recent incidence among patients with AIDS in the United States and Western Europe is <1

case per 100 person-years [47]. Most of the recent cases occur in patients who are unaware of their HIV-status or are not receiving ongoing care for HIV due to non-adherence to treatment, those with active substance abuse or psychiatric illness, those who are not receiving or responding to antiretroviral therapy or prophylaxis because of factors related to pharmacokinetics, or unexplained biologic factors, and in those with advanced immunodeficiency (CD4+ T cells <100 cells/mm^3) [48–50]. Data from the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) Europe point to PcP as the most common AIDS-defining illness in Europe in 2015, recorded in 20.0% of cases, followed by esophageal candidiasis (11.2%) and wasting syndrome due to HIV (10.8%) [51].

In developed countries, the marked decrease in incidence of PcP in HIV-infected patients is being counterbalanced by the emergence of the disease in non-HIV-infected populations, namely haematologic patients; solid organ transplant recipients, including renal transplants; solid malignancies; rheumatoid conditions; patients with connective tissue disorders; those receiving immunomodulatory therapies; or patients with pre-existing chronic lung conditions or with less typical scenarios, such as Dengue fever [27, 28, 52–58]. In developing countries, where most HIV-infected persons reside, PcP is an emerging disease with high prevalence and is poorly controlled, since the access to cART and PcP chemoprophylaxis is still limited; and possibly also due to lack of PcP diagnostic resources and expertise [59–62]. Recent reports described an increased number of PcP cases in Africa, Asia and South America, with differences in the observed frequencies [61]. These variations are possibly due to differences in study design. Including heterogeneity in the patient population and the diverse laboratory methods used [61, 62].

7.2.2 *Circulation and Transmission of* P. jirovecii

P. jirovecii is a ubiquitous human-specific pathogen. Initial infection with *P. jirovecii* usually occurs in early childhood; about two-thirds of healthy children have antibodies to *P. jirovecii* by ages 2–4 years [63, 64].

Transmission of *Pneumocystis* can be aerial, and has been suggested based on rodent studies. An article published recently, using the rodent model, shows that the cystic forms (but not the trophic forms) can be transmitted by aerial route from host to host [65]. Also, the detection by molecular methods of *Pneumocystis* DNA in air samples collected in rooms and wards where patients with PcP were hospitalized [66, 67], and case clusters in immunocompromised patients support this transmission route [38, 65, 68, 69]. Vertical transmission of *P. jirovecii* in humans, although controversial, was suggested by some authors [70–73]. *P. jirovecii* is human specific and cannot be acquired from other animal reservoirs [10]; but, since it is accepted to be ubiquitous, the existence of environmental reservoirs of this pathogen cannot be set aside [74].

Pneumocystis pneumonia may probably occur by both de novo acquisition of *P. jirovecii* infection and reactivation of a latent infection [75, 76]. Due to the high

seroprevalence of anti-*P. jirovecii* antibodies in children, it was initially assumed that latent organisms were acquired by the host early in childhood [63]. Once acquired, the microorganism remained in the lungs of the immunocompetent host without causing symptoms, since the immune system was able to successfully control the infection, although it was not able to eliminate the microorganism [77, 78]. However, in a situation of immunosuppression, latent infection was reactivated and the disease was manifested. Other studies support the idea that *P. jirovecii* can be acquired de novo, contrary to the theory of latent infection as a primary infection mechanism. Wakefield et al. used sequential sampling and observed that latency is not the rule after an episode of PcP [79]. Other studies showed that genotypes distribution patterns differed at each of the cities where the study was performed and correlated with the patients' place of diagnosis but not by place of birth, suggesting that most cases of PcP do not result from infections acquired early in life [80]. It suggested that at least some infections must be actively acquired from a relatively common source (humans or the environment).

Meanwhile, pulmonary colonization with *P. jirovecii* in patients with diverse levels of immunodeficiency, primary respiratory disorders, or even in the immunocompetent general population, has been reported [18, 79, 81, 82]. *Pneumocystis* colonization occurs in persons without signs and symptoms of acute pneumonia. Because of the low organism burden associated with colonization, polymerase chain reaction (PCR)-based techniques are most often necessary to determine the presence of the organism's DNA [83, 84]. This is an important epidemiological issue, especially in terms of transmission, since colonized populations may be a potential reservoir of the fungus and pass on to others, putting those people at risk especially if they are immunocompromised.

7.2.3 Molecular Epidemiology of P. jirovecii

In the absence of a well-established culture system to isolate and maintain alive *P. jirovecii* organisms, previous efforts to describe differences among isolates have been performed using molecular methodologies. It has been shown that specific single-nucleotide polymorphisms (SNPs) in *Pneumocystis* genes are highly informative markers for comparative analyses between the given genotype and specific clinical or epidemiological data [80, 85–89].

A variety of typing methods have been used for *Pneumocystis* genetic analysis. Single-strand conformation polymorphism [90–92], restriction fragment length polymorphism [93] and type-specific oligonucleotide hybridization [94] were initially used, but have been replaced by DNA sequencing schemes. The multilocus sequence typing approach has become the most frequently used method [95], since it offers many advantages, such as reproducibility, possibility of exchanging data between different laboratories, as well as observation of polymorphisms at many loci at the same time [88]. However, technologies based on Sanger sequencing have difficulty to detect infections with more than one genotype so, amplification of

genomic DNA associated with single base extension technology centered on the detection of specific SNPs was developed [96, 97]. This technique detects mixtures of genotypes with a higher sensitivity than technologies based on Sanger sequencing. Recent studies have also proposed the analysis of microsatellite markers, also called short tandem repeat analysis, to genotype *P. jirovecii* [98, 99].

With these techniques, several polymorphic loci have been identified and characterized. Two loci have been most widely studied with the purpose of determining the genetic diversity of this organism: the mitochondrial large subunit ribosomal RNA (*mtLSUrRNA*) and the internal transcribed spacer (*ITS*) regions (ITS1 and ITS2) of the nuclear rRNA operon [88]. The *mtLSUrRNA* gene is also the locus used most widely for PCR-based detection of *P. jirovecii*, as it is present in a large number of copies [100]. *ITS*1 and *ITS*2 show the highest level of sequence variation in *P. jirovecii*, and are considered to be the most discriminatory DNA regions for genotyping studies [101]. Nevertheless, the cytochrome b (*CYB*), the β-tubulin (*β-TUB*), the thymidylate synthase (*TS*), the thioredoxin reductase (*TRR*1), the dihydrofolate reductase (*DHFR*), the dihydropteroate synthase (*DHPS*) and the superoxide dismutase (*SOD*) loci, are also variable regions of the *P. jirovecii* genome suitable to be used in identification and characterization of *P. jirovecii* haplotypes correlated with drug resistance and PcP outcome [86, 88, 89, 96, 97, 102–111]. The major genetic variations in these loci associated with clinical outcome are summarized in Table 7.2.

These specific polymorphic loci have been studied with the purpose of understanding important properties of isolates like biodiversity [80, 85, 91, 92, 115, 116], virulence [87, 89], drug susceptibility [103, 104, 106, 113, 117], geographical

Table 7.2 The major genetic variations of *Pneumocystis jirovecii* associated with clinical outcome to date

Locus	SNP position or Genotype	Clinical outcome	References
DHPS	165, 171	Associated with sulpha drugs prophylaxis failure; Failure of both trimethoprim-sulfamethoxazole treatment and trimethoprim-sulfamethoxazole or dapsone prophylaxis.	[86, 104, 107, 111, 112]
	55, 57	Associated with trimethoprim-sulfamethoxazole resistance.	[105, 106, 109, 112]
DHFR	312, 381	Associated with PcP infection high burden and drug resistance.	[89, 105, 113]
SOD	110, 215	Associated with severity of PcP episodes.	[89, 96, 97, 114]
mtLSUrRNA	85	Associated with high *P. jirovecii* burden levels and unfavourable follow up of infection.	[96, 97]
CYB	SNPs in the Q_o site	Associated with atovaquone prophylaxis failure.	[89, 103, 108]
ITS regions	Genotype Ne	Associated with treatment failure and bad clinical outcome	[110]

distribution of genotypes [80, 114, 117–119], and modes of transmission [67, 68, 71, 73, 75, 80, 82, 120]. Concerning biodiversity, studies demonstrated that patients could be infected with a single genotype, while others could be coinfected by more than one *P. jirovecii* genotype [80, 85, 91, 92], and that new *P. jirovecii* genotypes have been identified in several loci [116]. In terms of virulence and drug resistance, data suggest that these factors may be dependent on multiple *P. jirovecii* genotypes and therefore on the association of multiple polymorphisms that occur in various regions of the genome of the organism. The different associations of polymorphisms, could give the organism distinct characteristics, influencing the presentation and clinical course of PcP [89, 105, 114, 116]. However, studies performed on drug susceptibility strongly suggest that mutations in *P. jirovecii* therapeutic targets are selected by drug pressure [86, 88, 89, 102, 104, 105, 107, 108, 111, 112]. Gene mutations that confer clinical resistance to sulpha/sulphone agents on therapeutic dose are not yet reported [60].

Studies on geographical distribution of genotypes have shown that the isolates could be clustered due to geographic differences but are also dependent on clinical characteristics of the populations studied [88, 118, 119]. On the other hand, studies in transmission of *P. jirovecii* have suggested and supported theories about airborne route [67, 120], the existence of asymptomatic carriers [18, 79, 81, 82], vertical transmission [71, 73], and de novo acquisition of the infection [18, 79, 80, 114]. These advances in the understanding of the genetic diversity of *P. jirovecii* raised important questions, such as whether some organisms are more pathogenic than others and whether resistant or more virulent genotypes spread between hosts. However, despite the wide application of molecular methods to study this organism, inconsistent results in association of specific genotypes to the clinical course or outcome of PcP are common [88]. This is due to the lack of guidance and standardization regarding the appropriateness of different methods and loci to apply in these studies. The standardization of molecular typing guidelines would facilitate comparing results reported by different laboratories and, consequently, the analysis of the correlation between genotypes and a specific clinical picture caused by their invasion may be used to predict the PcP outcome, as well as to facilitate the decision about treatment and prophylaxis of PcP.

7.3 Pathogenesis and Host Immune Response During *P. jirovecii* Infection

7.3.1 Pathogenesis of P. jirovecii *Infection: Lung Injury*

The pathogenesis of *Pneumocystis* pneumonia can be divided into four stages: establishment of the infection, organism proliferation, changes within the alveolar microenvironment and host immune response [121, 122].

P. jirovecii is an atypical fungus exhibiting a highly defined tropism for human lungs, where it exists primarily as an extracellular alveolar pathogen [122, 123]. Thus, the initial event in the establishment of the infection is the preferential attachment of the trophic forms of *Pneumocystis* to type-I epithelial cells and, to a lesser extent, to alveolar type-II epithelial cells, through interdigitation of their cell membranes with those of the host cells [124, 125]. This attachment is facilitated by both fibronectin and vitronectin that are present in the alveolar lung fluid [126–128]. Integrins, which are fibronectin receptors, may also play a role in *Pneumocystis*-epithelial cell interactions [129].

This binding of *Pneumocystis* to cell matrix proteins, triggers altered gene expression that results in increased *Pneumocystis* proliferation and also inhibits the growth of lung epithelial cells [125, 130]. Otherwise, the impaired cellular immunity is generally considered the major predisposing factor for organism proliferation.

Consequently, as infection evolves, the alveolar microenvironment is impaired by direct *Pneumocystis*-mediated lung damage and also by initiation of subsequent host immune and inflammatory response that injures the lung [131]. In the first days of infection, the inflammatory reactions result in cellular alterations with limited lesions. As the infection evolves, characteristic features of the infection begin to appear, such as the presence of an exudate that fills the alveoli uniformly and the observation of focal lesions of necrosis in the alveolar type-I epithelial cells, hypertrophy of the alveolar cells and filling of the alveolar space by injured cells and *Pneumocystis* forms [121]. Although the mechanisms of direct injury are unclear, studies indicate that the release of several *Pneumocystis*-derived proteolytic and glycolytic enzymes may mediate lung collateral damage while performing their intended functions in the life cycle of this pathogen [132–136]. On the other hand, another mechanism by which *Pneumocystis* evades its host is by antigenic variation. This microorganism has a major surface glycoprotein (Msg) represented by a family of related glycoproteins encoded by about 50–80 genes depending on the species [137, 138]. Therefore, *Pneumocystis* is able of expressing one isoform of Msg at a given time of infection and switch to another in the course of infection, which allows it to hide from the host immune system and to proliferate.

Finally, the host immune response elicited by *Pneumocystis* infection is proved to be an important determinant of the ultimate outcome of the infection. Although infection is necessary to cause PcP, certain aspects of the immune response cause or exacerbate disease symptoms. Although all patients that develop clinical PcP are immunocompromised, patients with greater immune competency may better control the number of the organisms, but suffer greater inflammatory injury. Conversely, patients with more severe immunosuppression may suffer fewer inflammatory complications but are more susceptible to direct effects of higher organism burdens. That is why immunocompromised non-HIV-infected patients usually have a more rapid onset of the disease with exacerbated clinical manifestations than those infected with HIV, in whom PcP usually has a subacute presentation with more insidious involvement [123, 139, 140].

Thus, although the specific mechanisms of PcP-related immunopathogenesis are not fully defined, the contribution of inflammation and the immune response to this disease process seems to be clear.

7.3.2 Immunity Against P. jirovecii *Infection*

Because of the inability to reliably culture *Pneumocystis* in vitro, investigations into events associated with immunity against *Pneumocystis* has been relegated to insights gained from animal models. These studies have shown that immunity to *Pneumocystis* is a dynamic interplay between nearly every arm of the immune system. Innate cellular immunity is absolutely critical to providing the initial recognition event that precipitates an immune response and to clearing the pathogenic organism from the host. Adaptive immunity, in turn, is crucial for amplifying the innate cellular response, controlling the invader as well as maintaining long-term memory protection.

In the beginning of the infection, as *Pneumocystis* has tropism for the alveolar spaces of the lung, the alveolar macrophages (AM) are the first line of host defence to control this microorganism. There are many studies showing an inverse correlation between macrophage numbers and severity of PcP [141–143] since AM serve as an important host component to recognize the organism and play a central role in directing the inflammatory and adaptive immune responses to this pathogen. The recognition and uptake of *Pneumocystis* by AM enables them to directly kill both trophic and cystic forms, and occurs through multiple receptor systems, such as the mannose receptor that interacts with gpA/Msg on the surface of *Pneumocystis* and dectin-1 receptors that recognize the β-glucan moieties in the fungal cell wall [144–147]. Once internalized, several studies have shown that *Pneumocystis* is incorporated into phagolysosomes and subsequently degraded by induction of the oxidative burst and hydrogen peroxide generation [143, 145, 147, 148]. In addition to degrading *Pneumocystis*, AM also produce a wide variety of pro-inflammatory mediators that, in addition to assisting in the eradication of this organism by the host and in the activation of the adaptive immune system, also promote inflammatory responses that lead to lung injury [143].

There have been fewer studies on the role of other phagocytes participating in the innate response to *Pneumocystis*; however, dendritic cells have shown ability to protect mice from *Pneumocystis* pneumonia, since these cells can induce anti-*Pneumocystis* IgG antibodies and protect $CD4^+$ T cell-depleted mice from *Pneumocystis* pneumonia [149]. Also, neutrophils are thought to play a potentially negative role in the innate responses against this pathogen, since HIV-infected patients had significantly fewer neutrophils recovered in their bronchoalveolar lavage compared to non-HIV-infected patients, which corroborates with the exacerbated clinical manifestations and inflammatory complications of PcP in non-HIV-infected patients [139, 150]. Even more, episodes of severe inflammation caused by

Pneumocystis infection in the lungs of HIV-infected individuals have been synonymous with the presence of elevated neutrophil counts [151, 152].

When the adaptive immunity is activated, the $CD4^+$ T cells have the central role, having this been well-established with a strong correlation between their diminished numbers in infected individuals and an increased risk of PcP development [22, 32]. While the level of $CD4^+$ T cells clearly correlates with the control of *Pneumocystis* infection, the mechanism by which these cells contribute to clearance of the organism is less clear. Studies with severe combined immunodeficiency (SCID) mice showed that $CD4^+$ T cells play an essential role in the recruitment and activation of effector cells, which are responsible for eliminating the organism. These animals develop severe *Pneumocystis* infection despite the presence of functional neutrophils and macrophages [153, 154]; however, when reconstituted with $CD4^+$ T cells from the spleen, they regain the ability to effectively clear the infection, indicating that $CD4^+$ T cell-mediated activation of macrophages is required for effective host defence against *Pneumocystis* [155, 156]. On the other hand, the specific T-cell subsets required for successful resolution of the infection remain unclear, as authors show the increased recruitment of T helper (Th) lymphocytes, both type 1 (Th1) and type 2 (Th2), to the lungs following experimental *Pneumocystis* infection in mice, besides a predominant frequency of Th2 cells response [157, 158].

Relatively to $CD8^+$ T cells, they work in cohort with $CD4^+$ T cells in the normal, effective immune response to *Pneumocystis* infection. However, there has been much debate on whether their involvement is protective or detrimental, particularly in the setting of $CD4^+$ T-cell deficiency. Authors have shown that in mice selectively depleted of $CD4^+$ T cells, infection with *Pneumocystis* leads to an influx of $CD8^+$ T cells in the lungs, though the mice fail to control the infection [159]. However, mice depleted of both cell types had a higher organism burden than those depleted of $CD4^+$ T cells alone, which suggests that $CD8^+$ T cells have a partial protection role in the context of $CD4^+$ T-cell deficiency or dysfunction [160].

The role of gamma delta (γδ)-T cells in *Pneumocystis* infection has not been studied so extensively. However, recent studies that investigate *Pneumocystis* infection in wild-type mice observed that γδ-T cells were recruited in high numbers to the lungs during *Pneumocystis* infection [161]. They demonstrated that γδ-T cells-deficient mice had augmented resolution of *Pneumocystis* pneumonia correlated with augmented $CD8^+$ T-cell recruitment as well as higher production of interferon gamma (IFN-γ). These results suggest that one role of γδ-T cells may be to influence recruitment of $CD8^+$ T cells to the lungs and possibly modulate their inflammatory effect.

The importance of B cells in protection from *Pneumocystis* infection is evident from experimental animal models of B cell deficiencies [162–164], as well as clinical studies where PcP has been reported following B cell-targeted chemotherapy [165, 166]. In addition to obvious effects on antibody production, the increased susceptibility of these patients may also be due to the fact that B cells can function as critical antigen presenting cells during the infection [164]. Thus, the question arises as to whether B-cell-mediated protection against *Pneumocystis* is primarily antibody mediated, or is produced through B-cell activation of $CD4^+$ T cells. Reports

of PcP in patients with genetic mutations affecting immunoglobulin production [167], along with the observations that the majority of healthy adults are seropositive for *P. jirovecii*, rarely presenting symptomatic infection [84], provide strong evidence that anti-*Pneumocystis* humoral immunity is a critical component of protective immunity to this pathogen. On the other hand, studies demonstrated that SCID mice that received $CD4^+$ T cells from normal mice were able to clear *Pneumocystis* infection more efficiently than mice that received $CD4^+$ T cells from B cell-deficient mice [164]. Together, these studies show that antibodies play a crucial role in clearing *Pneumocystis* from the lungs, but also indicate that B cells play a critical role in priming and activating $CD4^+$ T cells, and that this interaction is required for clearance of *Pneumocystis* in the lungs.

In addition to all these cellular immunity participants, various cytokines and chemokines, released by all nucleated cells, participate in the host response and provide a mechanism for intercellular communication that is not dependent on cell contact [122, 147, 168]. They are involved in every aspect of host defence against infection and play a determinant role during resolution of PcP. They also participate in the maintenance of a crucial balance between the clearance of *Pneumocystis* from the lungs and modulate the inflammation response to this pathogen. The main groups of cytokines and chemokines implicated in host defence against *Pneumocystis* are tumour necrosis factor alpha (TNF-α) [169, 170]; interleukin-1 (IL-1), IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) [171–173]; IL-10, IL-12 and IL-23-IL-17 axis [174–176]; monocyte chemoattractant protein-1 (MCP-1), lymphotactin and macrophage inflammatory protein 1 and 2 (MIP-1, MIP-2) [177]; and IFN-γ [178, 179].

The interplay of the organism, host inflammatory cells, release of cytokines, generation of toxic metabolites, and involvement of both cellular and humoral immunity is complex. However, the understanding of the implications of the host immune response in the inflammatory process to *Pneumocystis* infection is important because there is strong evidence that pulmonary impairment and mortality are more closely associated with lung inflammation than organism burden [131].

7.4 Clinical Manifestations of PCP

P. jirovecii produces an often-fatal pneumonia in a variety of immunocompromised individuals. It became evident in the HIV-infected population that the infection can be disseminated and, although rare, almost any organ can be involved. The frequency of extrapulmonary *Pneumocystis* in HIV-positive patients seems to be <2.5% [180]. This can occur without concurrent pulmonary involvement and *P. jirovecii* has been found invading lymph nodes, spleen, liver, gastrointestinal tract, pancreas, eye, ear, adrenal and thyroid glands, heart, central nervous system and others [17, 180–182].

The diagnosis of PcP depends on a high index of suspicion. PcP should be suspected in the presence of conditions known to be associated with the disease in HIV-infected and non-HIV-infected patients [183] (Table 7.3).

Table 7.3 Main underlying conditions of *Pneumocystis jirovecii* pneumonia in non-HIV infected patients [183]

Underlying conditions	Percentage of total cases in non-HIV-infected patients
Haematological malignancies	32.5
Solid tumours	18.2
Inflammatory diseases	14.9
Solid organ transplant	12.3
Vasculitis	9.7
Miscellaneous	12.3

Table 7.4 Clinical and laboratory features at presentation of *Pneumocystis jirovecii* pneumonia in HIV-infected and non-HIV-infected patients [185]

	HIV-infected patients	Non-HIV-infected patients
Temperature ≥ 38.0 °C	76%	92%
Dyspnoea	68%	66%
Cough	81%	71%
Sputum	23%	21%
Median duration of symptoms (days)	28	5
Rales	30%	34%
Abnormal chest radiography	96%	92%
Hypoxemia (mmHg)	69	52

Before PcP associated with AIDS, two distinct modes of onset were described. One was the insidious onset of either cough or tachypnea progressing to dyspnea and cyanosis with either no fever or a mildly elevated temperature, always associated with a clinical course of 3 or more weeks, and in some instances lasting over 2 months. This clinical pattern was described in children with congenital immunodeficiency [184]. The other mode of onset was abrupt, with symptoms, usually of fever, often spiking, cough and tachypnea all having their onset within few days of each other. Almost all children and adults on high dose of steroid therapy at the time symptoms began had this clinical picture [184].

In HIV-infected patients, the most common manifestations of PcP are subacute onset of progressive dyspnea, variable temperature elevation, nonproductive cough, and evidence of respiratory distress. In mild cases, the chest is clear, though there may be scattered rales or rhonchi, wheezes, or a change in breath sounds. Acute dyspnea with pleuritic chest pain may indicate the development of a pneumothorax. Oral thrush is a common co-infection. This clinical picture is similar to those described in children with congenital immunodeficiency [184]. Patients who are immunocompromised non-HIV-infected with PcP typically present an abrupt onset of respiratory insufficiency that may correlate with a tapered or increased dosage of immunosuppressant medications [28, 185] (Table 7.4). PcP have a more insidious course in patients with AIDS than in patients with other immunodeficiencies [186].

PcP is often the AIDS-defining illness in patients infected with HIV, occurring most frequently when the $CD4^+$ T cells count <200/mm^3 blood. The mortality rates

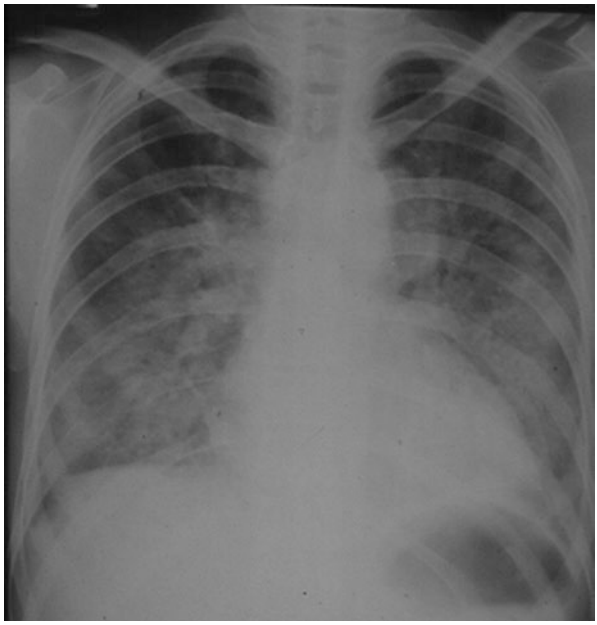

Fig. 7.2 Posteroanterior chest radiograph of a HIV-infected patient with *Pneumocystis jirovecii* pneumonia, demonstrating diffuse bilateral infiltrates

reported in HIV-infected patients for first episodes of PcP is 10–20%, but the rate increases with the need of mechanical ventilation [187]. PcP in non-HIV-infected patients have more neutrophils and fewer *Pneumocystis* organisms in their lungs than patients with PcP associated with AIDS [139]. Despite the availability of effective antimicrobial agents, the mortality rate among patients with PcP in the absence of AIDS is 30–60%, depending on the population risk [28, 188]. Hypoxemia is the most characteristic laboratory abnormality, and elevation of lactate dehydrogenase (LDH) levels to 500 mg/dL is common but nonspecific [189, 190].

Chest radiographies typically demonstrate diffuse, bilateral, symmetrical interstitial infiltrates, spreading from the hilar region to the periphery with time, in a butterfly pattern [191] (Fig. 7.2). A chest radiograph may be normal in patients with early diseases [192]. Atypical radiographic presentation also occurs (nodules, blebs and cysts, asymmetric disease, and upper locations). In the classic diffuse PcP, all lobes of both lungs are generally involved although segmental distribution has been described. Absence of hylar adenopathy and pleural effusion is also characteristic unless PcP is a complication of a basic disease process in which adenopathy and/or pleural effusion are a part. Interstitial and mediastinal emphysema and pneumothorax may occur often with a fatal outcome [193]. In 13–18% of HIV-infected patients with PcP another concurrent cause of pulmonary dysfunction, such as tuberculosis, Kaposi sarcoma, or bacterial pneumonia should be sought [194, 195].

Radiographs of the lungs are often helpful in the diagnosis of a PcP at a time when there are virtually no physical findings and the only complaints are of cough, tachypnea, and fever. Since the haziness is nonspecific, the radiograph is only helpful in the context of clinical findings favouring the development of PcP. Thin-section

computed tomography (CT) demonstrating patchy ground-glass attenuation increases the likelihood that a diagnostic study, such as bronchoscopy, will demonstrate *Pneumocystis* in patients with mild-to-moderate symptoms and normal or nonspecific radiograph [196, 197].

In HIV-infected patients, the chest radiographs opacities are discrete and interstitial in distribution; in contrast, in patients with other immunodeficiencies radiographs were alveolar and diffuse [198].

Following therapy, radiographs show gradual clearing after a variable latent period, during which the radiograph may actually appear worse, finally becoming completely normal in 6–8 weeks [184].

7.5 Laboratory Diagnosis of PCP

The presumptive diagnosis of PcP is based on: clinical manifestations, pulmonary function testing, arterial blood gas testing (ABG) at rest and after exercise, and nonspecific radiological and laboratory tests. The history and physical examination are part of the evaluation of the patient with pulmonary symptoms. Over the years it was noticed that the presentation of PcP depends on the underlying disease. Thus, in HIV-infected patients the duration of the clinical manifestations is longer and the diagnosis is more difficult than in patients with other immune deficiencies [186]. Pulmonary manifestations are the most frequent in the natural history of PcP. Taking into account that PcP is an interstitial disease causing serious difficulties in gas exchange occurring in the lungs, a partial pressure of oxygen (PaO2) in peripheral blood ≤9.3 kPa (70 mmHg) is indicative of PcP in spite of the fact that 10 to 20% of cases of PcP have normal levels of PaO2 [199]. The measurement of LDH, which increases in the beginning phase of the infection, is a nonspecific laboratory test for PcP diagnosis that can be used as a prognostic tool, and to assess response to PcP therapy [200]. Also, the evaluation of the immune status of the patient is important to determine the risk of developing PcP. This disease occurs most frequently in patients with $CD4^+$ T cells count ≤200/mm^3 blood. This information applies to HIV-infected patients and to non-HIV-infected patients with other immunodeficiency, e.g., cancer patients receiving chemotherapy.

All these elements are useful but nonspecific, neither confirming or nor denying the diagnosis of PcP. For many years *P. jirovecii* could not be cultured –until a culture system to propagate *P. jirovecii* in vitro was developed only in 2014 [201]. Since this culture system still needs to be validated, disseminated and shown to be cost-effective for diagnostic purposes, microscopic visualization of cystic or trophic forms in respiratory specimens with cytochemical staining or immunofluorescent staining with monoclonal antibodies (IF-Mab) are the standard procedures to identify this microorganism and diagnose the disease.

7.5.1 Biological Specimens to Study

The definitive diagnosis of PcP is determined by cyto-histopathological examination of respiratory specimens. These specimens can be obtained by invasive techniques, such as open-lung biopsy (LB), transbronchial biopsy (TBB), bronchoalveolar lavage fluid (BALF) and bronchial secretions (BS), and by less invasive techniques such as induced sputum (IS), spontaneous sputum (SS), nasopharyngeal aspirate (NA) and oropharyngeal washing (OW). BALF and IS are the most widely used clinical specimens for the diagnosis of PcP.

The diagnosis of lung tissue fragments, allows the observation of the microorganism in more than 95% of cases of infection [202]. The BALF—obtained by fiberoptic bronchoscopy—analysis allows the diagnosis in more than 80% of all patients with PcP, and in more than 95% of patients with HIV co-infection [194, 203]. In turn, induced sputum (IS) that is obtained by inhaling 1.8% saline with the aid of an ultrasonic nebulizer, is widely used for patients with AIDS and PcP, but its utility for patients with other forms of immunodeficiency is less defined. Non-HIV immunocompromised patients with PcP have lower burden of organisms, and sputum induction may consequently have lower diagnostic yield in these patients [204]. This technique allows *Pneumocystis* detection in 30–55% of cases of infection, after nonspecific staining, sometimes difficult to interpret [205]. As an alternative to BALF and IS, upper respiratory samples (NA and OW) have been utilized [83, 206–208] and when studied by PCR technologies, have sensitivity above 75% [206, 209].

The diagnosis of extrapulmonary pneumocystosis is possible by demonstrating *P. jirovecii* cystic or trophic forms in affected tissues. Nonspecific or specific staining method can be utilized, or a PCR method to detect *Pneumocystis* DNA in the affected tissues.

7.5.2 Staining Methods

Several staining methods have been described for the diagnosis of PcP. Gomori-methenamine-silver (GMS), Giemsa, or rapid Giemsa-like stains such as Diff-Quik, toluidine blue O (TBO), cresyl echt violet, calcofluor white and IF-MAb. All these methods reveal the characteristic morphology of the cystic and of the trophic forms (Fig. 7.3).

GMS described by Gomori and modified by Grocott and Musto and collaborators [210, 211], has been considered the gold standard technique for the diagnosis of PcP for many years. This reagent selectively stains the *Pneumocystis* cystic form wall that appears dark brown [210] (Fig. 7.3a). TBO stain also has a good affinity for components of the cystic form wall, which stains metachromatically in reddish violet [212] (Fig. 7.3b). GMS, TBO and cresyl echt violet that is similar to TBO, all identify *Pneumocystis* cystic forms but not its other developmental forms. They can

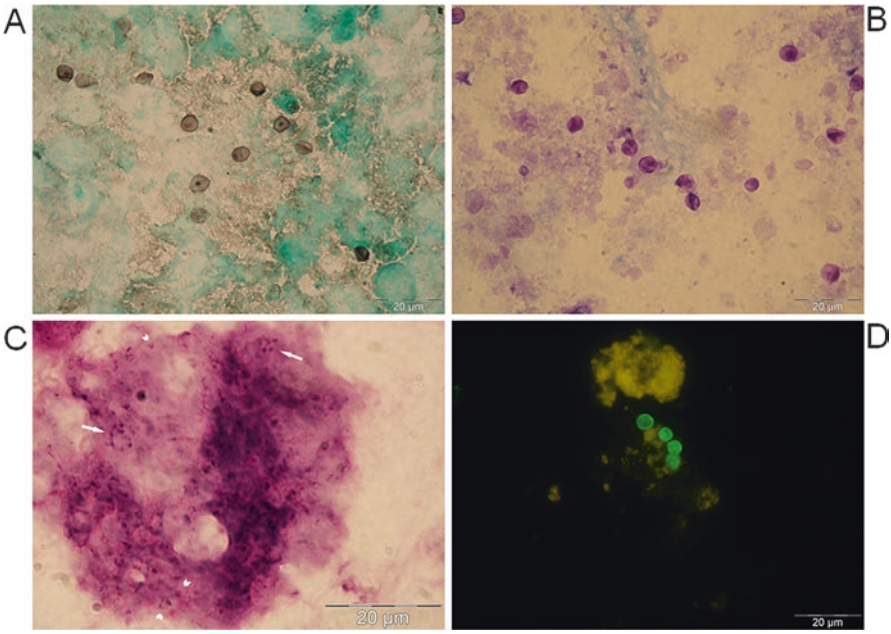

Fig. 7.3 Respiratory specimens with *Pneumocystis* after appropriate staining (magnification ×1000). (**a**) Rat-derived *Pneumocystis* cystic forms stained with Gomori-methenamine-silver (GMS). (**b**) Rat-derived *Pneumocystis* cystic forms stained with toluidine blue O (TBO). (**c**) Clustered trophic and cystic forms of *Pneumocystis jirovecii* in BALF stained with Giemsa. Mature cystic forms (*arrow*), and several trophic forms (*arrowhead*) are quite visible. (**d**) *Pneumocystis jirovecii* cystic forms in BALF stained with immunofluorescent-MAbs anti-*Pneumocystis jirovecii*

be used in any kind of clinical specimens, but they also stain the cell wall of yeasts and other fungi. Therefore, it is required an experienced technician for the reading of the microscopic slides. In turn, calcofluor white is a chemifluorescent agent that nonspecifically binds to β-linked polysaccharide polymers of the cell wall of *Pneumocystis* [213].

Giemsa and Diff-Quik stain the nuclei of all *Pneumocystis* life-cycle stages. They do not stain cystic or sporocytic walls, which appear like a clear peripheral halo around these forms [214] (Fig. 7.3c). These panoptic stains are also used for the diagnosis of other pathogens in the lungs. The reading of the microscopic slides is difficult, especially when dealing with specimens with low fungal burden, requiring the observation by an experienced technician.

The best strategy for a more accurate PcP diagnosis in laboratories that only have these staining methods is the parallel use of a method that stains the cystic form wall and the nuclei of the developing forms, in different smears from the same specimen.

Histologic examination of biopsy specimens for recognition of foamy eosinophilic material in which *P. jirovecii* cystic or trophic forms are embedded can be

achieved by performing GMS, Giemsa or Diff-Quik, TBO, or IF-MAb, that show the morphology of the cysts, easier to identify than the trophic forms in paraffin-embedded tissues.

The IF-MAb specific for *P. jirovecii* began to be implemented in the diagnosis of PcP, since their development in 1986 [215, 216]. They are considered more sensitive and specific than other cytochemical staining methods, which makes them the preferred method to apply in specimens with lower diagnostic yield (IS, BS, NA and OW) [205, 216] (Fig. 7.3d). Actually, because of its reliability, IF-MAb is the most commonly used technique in the diagnosis of PcP [217].

7.5.3 Molecular Diagnosis

In the last two decades, the detection of *P. jirovecii* DNA in clinical specimens by using molecular tools has brought important advances in the diagnosis, epidemiology and management of PcP. PCR assays showed high efficiency to amplify *P. jirovecii* DNA in clinical specimens [80, 117, 217–219].

Molecular techniques play an important role when used in association with less invasive or even noninvasive respiratory specimens (IS, NA, OW) [206, 217, 220]. However, the sensitivity of the PCR assays is directly affected by the implicit lower sensitivity of the noninvasive respiratory specimens, due to their lower fungal burden. In BALF, the sensitivity of PCR assays for detection of *P. jirovecii* DNA, such as the *mtLSUrRNA* nPCR, is usually very satisfactory (higher than 95%), with a detection threshold that can reach values of 0.5–1 organism/μL of sample [218]. Yet, in noninvasive specimens, those PCR assays result in lower sensitivity values (less than 80%) [206, 221, 222].

The PCR tools have greatly improved the diagnosis of PcP, allowing the early detection of *P. jirovecii* infection. In fact, PCR assays can detect *P. jirovecii* DNA in respiratory specimens from PcP patients that were negative microscopically [83, 218, 223, 224]. The use of these techniques may lead to a decreased time period from onset of symptoms to treatment, which provides a recognized positive effect on prognosis by avoiding the evolution of early PcP cases to a severe disease with associated respiratory failure and significant mortality [200, 217]. The ambiguity created by the fact that a positive PCR test associated with a negative microscopy may be the result of either PcP or *P. jirovecii* colonization [19, 218]. can be solved by combining the test results with the etiologic features of *P. jirovecii* as opportunistic pathogen and the patient clinical data. Only the careful assessment of the patients' clinical signs and symptoms, radiological and laboratorial complementary tests, in combination with the PCR data analysis, will shed light on the effective state of a specific *P. jirovecii* infection [217].

In clinical practice, colonization or subclinical carriage of *P. jirovecii* is usually identified when the DNA of the pathogen is detected by a PCR assay in respiratory specimens from immunocompromised or immunocompetent persons without clinical manisfestations of PcP [19, 54, 199]. Usually, in these situations, the microscopic

examinations are often false negative due to the very low fungal burdens, difficult to detected in those subjects [225].

7.5.3.1 Molecular Techniques for *P. jirovecii* DNA Detection

Several PCR protocols for detection of *P. jirovecii* DNA in clinical specimens have been reported, using different PCR techniques and targeting a variety of *P. jirovecii* genes such as the *mtLSUrRNA*, mitochondrial small subunit rRNA (*mtSSUrRNA*), the *ITS* regions of the rRNA operon, 5S ribosomal RNA (*5S rRNA*), 18S ribosomal RNA (*18S rRNA*), *DHPS*, *DHFR*, *TS*, cell-division-cycle *cdc2* gene, major surface glycoprotein (*MSG*), upstream conserved sequence of the MSG (*UCS*), *KEX*1, *CYB*, *SOD*, α and β tubulin (α- β-*TUB*), *TRR*1 and *arom* [102, 103, 105, 136, 217, 226].

The *mtLSU rRNA* nested-PCR was the most sensitive method among nine PCR assays evaluated for detection of *P. jiroveii* DNA. It produced less false-negative results, and displayed higher degree of concordance with microscopic data than *mtLSU rRNA* single-PCR, *ITS* regions nested-PCR, *DHPS* single- and nested-PCR, *DHFR* nested-PCR, *MSG* heminested-PCR, *18S rRNA* 1-tube nested-PCR and *5S rRNA* real-time quantitative PCR (RT-qPCR) [227]. The fact that several genes used for molecular detection of *P. jirovecii* (e.g. *DHPS*, *DHFR*, *ITS*) are present as single nuclear encoded genes, whereas the mitochondrial *mtLSU rRNA* is a multicopy gene in the *P. jirovecii* genome, contributes largely to the higher successful amplification rates of the *mtLSU rRNA* PCR assays, especially the nested-PCR technique [97, 217, 228]. A bivariate meta-analysis and systematic review assessed the usefulness of PCR-based assays in BALF for diagnosis of PcP. It included 16 studies with a total 1857 BALF samples from 1793 patients published between 1994 and 2012, pointing out for overall pooled sensitivity of 98.3% (95% CI, 91.3%–99.7%) and specificity of 91.0% (95% CI, 82.7%–95.5%), which predicts PCR in BALF as a very accurate method for diagnosis of PcP [229]. Although nested-PCR seems to improve the sensitivity of the detection of *P. jirovecii* in respiratory specimens, the clinical relevance of a positive result remains to be investigated [230]. The lower specificity rates found can be explained by the asymptomatic carriers or colonized patients with positive nested-PCR tests incorrectly diagnosed as having PcP (false positive PcP cases). Again, this leads to the intriguing issue that positive PCR tests associated with negative microscopic examinations may correlate with either PcP or *P. jirovecii* colonization, or even could be an effect of contamination prone process [229]. The use of less invasive samples from the upper respiratory tract in association with highly sensitive and specific molecular tools, such as nested-PCR or RT-qPCR, provide a more cost-effective option than the standard procedures using BALF [223, 231–233].

Molecular strategies for detecting and quantifying *P. jirovecii* burdens were explored by several authors. Several RT-qPCR protocols for detection of *P. jirovecii* DNA in clinical samples have been reported, targeting the MSG multigene family of *P. jirovecii*, *β-TUB*, *KEX1* genes, *mtLSUrRNA* and *cdc2* genes [97, 224, 234–237]. A meta-analysis study assessed the use of RT-qPCR for the diagnosis of PcP

in immunocompromised patients, including 10 individual studies from 1990 to 2010 [238]. Overall, the sensitivity of RT-qPCR was 97% (95%CI: 93%–99%) and specificity was 94% (95%CI: 90%–96%). In the subgroup of HIV-infected patients, the sensitivity and specificity were 97% (95%CI: 93%–99%) and 93% (95%CI: 89%–96%), respectively. Regarding *P. jirovecii* DNA detection in BALF, the sensitivity was 98% (95%CI: 94%–99%) and specificity 93% (95%CI: 89%–96%) [238]. Although RT-qPCR demonstrated good diagnostic accuracy, further studies are needed in order to identify any differences in the diagnostic performance of RT-qPCR in HIV-infected and non-HIV-infected immunocompromised patients and in differentiating colonization from disease [217]. In order to achieve more accurate tests, especially in the management of PcP in non-HIV immunocompromised patients, thresholds should be assessed according to underlying diseases and other clinical and radiological parameters [237]. Also, when interpreting the significance of the fungal burden, it is not only important to consider the underlying condition of the patient, but also the quality of the biological specimen under study.

A comparative study of three commercial assays (*Pneumocystis jirovecii* [*carinii*]—FRT PCR Kit [AmpliSens], MycAssay *Pneumocystis* [Myconostica] and real-time PCR *Pneumocystis jirovecii* [Bio-Evolution]) and an in-house PCR was performed [239]. The sensitivity and specificity when testing proven/probable PcP was 100%, 100%, 95% and 83%, 93% and 100%, respectively, and sample concordance between the Amplisens and MycAssay were excellent (Kappa: 0.85) [239]. Also a commercial RT-qPCR for both the detection of *Pneumocystis* and of DHPS point mutations associated with resistance to sulfa-based drugs such as trimethoprim-sulfamethoxazole (TMP-SMX) and dapsone, used for both prophylaxis and treatment of PcP was developed [240].

In conclusion, several studies consistently pointed the nested-PCR and the RT-qPCR assays, especially the ones targeting the *mtLSUrRNA* gene, as the most sensitive and specific molecular tools for detection of *P. jirovecii* [67, 218, 225, 226, 234, 235, 241]. Given the great variety of PcP PCR assays available, it may be wise for diagnostic laboratories to use commercial and standardized tests that have developed an understanding of how to interpret results, in particular low level positives.

PCR-based approaches, namely multilocus sequence typing, can also be used to determine the epidemiology and transmission of infection and to investigate potential outbreaks [80, 121, 242].

7.5.4 New Alternatives for Diagnosis of PcP

7.5.4.1 Usefulness of Blood

The success of microscopy and molecular techniques in the diagnosis of PcP depends on the available resources and technology of the laboratory, the team's experience, as well as the type of biological specimen analyzed. The demand for lesser exclusive technologies and the evidence that invasive techniques to obtain the standard

respiratory samples are not always possible to perform and carry associated risks of complications [199], launched the interest in blood and serum specimens. Therefore, these specimens have been tested as an alternative to respiratory specimens since the 1990s. At first, the attention was turned to the detection of *P. jirovecii* in blood samples through molecular methods such as PCR, once a blood-borne phase of the infection was suggested although never demonstrated [243]. However, most studies that experienced this method (5/7) showed low to very low sensitivity (0–30%), depending on the locus in analysis [243–249]. Then, new alternative strategies for the diagnosis of PcP emerged through the measurement of blood biomarkers that reflect the host-pathogen interaction, as noninvasive interventions [250, 251]. Most recently, the interest in the serum antibodies has increased as promising studies using recombinant antigens of *P. jirovecii* and antibody immunodetection techniques, such as immunoenzymatic or immunoblotting assays, have shown potential application in the diagnosis and epidemiological studies of PcP [252–255].

7.5.4.2 Blood Biomarkers

Elevated serum levels of the LDH, beta-d-glucan (BG) and Krebs von den Lungen-6 antigen (KL-6), as well as low serological levels of S-adenosylmethionine (SAM), have been related to PcP and proposed as markers of the disease [223, 250, 251, 256–258]. However, the use of these metabolites in PcP diagnosis is complex because their serum levels are not strictly specific to *P. jirovecii* infection. The measurement of serum BG, the main structural component of the cell wall of *Pneumocystis* cystic forms is the most promising procedure for establishing the serologic diagnosis of PcP [223, 250, 258, 259]. However, BG is also a structural component of fungi and, as such, may be increased in patients with other fungal infections. The combination tests BG/KL-6 was demonstrated to be the most accurate serologic approach for PcP diagnosis, less onerous and minimally invasive, in comparison with the classic diagnostic approach (BALF followed by microscopy or DNA detection) [223, 258].

Two meta-analysis studies performed with a majority of HIV-infected patients estimated that the measurement of BG serological levels for PcP diagnosis presents a high sensitivity (95–96%), a medium specificity (84–86%) and a negative predictive value varying between 98.5% and 98.9% for a PcP prevalence of 20% [259, 260], although recent studies reveal a lower sensitivity for non-HIV-infected patients (85%) [261]. Given the high sensitivity of BG detection in serum, a negative serum BG result can exclude PcP in a patient at risk of the disease, but a positive BG test cannot be used to confirm the diagnosis of PcP. So, a BG positive test alone cannot be considered diagnostic of PcP due to the lack of BG's specificity to *P. jirovecii* infection and also because there is not a consensus threshold for its application in PcP diagnosis [258, 262]. Though, the results of BG tested alone should be interpreted with caution. BG can still contribute to the diagnosis of PcP and to exclude the disease, in combination with PCR of upper respiratory tract specimens—when BALF is not available or difficult to obtain [118], and with other clinical diagnostic criteria indicative of PcP, especially in non-HIV-infected immunocompromised patients.

7.5.4.3 Immunodiagnosis

In the state of the art of immunodiagnosis of PcP, different studies have showed that *P. jirovecii* recombinant antigens are recognized by human serum antibodies, which gives them an enormous potential for application in serological diagnosis of PcP [252–255]. Nowadays, the *Pneumocystis* antigen that has received the most attention is the major surface glycoprotein (Msg), which is highly specific of this pathogen, contains shared and species-specific epitopes, elicits humoral and cellular protective immune responses and plays a central role in the interaction of *Pneumocystis* with its host [263]. Studies that evaluated recombinant fragments covering the entire length of Msg (MsgA, MsgB and MsgC) showed that the carboxyl-terminal domain (MsgC) is the most conserved and reactive region of Msg [252–255]. These data suggest that epitopes that stimulate at least part of the human antibodies against *P. jirovecii* may be located in this region. That was proved recently, in a study where the researchers designed and produced a recombinant synthetic antigen with three antigenic regions of the Msg protein—one from the terminal portion of MsgB and two from MsgC [264]. The authors purified this antigen and used it as an antigenic tool in an ELISA technique, which showed that the IgM anti-*P. jirovecii* levels were statistically increased in patients with PcP ($p = 0.001$), compared with patients without PcP. The ELISA test presented a sensitivity of 100% and a specificity of 80.8%, when associated with the clinical diagnosis of PcP of each patient, showing as an immunodiagnostic tool for PcP diagnosis and a possible improvement in the clinical management of this disease can be near.

7.6 Management of PCP

7.6.1 Treatment

If PcP is left untreated, associated mortality is approximately 100%, especially in non-HIV-infected patients. Mortality from PcP increases with abrupt onset of respiratory failure and delay in PcP diagnosis. Disease can be stratified according to mild, moderate or severe, depending on presenting symptoms, oxygen saturation and chest radiographic changes. Requirement for mechanical ventilation and vasopressors is a poor prognostic feature.

Clinicians should start treatment as soon as possible, even before a definitive diagnosis has been given, because *Pneumocystis* persist in clinical specimens for days or weeks after effective therapy is initiated [265]. Most information on treatment and duration of therapy is extrapolated from HIV guidelines, with limited information in the non-HIV population [266]. TMP-SMX is the preferred agent for treatment because of its low cost, clinical efficacy against PcP, and bioavailability in both intravenous and oral formulations [266]. Oral outpatient therapy with TMP-SMX is highly effective in patients with mild-to-moderate disease. If the patient has

severe PcP infection (acute respiratory failure, hemodynamic instability, need of ventilatory support), should be treated with intravenous TMP-SMX, because of the possibility of decreased drug absorption in the critically ill patients [267]. Corticosteroids are used adjunctively with TMP-SMX to reduce the likelihood of respiratory failure and death. Costicosteroids could suppress the acute inflammatory process associated with PcP [266]. If corticosteroids are institute in PcP HIV-infected patients who developed respiratory failure, prednisone 40 mg twice daily for 5 days, 40 mg daily for 5 days, then 20 mg daily for the duration of anti-Pneumocystis therapy is recommended [268]. Among non-HIV immunocompromised patients, corticosteroids use in the treatment of PcP is controversial. However, in severely ill PcP patients corticosteroids could be useful to reduce the duration of mechanical ventilation and ICU admissions [188].

If the patient developed a mild rash while being treated previously with TMP-SMX, desensitization should be first considered [266]. Yet, if the patient develops a severe rash such as Steven–Johnson's syndrome desensitization is not recommended, and TMP-SMX is contraindicated. Alternative therapeutic regimens for mild-to-moderate disease include primaquine plus clindamycin and atovaquone, and alternative therapeutic regimens for patients with moderate-to-severe disease include intravenous pentamidine or intravenous clindamycin plus oral primaquine. Second line PcP therapeutic options have inferior efficacy or similar to that of TMP-SMX but with increased prevalence of adverse effects or increased mortality rates [269–271]. The recommended duration of therapy for PcP is 21 days. Table 7.5 summarizes the various treatment regimens for PcP.

The rate of response to therapy depends on the agent used, number of previous PcP episodes, severity of pulmonary illness, degree of immunodeficiency, timing of initiation of therapy and co-morbidities. Clinical treatment failure is defined as a worsening of or no improvement in lung/respiratory function, evidenced by a decline in oxygen saturation after at least 4–8 days of anti-PcP treatment [23]. Failure attributed to lack of drug efficacy occurs in approximately 10% of those with mild-to-moderate disease. Clinicians should wait at least 4–8 days before switching therapy due to lack of clinical improvement. In the absence of corticosteroid therapy, early and reversible deterioration within the first 3–5 days of therapy is typical. Other concomitant infections must be excluded as the cause of clinical

Table 7.5 Drugs for treatment of *Pneumocystis jirovecii* pneumonia

Drug	Dose	Route	Comments
Trimethoprim–sulfamethoxazole	15–20 mg/kg 75–100 mg/kg daily in divided doses	Oral or intravenous	First choice
Primaquine plus clindamycin	50 mg daily 600 mg three times daily	Oral	Alternate choice
Atovaquone	750 mg two times daily	Oral	Alternate choice
Pentamidine	4 mg/kg daily 600 mg daily	Intravenous Aerosol	Alternate choice

failure [194, 196]. Treatment failure attributed to treatment-limiting toxicities occurs in up to one-third of AIDS patients [272].

7.6.2 Prophylaxis

The most effective way of preventing PcP in HIV-infected people is by immune-reconstitution through the administration of effective antiretroviral therapy. Prophylaxis should be administered until immune reconstitution has been achieved.

HIV-infected adults should receive chemoprophylaxis against PcP if they have $CD4^+$ T cells counts <200/mm^3 or a history of oropharyngeal candidiasis [22–24]. Persons who have a $CD4^+$ T cells percentage of 14% or a history of an AIDS-defining illness, but who do not otherwise qualify, should be considered for prophylaxis [22–24].

For prophylaxis of PcP in non-HIV-infected patients with compromised immune systems there are data related to specific conditions where PcP may have higher prevalence and when prophylaxis could be utilized [58] (Table 7.6). Recommendations for prophylaxis are reviewed in the ECIL guidelines, although especially focused on patients with haematological malignancies and undergoing stem cell transplantation (SCT) [26].

TMP-SMX are the recommended prophylactic agents. This combination therapy at a dose of one double-strength tablet daily confers cross protection against toxoplasmosis and many respiratory bacterial infections [23, 279–282].

TMP-SMX chemoprophylaxis should be continued in patients who have non-life treating adverse reactions. Yet, it should be permanently discontinued in patients with life treating adverse reactions, including possible or definite Stevens–Johnson syndrome or toxic epidermal necrolysis. For patients who cannot tolerate TMP-SMX, alternative prophylactic regimens include dapsone, dapsone plus pyrimethamine plus leucovorin, aerosolized pentamidine, and atovaquone (Table 7.7).

In HIV-infected patients, primary PcP prophylaxis should be discontinued for those patients who have responded to cART with an increase in CD4+ T cells count from <200/mm^3 to ≥200/mm^3 for more than 3 months. Prophylaxis should be reintroduced if CD4+ T cells count decreased to <200/mm^3.

HIV-patients who have a history of PcP should be given chemoprophylaxis for life with TMP-SMX (secondary prophylaxis or maintenance therapy/unless immune reconstitution occurs as a result of cART) [283]. Secondary prophylaxis should be discontinued following recommendations for discontinuation of primary prophylaxis.

Table 7.6 Prophylaxis for *Pneumocystis jirovecii* pneumonia in non-HIV-infected patients

Specific conditions	Comments
Hematopoietic cell transplantation	
Allogeneic stem cell transplant recipients	• PcP prophylaxis until at least 6 months after HCT. For those who continue to receive *immunosuppressive drugs* prophylaxis should be longer than 6 months [273].
Autologous stem cell transplant recipients	• PcP rate is much lower than among allogeneic recipients. PcP prophylaxis should be required in high-risk populations, including underlying lymphoma, leukaemia, or myeloma, especially on intensive treatment with purine analogues or high-dose corticosteroids. Extend PcP prophylaxis should be 3–6 months, or longer for patients with ongoing immunomodulatory therapy post-transplantation [273].
Solid organ transplant recipients	• PcP overall incidence varied in the range of 5–15%, but appeared highest in lung transplant recipients. PcP prophylaxis is recommended for at least 6–12 months' post-transplantation, though longer durations should be considered. Shorter duration of at least 4 months post renal transplant [33, 274].
Acute lymphoblastic leukaemia and prolonged $CD4^+$ counts < 200/μL	• PcP prophylaxis recommended in a broad range of haematological malignancies if the risk is ≥3.5%, including patients on more intensive chemotherapeutic regimens. These patients appear to have a higher risk of PcP associated mortality. Non-neutropenic cancer patients given steroids for prolonged periods of time carry substantial risk of PcP. PcP prophylaxis should continue until $CD4^+$ > 200/μL [275].
Inflammatory bowel disease	• Treatment of IBD with immunomodulators and biologic agents the potential for OI is a key safety concern. PcP prophylaxis is recommended for patients receiving three or more immunomodulatory drugs, including calcineurin inhibitors and/or anti-TNF. The duration of this prophylaxis is currently being reviewed and studied [276].
Primary immunodeficiency diseases, severe protein malnutrition collagen vascular diseases on cytotoxic or immunosuppressive therapy, conditions treated with daily equivalent of 20 mg or more of prednisone for ≥1 month	• PcP prophylaxis in Wegener's granulomatosis when $CD4^+$ < 300/μL. Others AID the risk of infection is related to treatment with systemic steroid, and if the annual risk of PcP is >9% the use of PcP prophylaxis should be recommended if $CD4^+$ < 200/μL [277]. In other immunodepression conditions the risk-benefit ratio of prophylaxis remains unclear. Prophylaxis is warranted when the risk for PcP is ≥3.5%. In children at lower PcP incidence rates [278].

Table 7.7 Drugs for prophylaxis against *Pneumocystis jirovecii* pneumonia

Drug	Dose	Route	Comments
Trimethoprim–sulfamethoxazole	1 double-strength tablet daily or 1 single-strength tablet daily	Oral	First choice
	1 double-strength tablet 3 times per week		Alternate choice
Dapsone	50 mg twice daily or 100 mg daily	Oral	Ensure patient does not have G6PD deficiency
Dapsone plus Pyrimethamine plus Leucovorin	50 mg daily 50 mg weekly 25 mg weekly	Oral	
Dapsone plus Pyrimethamine plus Leucovorin	200 mg weekly 75 mg weekly 25 mg weekly	Oral	
Pentamidine	300 mg monthly	Aerosol	
Atovaquone	1500 mg daily	Oral	Give with high-fat meals, for maximal absorption

G6PD, glucose-6-phosphate dehydrogenase

7.7 Conclusions

Pneumocystis jirovecii is a worldwide pathogen implicated in interstitial pneumonia. Important advances have been made in the past 20 years in understanding this organism, especially with regard to its taxonomy, epidemiology, host specificity, transmission, and pathophysiology. In developed countries, PcP is still a major concern among people infected with HIV, although the incidence of this disease in this population group has dropped dramatically due to successful implementation of anti-PcP prophylaxis and cART. However, we are witnessing an increase and change in the population of patients at risk of PcP, based on immunocompromised non-HIV-infected patients. As the host defence against *Pneumocystis* requires virtually every arm of the immune system to clear the organisms from the lungs, the clinical presentation in HIV-infected patients is different from that in other immunocompromised non-HIV-infected patients. In high-risk patients, clinical presentation and radiology are sufficient for initiating empirical therapy but should not be used as definitive diagnosis that is dependent on specific laboratory diagnostic methodologies, which have improved dramatically. Less invasive samples from the upper respiratory tract in association with highly sensitive and specific molecular methods, such as nested-PCR or RT-qPCR, have been tested intensively in comparison to the standard procedures. Also new diagnostic tools based on the measurement of blood biomarkers of infection and detection of antibodies have been and continue to be developed. Yet, none of the methods developed until now are able to distinguish PcP cases from colonization cases. In addition to diagnosis, molecular based methods can be used for transmission studies, and epidemiological and evolution studies of the organism. The standard first line therapy/prophylaxis for PcP, in HIV- and

non-HIV-infected patients, is the combination therapy TMP-SMX. Despite being effective, there are significant prophylactic and treatment failures as well as intolerance or side effects associated with this anti-folate inhibitors combination that may hamper the clinical outcome of the disease. Therefore, optimal strategies for prevention, diagnosis and treament of *P. jirovecii* symptomatic infections are fundamental to providing comprehensive, high-quality care for these patients.

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Chapter 8
Coccidioides and Coccidioidomycosis

Marcus M. Teixeira and Bridget M. Barker

Abstract *Coccidioides immitis* and *C. posadasii* are the causative agents of coccidioidomycosis, or San Joaquin Valley Fever. The disease was first described in 1892 in Argentina, and its distribution and frequency investigated into the mid-1900s. National reporting has measured a recent increase in disease burden in the United States. All mammals appear to be susceptible to infection; however disease manifestation varies from asymptomatic to lethal. Several thousand cases of the disease are reported annually in the United States. We discuss the epidemiology, diagnostics, virulence factors, and treatment options for coccidioidomycosis.

8.1 History and Taxonomy

Coccidioidomycosis or Valley fever is a deep systemic mycosis reported in arid and semi-arid areas of the Americas. The disease is caused by the geo-zoo-anthropophylic fungal species nested in the *Coccidioides* genus [1–3]. The history of coccidioidomycosis began in 1892 when Alejandro Posadas evaluated the cavalry soldier Domingos Ezcurra [4]. He had disseminated verrucous lesions after suffering from an unknown disease for a year while battling on the border of the Chaco region of Argentina. He also presented lymphadenopathy, and several attempts to use treatments and surgical interventions were performed, but did not halt the progression of the disease. Domingos Ezcurra's death in 1889 and a complete autopsy demonstrated lesions in the skin, lymph nodes, lungs, liver, and spleen. Histopathological investigation of

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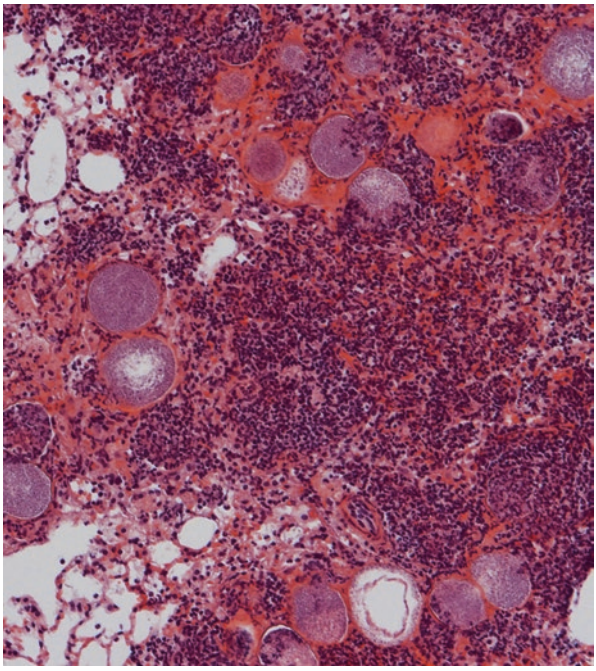

Fig. 8.1. CD-1 mice infected with 10^6 arthroconidia instilled intranasally show significant infection and lung damage by day 5. H&E staining reveals large spherules and massive influx of host immune cells

involved organs revealed the presence of epitheliod granulomas, infiltrates of mononuclear cells as well the presence of spherules as shown in Fig. 8.1 [5, 6].

For more than a century, *Coccidioides immitis* was considered a singles species; however, molecular systematics revealed that at least two species complexes, containing distinct populations with limited gene flow [7–10]. *Coccidioides posadasii* was proposed by [11] in a tribute to Alejandro Posadas, the discoverer of coccidioidomycosis [11]. *C. immitis* is prevalent in central/southern California, Eastern Washington and north Mexico while *C. posadasii* is endemic in Arizona with a discontinuous distribution extending into Utah, Texas, Mexico, Central and South America [12, 13]. Recent comparative genomic and population genetic studies suggested that *C. immitis* is composed of three subpopulations, divided into San Diego/Mexico, San Joaquin Valley and Washington. These studies also proposed that *C. posadasii* is divided into Arizona, Mexico, Guatemala, and Texas/South America populations [14, 15]. It is likely that *C. immitis* and *C. posadasii* co-occur in nature, as both species have been found in patients from both San Diego/USA and Mexico, and hybrid strains have been discovered [15, 16].

8.2 Clinical Aspects of Disease

The disease affects both healthy and immune compromised human populations, as well as many other mammals [1, 17, 18]. Populations at greatest risk for severe disease are persons with underlying immunocompromising conditions. These

include HIV/AIDS, immunomodulatory therapies for cancer, organ transplant, and autoimmune diseases [19–25]. Males more than females, African American and Filipino more than European ancestry, and pregnancy also have greater risk of dissemination [26–30]. Mutations of IL-12rβ1, IFN-γr1, STAT1, and STAT3 have also been associated with disseminated disease [31].

Coccidioides sp. infections primarily affect the lungs. Most commonly, infection is initiated by the inhalation of infectious propagules of the fungus (arthroconidia), but can be a result of dermal invasion [32]. The disease is asymptomatic in approximately 60% of infections. The remaining cases are usually characterized by a self-limited respiratory illness [33–37]. The disease may be confounded with several other upper respiratory infections and requires specific laboratory tests including culture and serology, along with associated travel history or inhabitation of endemic areas of the disease [38–40].

For those with symptoms, the most common forms are acute and sub-acute disease, which manifests as mild respiratory illness (often flu-like) with fever, cough, and is frequently associated with rib cage pain and/or fatigue [39, 41]. In the majority of acute pulmonary cases the use of antifungal drugs is not required [38, 39]. However, pneumonia may be observed in those who develop a severe acute respiratory disease and chemotherapy may be necessary for disease management. Clinical conditions in those patients include intense and elevated fever, dyspnea, hypoxemia, and are frequently observed in immunocompromised individuals [2, 3, 38, 39].

Several factors may contribute to those cases, including the inoculum, pathogen variation, and host genetic background [42–46]. Skin lesions are the most frequent extra-pulmonary manifestation and are present in 10–50% of cases [47, 48]. Lesions frequently observed include papules, nodules, pustules, abscesses, warts or ulcerations. Lymphohematogenous disease is associated with high morbidity and mortality rates, and may require life-long antifungal therapy in most cases. One percent of cases are diagnosed as disseminated coccidioidomycosis; however, in susceptible populations the risk of dissemination can reach 75% [31]. Dissemination usually onsets within a few weeks to months following the primary infection [49]. A widespread septic fungemia may occur in some cases and is often fatal without treatment [2, 33, 50, 51].

8.3 Epidemiology

Coccidioidomycosis disease range exists across arid to semi-arid regions of the Americas. The disease is endemic to many desert biomes across the western United States, Mexico, Guatemala, Venezuela, Colombia, Paraguay, Argentina, and Brazil [1, 3, 52–55]. Those areas are generally characterized by having elevated temperatures and low annual rainfall with sporadic seasonal rainy periods. During seasonal rains, is believed that the fungus grows in the filamentous form. Upon maturation, arthroconidia are produced asexually and become airborne during drought, wind storms, and haboobs [32, 56–59].

This theory of "grow and blow" may be responsible for the annual peaks of infection in the western USA during summer months [60]. Soil perturbation by natural phenomenon such as windstorms and earthquakes; or by anthropogenic actions such as agriculture, excavation of archeological sites, deforestation, or hunting armadillos, which are known risk factors for coccidioidomycosis [2, 61–67]. In addition, microenvironmental conditions such as soil type and available nutrients may be key factors for fungal development and may explain the discontinuous distribution of the fungus in specific endemic areas [59, 68, 69]. Upon inhalation of an arthroconidium into a mammal lung, both *Coccidioides immitis* and *C. posadasii* will shift into a parasitic spherule cycle. Spherules became mature in tissue initiating an encasement for multiple uninucleate endospores that after rupture can disperse throughout the body (Fig. 8.2; [32]).

The genus *Coccidioides* is nested within the Onygenales order (Ascomycote) and shares with *Blastomyces*, *Paracoccidioides*, *Histoplasma,* and Dermatophytes the ability to degrade animal derived material such as keratin [70]. Genomic analyses show that these human fungal pathogens co-inherited genes that allow the degradation of proteins present skin, carcasses, bones, and other animal tissues [71–73]. *Coccidioides* was found associated with different wild rodents in Arizona and California, and was isolated from armadillos in Brazil, which suggests that burrow-

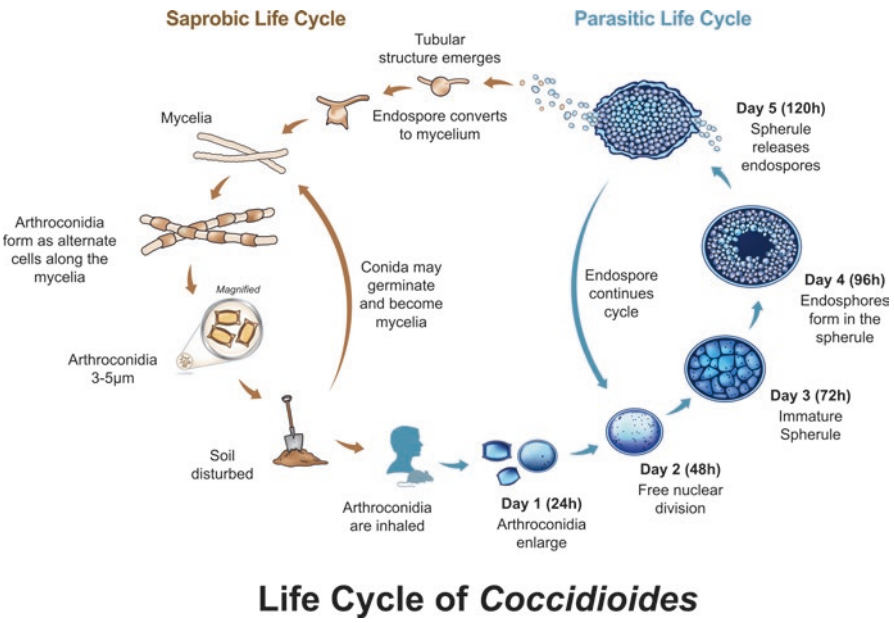

Fig. 8.2. Upon inhalation of an arthroconidium into a mammalian lung, both *Coccidioides immitis* and *C. posadasii* will shift into a parasitic spherule cycle. Depending on host and fungal factors, the spherules became mature within 5–10 days in tissue initiating an encasement for multiple uninucleate endospores, which are released after rupture and can disseminate throughout the body [32]

forming mammals may be direct (i.e. infection) or indirect (i.e. thriving in the burrow environment) hosts and responsible for the maintenance and dispersion of the fungus [62, 74–77].

All mammals are susceptible to infection. Primates (humans and other primates) and canids (wolves and domestic dogs) appear to have more severe disease on average than other mammals [18, 78–85]. Agricultural animals in the endemic region; including cattle, bison, pigs, sheep, and goats, have high rates of infection/exposure, but do not appear to have frequent pulmonary symptoms or severe disease [86–88]. Equine disease is rare, but severe and complicated to treat [89–91]. Infection of lizards and snakes is thought to be rare, but is reported [92, 93]. Bats, sea mammals, cats, zoo animals, and many other animals, all seem to have some level of infectivity, but disease rarely manifests [92, 94–99]. Birds do not appear to be infected, as we can find no reported cases.

In the 1990s in the United States, cases became reportable to the CDC and at the state level in California, Arizona, Nevada, New Mexico, and Utah [100]. The reported annual incidence of coccidioidomycosis in USA is estimated to be 150,000 cases annually. Since being tracked, the reported rates have increased to a high of 25,000 cases in 2011 [2, 101]. There were changes in reporting at this time in Arizona and the cause of the rise that year is still debated. From 2012 to 2014 the reports declined; however, a recent increase was reported in 2015 [102, 103]. Therefore, residents of and recent travelers to regions where *Coccidioides* is endemic that are diagnosed with community acquired pneumonia should be evaluated routinely for coccidioidomycosis as a possible etiologic agent [40, 104]. It was thought that dust storms and large-scale soil disturbance led to increased disease, but the results are mixed. The Northridge earthquake resulted in spike in the region near the earthquake origin; however, no increase in disease was observed after haboobs in Arizona [56, 59, 60, 65, 67, 105].

8.4 Virulence Factors

The species from the genus *Coccidioides* are dimorphic fungi and the shift to spherule cell types promotes a gene reprograming, which allows the fungi to proliferate or stay quiescent inside mammalian tissues [106–108]. Auxotrophic mutants of *Coccidioides* have been reported to decrease virulence or are avirulent in a murine model, but few virulence factors have been investigated for *Coccidioides* to date [109, 110].

Upon infection, the arthroconidia differentiate, resulting in the formation of specialized endosporulating spherules (Figs. 8.1 and 8.3). This morphological shift is one mechanism that allows *Coccidioides* to survive in harsh conditions imposed by the immune system even in immunocompetent hosts [111–113].

Three functional genomic studies based on transcriptomic analysis have identified genes and pathways expressed differentially in the spherule phase compared to the saprophytic stage of both *C. immitis* and *C. posadasii*. These studies revealed an

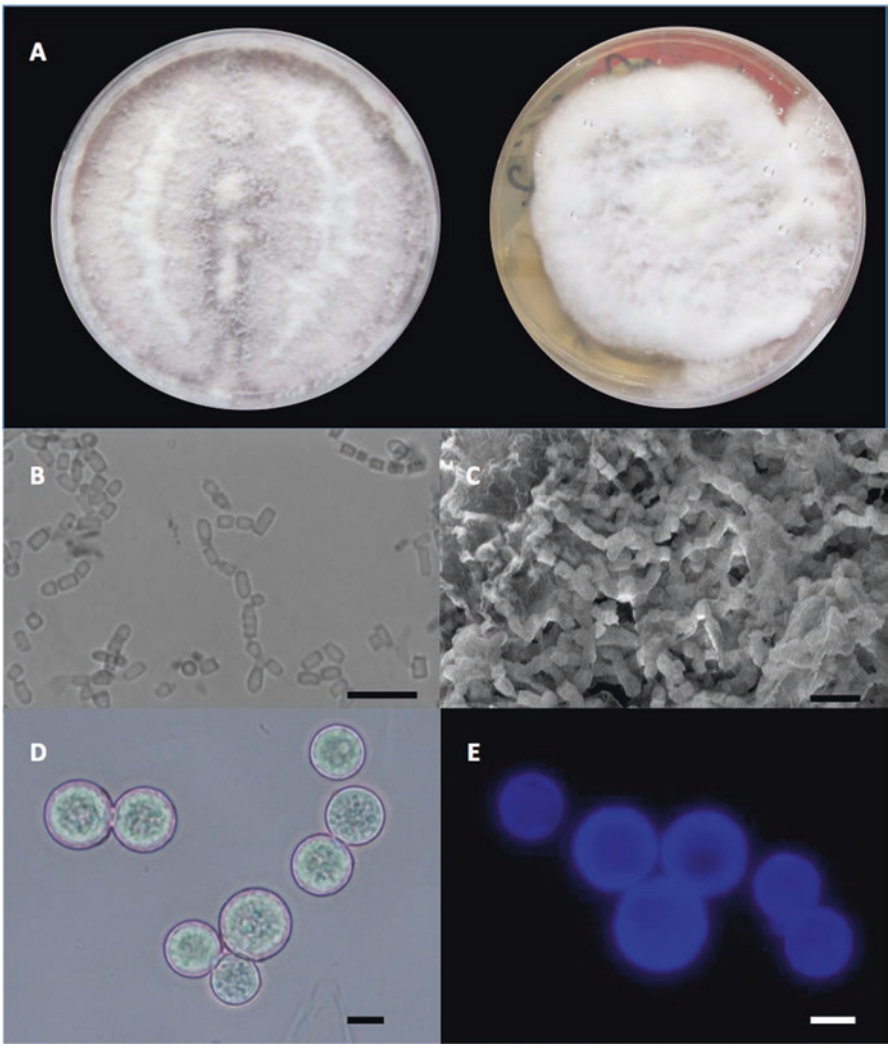

Fig. 8.3 (**a**) Optimal fungal growth is between 28 and 37 °C and white/beige cotonous mycelial colonies are expected to appear between days 2 and 7 (or potentially longer) surrounding the cultured biomaterial. Older *Coccidioides* colonies can also present dark pigmentation and a wrinkled surface. (**b**) Microscopically, the filamentous form of *Coccidioides* sp. is characterized by hyaline and septate hyphae harboring barrel-shaped arthroconidia ranging from 2 to 5 um. (**c**) Using Scanning Electron Microscopy (SEM) the barrel shape of the conidia can be easily observed. (**d**–**e**) Spherules and endospores cells can be visualized from BALF's and other biofluids. Direct mycological examinations are usually prepared using potassium hydroxide (**d**), and/or calcofluor white labeling (**e**) *Black bars* represent 5 μM

upregulation of spherule-specific genes controlling the dynamic and complex morphological switch that contributes to the development of infection, which codify for cell wall components (chitin and glucan modeling), oxidative stress, pH regulation, lipid metabolism, sugar transporters, and proteases [106, 108, 114]. Recently, the proteomic composition of spherulin (lysate of the spherule phase) was characterized and revealed the presence of important metabolic enzymes and lectin-binding glycoproteins of the infective phase of the fungus [115]. Structural and functional genomic studies of *C. immitis* and *C. posadasii* coupled with efficient gene disruption and insertional mutagenesis methods allowed the deletion and validation of potential virulence factors, listed below.

SOWgp. The outer cell wall portion of spherules is coated with several immunomodulatory proteins [116–119]. A specific glycoprotein (SOWgp) was shown to be dominant in the endospore surface (early spherule phase) and plays an important role in the cellular and humoral immunity in patients infected with *Coccidioides* [120–122]. The function of this gene was determined by targeted gene replacement and bioinformatics methods. The SOWgp gene encodes for a 422-amino acid protein harboring six blocks of proline-rich sites [123, 124]. The specific expression of this gene in the beginning of the parasitic phase of *Coccidioides* was first demonstrated by specific RNA hybridization and later confirmed by RNA-seq experiments [108]. The adhesin function of this antigen was determined by the ability of the recombinant SOWgp to bind to extracellular matrices (laminin, fibronectin, and collagen type IV). The disruption of SOWgp gene in the wild type *C. posadasii* C735 strain resulted in a lack of adhesion and a significant reduction in the virulence in a murine model of infection [123].

Mep1. Another important protein identified in the lysates of the parasitic phase of *Coccidioides* is the Mep1 protease. Early proteomic analysis revealed that spherule glycoproteins were naturally cleaved [125, 126]. It was shown that Mep1 cleaves the major *Coccidioides* spherule antigen SOWgp, thus protecting the pathogen from phagocytosis by innate immune cells [125]. This study revealed that SOWgp may play a role as a pathogen-associated molecular pattern (PAMP) molecule. Mep1 belongs to the metalloprotease subfamily M43B, containing 276 amino acids and is potentially secreted to outer cell space. Two conserved zinc-biding sites were identified being important to the structural stability of Mep1 [127]. The Mep1 gene was found to be highly expressed in the parasitic phase of *Coccidioides* and this was later confirmed by RNA-seq experiments. In vivo studies showed that the gene disruption of Mep1 in the C735 strain increased C57BL/6 mice survival, compared to infection with the native strain. This effect was more evident when mice were previously challenged with SOWgp antibodies. The experiments proved that the exposure of SOWgp could increase the phagocytic activity of alveolar macrophages allowing the fungal proliferation in the host [122, 123, 127].

Cps1. The Cps1 gene encodes for an AMP-binding protein, and may be part of non-ribosomal peptide synthase (NRPS) complex conserved among Ascomycetous fungi [128]. The novel peptide synthetase was initially discovered in the plant pathogen *Cochliobolus heterostrophus*, shown to be necessary

for virulence, and the depleted Cps1 strain was protective in plant infection models [128]. The gene knockout of the homologous Cps1 copy in the *C. posadasii* genome produced a reduction in the virulence effect as compared to the wild type strain. The Cps1 mutant was not able to produce disease, or persist in tissue in mice models. The role of Cps1 was not identified; however, RNA-seq experiments demonstrated that this transcript regulates at least 33 other *Coccidioides posadasii* genes [129].

Urease/Ugh. Urease acts in the conversion of urea into ammonia in almost all fungal species and is responsible for pH regulation during infection [130]. Urease was previously shown to be released from spherules during endosporulation. The urease gene of *Coccidioides* was initially characterized in 1997 and encodes for a 2517-bp ORF that translates to protein of 91.5 kDa containing 839 amino acid residues [131]. Despite the lack of signal peptide the protein is carried from the cytosol to central vacuole via vesicles of spherules and promotes the alkalization of the infection site [132]. The loss of function of urease gene resulted in a low virulent strain, allowing the clearance of *Coccidioides* infection more efficiently than native or reconstituted strains in murine infection experiments. Finally, immunization of mice with recombinant *Coccidioides* urease was protective against arthroconidia challenge by stimulating the T helper type 1 cells (Th1) response [133]. Fungi also metabolize ureidoglycolate into glyoxylate, generating CO_2 and ammonia. This process is mediated by the enzyme ureidoglycolate hydrolase and the lack of the Ugh gene in *Coccidioides* significantly decreases the virulence of this pathogen [134].

Chitin synthase and Chitinases. Chitin is an indispensable molecule of the cell wall of fungal pathogens, and is metabolized in *Coccidioides* by a subset of seven different classes of enzymes (CpCHS1-7), which may play an important role in fungal morphogenesis [116, 135]. Gene expression comparisons between spherule and mycelium revealed that transcripts of CpCHS1 and CpCHS4 are more abundant in the pathogenic phase [135]. Nikkomycin Z seems to be effective against *Coccidioides* by inactivating chitin synthases of the pathogenic phase of the fungus [136–138].

Chitinases also play an important role in spherule formation by cleaving the septal wall complex during endosporulation [116]. Two chitinases (CTS2 and CTS3) out of eight tested were found to be highly expressed during spherule development [139, 140]. These two genes are located on different chromosomes of *C. posadasii* and were disrupted to observe effects on virulence in a murine model of coccidioidomycosis. A third gene (ARD1) located next to the CTS3 was also disrupted during the homologous recombination of the insert creating a triple deletion strain [140]. This *C. posadasii* mutant strain has the ability to grow in the filamentous phase, producing hyphae and arthroconidia, but lacks the ability to endosporulate in vitro or within the host, and does not induce disease. This strain was later used in immunization trials in susceptible BALB/c mice. Mice immunized with the attenuated strain were protected against a secondary infection with wild type *C. posadasii* [140].

8.5 Immunopathology

Whereas adaptive immunity to infection has been studied in the development of vaccines, the humoral response is less well characterized. For most fungal infections, phagocytes such as neutrophils, macrophages and dendritic cells are involved in the initial control of infection [141]. For *Coccidioides*, these cell types have been implicated in the control of disease.

Human polymorphonuclear neutrophils (PMNs) have phagocytic activity against *C. immitis* in in vitro infection models when exposed to *Coccidioides* antigen, such as spherulin or coccidioidin [142, 143]. This process is also efficient for early released endospores, but not in mature spherules, primarily due to size [144]. Recent work showing frustrated phagocytosis reveals strong interactions and adherence, but lack of phagocytosis for large spherules [145]. Although PMN readily engulf arthroconidia and small endospores, they do not appear to be efficient in killing cells, as less than 20% mortality was observed in studies [146–148]. PMN infiltrates are usually observed in histopathological findings and these cell types appear to be rapidly recruited in the lungs in experimental pulmonary coccidioidomycosis [140, 149]. This is characterized by an intense inflammatory response in lungs that may contribute to tissue damage and granuloma formation that occurs during fungal infection (Fig. 8.1; [149, 150]).

Coccidioides endospores and arthroconidia are also phagocytized by alveolar and peritoneal macrophages from naive macaques, humans, and mice [146, 147, 151, 152]. In these studies, both cell types were phagocytosed, however, killing was less than 1% for unstimulated cells. For alveolar and peritoneal macrophages from BALB/c mice incubated with gamma-interferon (IFN-γ), a reduction of 50% of recovered CFU was observed [146]. In addition, a few studies suggest that lysosome fusion is inhibited by the fungus in naive lymphocytes; however, lysosomal fusion was observed in lymphocytes that were harvested from mice immunized with formalin-killed spherules [147].

Comparisons between more (DBA/2) and less (BALB/c) resistant mouse strains have revealed the resistance to coccidioidomycosis may be associated with higher pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-1α (IL-1α), and interleukin -6 (IL-6) [153]. TNF-α is also induced in macrophages in response to exposure to formalin-killed spherules [154]. Additionally, IFN-γ is important for resistance to *Coccidioides* infection in DBA/5 mice, whereas higher interleukin-4 (IL-4) appears to increase fungal burden in BALB/c mouse lungs [155]. Thus, while the predominant Th1 protective response is initiated in DBA/5 mice via IFN-γ, BALB/c mice generate a T helper type 2 (Th2) response, which can be ameliorated by recombinant interleukin-12 (IL-12) [156].

Interestingly, the presence of SOWgp on the surface of spherules triggers the Th2 response in naive host cells, which produce IL-6 and IL-10, but SOWgp antigen induces a Th1 response in activated cells [122]. SOWgp is an immunomodulatory molecule that is expressed in endospores. In order to avoid the recognition by mac-

rophages, the fungus secretes the metalloproteinase, Mep1, that hydrolyses surface-bound SOWgp and consequently reduces phagocytosis [127].

Coccidioides antigens also induce antibody responses during infection. However, high antibody titers typically correlate with a poor clinical outcome [157]. Certain antibody subsets can act as opsonins against both arthroconidia and endospores [158]. Both T27K *Coccidioides* antigen derived from spherules and heat-killed spherules interact with immature dendritic cells (DCs) that function to capture antigen, activating lymphocytes [159]. This suggests that DCs may play a critical early role in the development of adaptive immunity and T cell activation [160].

Pattern recognition receptors (PRRs) recognize *Coccidioides* specific proteins, polysaccharides, or nucleic acid motifs, and activate various inflammatory and antimicrobial responses [161, 162]. Dectin-1, Toll-like receptor 2 (TLR-2) and IL-1βR are all PRRs involved in *Coccidioides* infection [161, 163, 164]. In the absence of Dectin-1, several cytokines are differentially expressed 14 days P.I., which are required for the protective Th1 response [162]. However, other common antifungal defenses shown to be important for *Candida*, *Aspergillus*, and *Cryptococcus*, such as Dectin-2, mannose receptor (MR), and reactive oxygen species, do not appear to play a role for coccidioidomycosis, so it is possible that *Coccidioides* may have alternative strategies to evade the host immune response [165–171].

Early studies showed that T-cells are crucial in conferring protection against early infection with *Coccidioides* in mice models [172]. Th1 responses have generally been correlated with protection in murine models of coccidioidomycosis [133], whereas a predominant Th2 response is thought to lead to more severe disease [156, 173]. This is a general trend that is observed for many fungi [174–180]. However, what has become apparent is the fact that ~~is it~~ it is the *balance* among the Th1/Th2/Th17 response that mitigates the protective response to infection [163]. Loss of either the Th1 or Th2 response did not affect vaccine-mediated protection; however, the loss of a Th17 response resulted in more vaccinated mice succumbing to infection. T helper type 17 cells (Th17) are activated in response to early stages of *Coccidioides* infection and further contribute to the recruitment of innate cells to the lungs. IL-17 is produced by Th17 cells, and induces neutrophil recruitment and is Dectin-1 dependent for both *Coccidioides* and *Blastomyces* [181]. These cytokines also stimulate innate immune cells to increase the release of IL-1β, TNF-α, and IL-6 [163]. Additionally, the roles of Myd88 and CARD9 are being investigated, and appear to be essential for the development of the protective Th17 response [182, 183]. Taken in total, it is clear that the Th1/Th17 response together provide protection against the development of disease, but many questions remain. Recent work shows that the strain type that is used for infection can result in variable immune response [184]. Thus, disparate results between research groups could be due in part to use of different wild type strains.

8.6 Diagnostics

Differential diagnostics for coccidioidomycosis are extremely important because acute disease can manifest as a community acquired pneumonia [40]. Moreover, delays in the diagnosis of coccidioidomycosis or inadequate or unnecessary therapy may allow the extra-pulmonary proliferation of the fungus, and may require longer antifungal treatments for infected patients [38, 185]. Although coccidioidomycosis can be caused by two different species, there are no known differences in the clinical diagnostics between patients infected with *C. immitis* and *C. posadasii.* We will discuss the most common approaches to detect and identify the causative agents of disease.

Microscopy and Culture. *Coccidioides* is a Risk Group 3 microorganism and any culture of this fungus should be manipulated in a BSL3 environment as laboratory acquired infections have been reported [186]. Cultures of *Coccidioides* spp. can be obtained by direct plating tissue biopsies, BALF or biofluids on Mycosel, Sabouraud dextrose agar, blood agar, or any common agar used in a medical mycology laboratory [2, 185, 187]. Optimal fungal growth is between 28 and 37 °C and white/beige cotonous mycelial colonies are expected to appear between days 2–7 (or potentially longer) surrounding the cultured biomaterial. Older *Coccidioides* colonies can also present dark pigmentation and a wrinkled surface (Figure 8.3a). Microscopically, the filamentous form of *Coccidioides* sp. is characterized by hyaline and septate hyphae harboring barrel-shaped arthroconidia ranging from 2 to 5 um (Fig. 8.3b,c). The use of a *Coccidioides*-specific fluorescent DNA probe (AccuProbe) can be used to rapidly identify suspected colonies; however, false-negative results in samples fixed with formaldehyde have been seen [187–189]. Coccidioidal spherules and endospores cells can be visualized from BALF's and other biofluids. Direct mycological examinations are usually prepared using potassium hydroxide, calcofluor, and Papanicolaou (Figure 8.3d, e). Fixed biopsies can also be used for histopathological diagnostics and the observation of spherules is commonly achieved using routinely stains such as Hematoxylin-Eosin or Gomori Methenamine-Silver [185, 190].

Serological testing. Immunodiagnostics of coccidioidomycosis are widely used in clinical practice, especially in endemic areas of the disease. These methods include tube precipitin, complement fixation, immunodiffusion, agar gel precipitin-inhibition test, counterimmunoelectrophoresis, latex particle agglutination, fluorescent antibody, radioimmunoassay and ELISA [2, 185, 190–192]. Immunoglobulin (Ig)M positive reactions are reported to be positive during early and acute infection while after 2–3 weeks of illness the IgG reaction becomes positive and values are usually followed in a patient as a disease prognostic. In most asymptomatic cases both IgM and IgG are initially detected but titers may drop to negative after infection resolution [33, 191]. Tube precipitin and latex particle agglutination methods were largely used to detect IgM, whereas complement fixation was employed to detect late and chronic/disseminated cases of the disease [193]. A hallmark serological inquiry utilizing the enzyme immunoassay method based on the combination of

IgM and IgG revealed specificity and sensitivity 98.5% and 95.5% respectively and so far, is the most common method employed for the diagnostics and prognostics of coccidioidomycosis [194].

Some studies have discussed difficulties in the antibody detection at early time-points of infection, as well as in immunosuppressed patients [195]. An alternative approach is the detection of fungal antigens in biofluids via immunoassay. Recently, studies using antibodies against coccidioidal galactomannan were revealed to be efficient in the rapid diagnosis of severe coccidioidomycosis [196]. A commercial version, MiraVista® *Coccidioides* quantitative antigen test, is available. Cross-reactivity with other endemic mycosis such as blastomycosis, histoplasmosis, and paracoccidioidomycosis was already reported, so further diagnostics may be required and interpretation of results should consider the possibility of infection with other etiologic agents [197].

Nucleic acid detection. About 90 *Coccidioides* sp. genomes have been sequenced to date, and are available in public databases [14, 72]. This allows the scientific community to develop sophisticated molecular tools to detect the fungal DNA during infection. rDNA (ITS1-5.8S-ITS2) sequencing is the most common fungal barcode and still used for fungal detection and genotyping. Specific *Coccidioides* rDNA primers were developed and tested by PCR in serum samples of 94 patients, as well as from paraffin embedded tissues revealing a low positivity [198, 199]. The ITS2 region was therefore targeted for the *Coccidioides* detection via real-time PCR. Sixteen samples out of 20 positive cultures were positive for *Coccidioides* and few samples with negative histopathology were positive suggesting being a potential useful diagnostic assay for the disease [200]. Other authors revealed the conventional and nested PCR methods targeting the antigen-2/proline rich antigen gene reveled to be promising in clinical practice [201, 202]. Recently a molecular assay based on real time qPCR was tested in clinical and environmental samples, which seems to accurately and sensitively detect both *C. posadasii* and *C. immitis* from a variety of sample types [13]. This assay uses a set of variable flanking primers and an internal probe targeting a specific *Coccidioides* LTR transposon and will be commercially available in the near future.

8.7 Therapeutics

Most coccidioidomycosis infections are self-limited without antifungal treatment, although this process may take many weeks or many months to completely resolve [39]. For those patients, periodic reassessment of symptoms and radiographic findings are important to follow-up the disease resolution without chemotherapy [2, 38, 39]. The genetic background of the patient (e.g. Filipino or African descent), immunosuppression status (e.g. HIV-infected, transplanted or under corticosteroid therapy), pregnancy or those with preexisting disease (e.g. diabetes mellitus or cardiopulmonary disease) may need early antifungal treatment upon diagnosis of coccidioidomycosis [19, 38, 39]. A recent finding regarding *Coccidioides* ssp.

antifungal susceptibility shows variability among isolates, and azole resistant strains have been detected [203].

A small percentage of patients progress to a chronic pneumonia or develop extrapulmonary dissemination involving the skin, bones and the meninges. In those cases, antifungal therapy is used to control the *Coccidioides* infection [38, 39]. Coccidioidomycosis clinical presentations vary widely and the proper management strategies must be specific to a patient. Commonly used indicators of severe disease that requires antifungal treatment include: weight loss of >10%, periodic night sweats (longer than 3 weeks), pulmonary infiltrates compromising more than one-half of one lung or portions of both lungs, prominent or persistent hilar adenopathy, high anticoccidiodial antibody concentrations in excess of 1:16, inability to work, symptoms that persist for >2 months, or age > 55 years [38, 39].

Azoles. The most common class of drugs used in the treatment of coccidioidomycosis include oral azoles: fluconazole, itraconazole, and ketoconazole. Fluconazole is preferred due to high bioavailability and low adverse drug reaction [38, 39]. According to the 2016 Infectious Diseases of America treatment guidelines for coccidioidomycosis, the advised doses and administration routes for azole treatment are: ketoconazole (400 mg every day administered orally), fluconazole (400–800 mg/day administered orally or intravenously), and itraconazole (200 mg twice per day or 3 times per day administered orally). The use of azole treatment is primarily recommended in unresolved acute infections that evolve to chronic pneumonia or disseminated infections including meningitis. Risk groups listed above that have a current acute infection identified by recommended diagnostic methods are advised to start azole antifungal therapy. The duration of azole therapy is unclear but is commonly recommended up to 6 months, with periodic follow-up visits every trimester for up to 2 years after clinical cure [38, 39]. More recently, the newer class of azoles, voriconazole and posaconazole were tested in small clinical trials. Posaconazole has been used in disseminated nonmeningeal coccidioidomycosis that previously failed to respond to azole and/or AmB therapy as well as in patients with short-term previous treatment. Voriconazole was shown to be effective in cases of azole resistance. At least 6–12 months of therapy is recommended for both Voriconazole and Posaconazole [209–213].

Amphotericin B. Deoxycholate and liposomal formulations of amphotericin B are effective, and an alternative for coccidioidomycosis treatment in the case of azole failure [209]. It is recommended in patients with rapidly progressing disease characterized by respiratory failure or rapid dissemination, and also co-diagnosis with HIV [19, 38, 39]. Again, the 2016 Infectious Diseases of America treatment guidelines advise doses and administration routes for amphotericin B. Amphotericin B deoxycholate (0.5–1.5 mg/kg per day or alternate day administered intravenously), and lipid formulations of amphotericin B (2.0–5.0 mg/kg or greater per day administered intravenously) are both effective. The combination of amphotericin B and azole therapy in severe cases was shown to improve the outcome of patients. Pregnant women diagnosed with valley fever should undergo amphotericin B treatment due teratogenicity of azoles. Administration of amphotericin B may be reduced over time as patient outcome improves [38, 39]. The deoxycholate formulation of

amphotericin B was show to be ineffective against CNS infections and this formulation seems to be more effective in treating disease [38, 210–213].

Novel agents. Several other molecules have been tested for in vitro and in vivo effects against the ethological agents of coccidioidomycosis. Nikkomycin Z (NikZ) is a natural inhibitor of chitin synthase, an essential structural component for fungal cell wall integrity [136, 214, 215]. This molecule has a strong in vitro activity against *C. immitis* and *C. posadasii* [136]. In vivo studies showed that NikZ is efficient in treating canine coccidioidomycosis, treating induced disease in murine models, and has undergone phase 1 studies in humans [137, 138]. NikZ has demonstrated sterilization of the lungs of experimentally infected mice. If confirmed in clinical trials, this would be a major advance for treatment of this disease. Recently, the effective dosage of this drug was determined in murine models of coccidioidomycosis infection for phase 2 and 3 clinical trials, and shows promise in treating this important fungal infection [138]. Recently, a new compound that inhibits CYP51 named VT-1161 showed antifungal activity in vitro against *Coccidioides* ssp., and was demonstrated to be effective in murine central nervous system models of the disease [216].

8.8 Vaccination Strategies

The population living or visiting endemic areas in the western USA and other desert areas of Latin America are at high risk, and are potential candidates for vaccination against coccidioidomycosis. At least 20 million people reside in or visit the endemic areas of the southwestern USA [158, 217, 218]. A majority of people resolve coccidioidal infections without complications, and acquire life-long resistance to a second infection [219, 220]. Thus, there is a strong public health argument for the value of the development of a protective vaccine.

The initial attempt for immunization against coccidioidomycosis was done in a five-year clinical trial using formalin-inactivated spherules. However, this study failed to produce a significant reduction in incidence or severity of disease in phase 3 trials and high levels of skin reaction among participants were observed when protective concentrations of the vaccine were administered [221]. A second study used auxotrophic *Coccidioides* mutants with attenuated virulence, and mice immunized with those strains showed protection against coccidioidomycosis [110].

One approach to developing vaccine strains is to use targeted gene deletion on putative virulence factors. One study generated an avirulent mutant deficient for two chitin synthase genes (CTS2 and CTS3), which are important for cell wall integrity of *Coccidioides posadasii* [140]. The mutant strain was avirulent and did not induce disease in a murine model due the inability of spherules to mature and produce endospores. The protective nature of this knockout strain was tested, and results indicated a protective response against the disease; however, fungal clearance was not achieved in recovered tissues after infection with wild type strains and tested animals presented with an exacerbated inflammatory response [140]. Similar results

were obtained using another avirulent strain of *C. posadasii*. This mutant lacked a single chitin synthase gene CHS5, grew atypically, had a slow development of spherules in vitro, and did not convert into the parasitic phase in vivo [222, 223]. Immunized mice survived beyond 45 days post-infection, compared to control mice; however, viable cells of *Coccidioides* were still recovered from the organs. However, one recent vaccine candidate has shown clearance and protection in a murine model of infection. Immunizations in both C57BL/6 or BALB/c mice with a live and attenuated *C. posadasii* strain lacking the CPS1 gene resulted in a survival of over 95% of mice infected with lethal doses. Few residual fungal colonies were recovered from the lungs, and this live attenuated strain is a strong candidate for coccidioidomycosis vaccination [129].

Purified antigens have also been tested as vaccine candidates and provided important advances in the knowledge of the immunopathology of these fungal pathogens. *C. immitis* purified urease and the urease-DNA ectopically expressed by a mammalian plasmid promotes an immunoprotective Th1 response against coccidioidomycosis in a murine model [133]. The purified recombinant *C. posadasii* Gel1 protein also conferred protection to C57BL/6 mice against coccidioidomycosis. However, a significant decrease in the level of Th1 that is fundamental to immune response to coccidioidomycosis was observed [224]. The proline-rich antigen combined with a coccidioidal antigen 2 (Ag2/PRA), which codify for a recombinant 194 amino acid protein, has been shown to be protective against *Coccidioides* infection. Recombinant Ag2/PRA confers protection from *Coccidioides* infection by inducing a T-cell $CD4^+$-type response and is a potent vaccine candidate for this important disease [33, 225]. Other antigens such as SOW, ELI1, HSP60, aspartyl protease, and *Coccidioides*-specific antigen (CSA) have been used to immunize mice and showed significant reduction of fungal burden, but none promote complete clearance of the pathogen [33, 158, 217]. Finally, fungal glucans purified from *S. cerevisiae,* a potent immunomodulator that binds to Dectin-1, has been shown to stimulate immunity in CD1 mice infected with *C. posadasii*. This same vaccine was effective against Aspergillosis, which leaves open the possibility of a pan-fungal vaccine [226].

There are several candidates for the vaccine of coccidioidomycosis; however, none of those compounds or attenuated strains is commercially available. Cell-mediated immunity is critical for defense; however, the role of the humoral response in coccidioidomycosis is still a gap that impairs vaccine development. Moreover, considering that disease may disproportionately affect a naive elderly population, new adjuvants that increase the priming of the immune system and improve the performance of vaccines are needed [227].

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Chapter 9
Paracoccidioides spp. and Paracoccidioidomycosis

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Abstract Paracoccidioidomycosis, the most prevalent deep mycosis of Latin America, is caused by *Paracoccidioides* spp., pleomorphic fungi which are present as multibudding yeast cells in infected tissues. Due to their importance to the region, these fungi and their related disease have been the subjects of intense research for

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over 50 years. The present chapter is an attempt to summarize the findings of those decades of research and clinical findings, with an update on the latest advances on taxonomy studies, species distribution and reproductive modes, diagnosis trends, virulence factors, as well as clinical manifestations, and updated treatment of the disease.

9.1 Etiologic Agents

For almost 100 years, since its first description in 1908 by Adolpho Lutz in Brazil [1], *Paracoccidioides brasiliensis* was considered the only causative agent of paracoccidioidomycosis (PCM), a human systemic mycosis geographically confined to Latin America, where it constitutes one of the most prevalent deep mycoses. However, in 2006, the first report on molecular phylogenetic data revealed the presence of cryptic species, namely *P. brasiliensis* S1, PS2, and PS3 [2]. To date, a fourth species within the *P. brasiliensis* species complex, PS4, and a newly identified species within the *Paracoccidioides* genus, *Paracoccidioides lutzii*, have been reported [3–6] (Fig. 9.1).

Paracoccidioides spp. are pleomorphic fungi that depend on the temperature of incubation to establish a specific cell morphology. In infected tissues, and in vitro cultures at 37 °C, these fungi grow in the form of spherical-to-oval cells of different sizes, which can go from a few nanometers in diameter for young, recently separated buds, to up to 30 μm in mature yeast (Y) cells [7]. Yeast cells multiply by multipolar budding and are multinucleate. Buds connect to the mother cell by narrow necks, giving the whole structure an appearance of a ship's wheel (Fig. 9.2), a shape considered

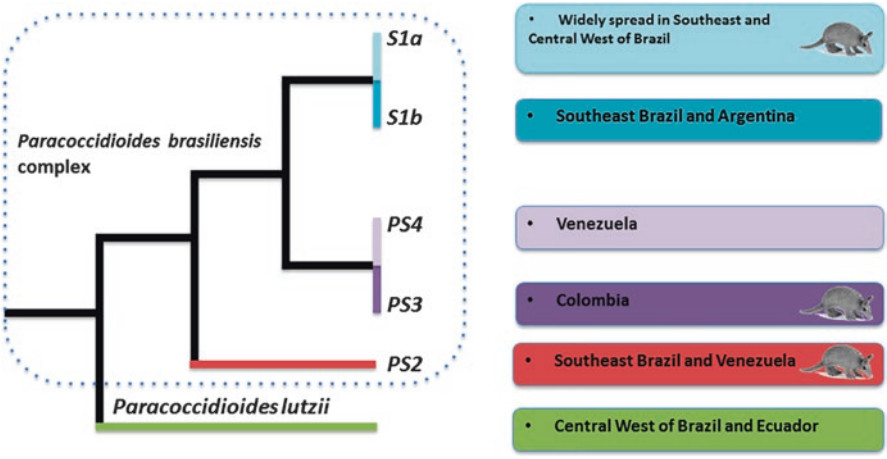

Fig. 9.1 Phylogenetic relationships of *Paracoccidioides* spp. Colored boxes indicate species geographic distribution

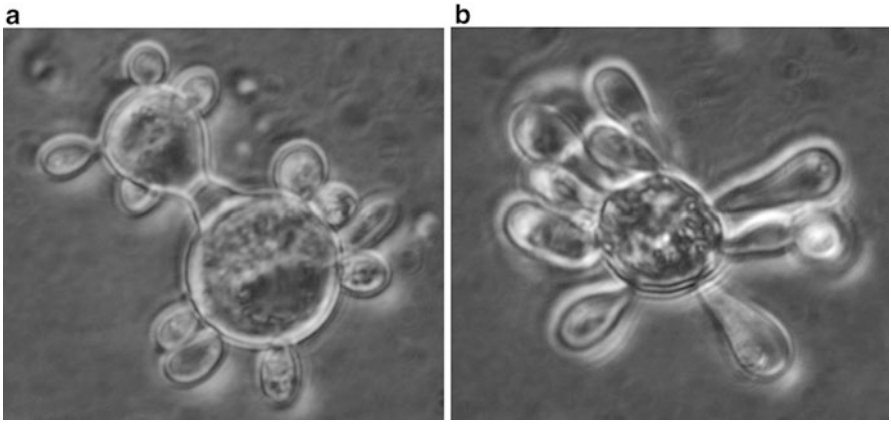

Fig. 9.2 Multibudding yeast phase of (**a**) *P. brasiliensis* and (**b**) *P. lutzii*. Cells were grown at 37 °C on YPD supplemented with 5% horse serum. Magnification ×400. Photos courtesy of Sabrina Rodríguez and Laura Barreto, IVIC, Caracas, Venezuela

as a diagnostic characteristic of *Paracoccidioides* spp. The mycelial (M) form grows slowly at room temperature. Microscopic observations show septate and freely branching hyphae, 1–3 μm in width [8], with the appearance of interwoven threads. Several types of conidia [intercalary, septate, pedunculate] are formed after 2 months of growth under conditions of nutritional deprivation [9, 10]. Conidia can germinate and produce Y cells at 37 °C, with the capacity to infect mice, or may produce germ tubes and branching mycelia if kept at 20–23 °C.

9.2 Taxonomy, Species Distribution, and Reproductive Modes of *Paracoccidioides* spp.

P. brasiliensis [1] was considered a single species for almost 100 years. With the advances in the molecular biology field and fungal genotyping techniques during the 90s, many authors demonstrated that the *Paracoccidioides* genus has an extensive genetic diversity deciphered by karyotyping profiles, RAPD, RFLP, and microsatellites typing methods [11–17]. Those molecular methods were very important for an initial assessment of the population diversity of this pathogen, and provided evidence for geographical and genetic isolation [14], and varied ploidy [11, 17]. The Sanger sequencing technology in the late 90s brought to the mycologist an opportunity to analyze clinical isolates at a nucleotide base level across multiple genomic loci. This allowed the emergence of an operational method proposed by [18] in which fungal species could be delineated by phylogenetic concordance and non-discordance of multiple genes (GCPSR – Genealogical Concordance for Phylogenetic Species Recognition). This new concept is extensively applied in the mycology field as a tool for species discrimination as well as giving support for

molecular epidemiology studies [19, 20]. The methodology was first applied in *P. brasiliensis* by [2] in a set of 65 isolates through eight gene loci, and at least three distinct phylogenetic species were proposed: ***P. brasiliensis* S1** – widely distributed species in Brazil, Uruguay, Peru, and Argentina; ***P. brasiliensis* PS2** - limited to southeast Brazil and Venezuela and ***P. brasiliensis* PS3** - geographically restricted to Colombia. This study was extremely important for species delineation and eco-epidemiology in *Paracoccidioides*; however, no differentiation between soil, veterinarian or human isolates was found. A fourth phylogenetic species was later proposed to be restricted to Venezuela, being named as ***P. brasiliensis* PS4** (Fig. 9.1) [3, 4]. One of the most studied isolates in the paracoccidioidomycosis field, Pb01, was not included in the dataset of Matute et al. [2], which later was found to present a unique multilocus phylogenetic composition, when compared with the three reported *P. brasiliensis* species S1, PS2, and PS3 [5]. More isolates sharing a genetic profile with Pb01 (*Pb01-like*) were later identified based on the same techniques, revealing a great genetic isolation from the previous identified *P. brasiliensis* complex [6]. In addition, *Pb01-like* strains displayed exclusive elongate rod-shaped conidia beyond the typical bicorn cocked hat- and barrel-shaped conidia produced by *P. brasiliensis* [21]. The strains that composed the *Pb01-like* clade were mostly recovered from Central-North areas of Brazil and from Ecuador. Recently the phylogenetic species *Pb01-like* was ranked as a new species apart from the *P. brasiliensis* species complex and named ***Paracoccidioides lutzii,*** as a tribute to the discoverer of PCM, Adolfo Lutz (Fig. 9.1) [3, 22].

All *Paracoccidioides* species described so far cause both, acute and chronic disease; however, some relevant clinical features and phenotypes were observed in those patients infected with *P. lutzii* and *P. brasiliensis* species complex [23]. (1) Serology-based diagnostics of PCM must be performed with species-specific antigens, since most sera recovered from patients infected with *P. lutzii* are not recognized by *P. brasiliensis* antigens and vice-versa [3, 24–26]. (2) Most of PCM infections reported in endemic areas of *P. lutzii* show frequent lymphatic-abdominal clinic manifestation [27, 28]. (3) A preliminary study reported that patients infected with *P. lutzii* isolates have a better response to trimethoprim-sulfamethoxazole therapy than those patients infected with *P. brasiliensis* [17]. (4) *P. brasiliensis* has lower interaction capabilities with hemocytes, when compared to the *P. lutzii* infection model in *Galleria mellonella*, which would indicate different phagocytosis mechanisms [29]. (5) *P. brasiliensis* displays higher adhesion and adhesin expression levels, and greater virulence than *P. lutzii,* in the infection model *G. mellonella* [30]. With the exception of serological achievements, those studies were based on few isolates, and studies on virulence models require more isolates from each group in order to improve knowledge of infection dynamics of both pathogens.

In recent years, next-generation sequencing (NGS) has decreased the cost of sequencing whole genomes and increased the amount of generated data per sequencing run. This allows an increase in the power of genetic discrimination between microorganisms by analysis of thousands of loci instead of just a few. The genomes of *P. brasiliensis* S1 (Pb18), PS2 (Pb3) and *P. lutzii* (Pb01) were fully sequenced, revealing unique adaptations of the *Paracoccidioides* lineage as well as reinforcing

the genetic divergences between those two species [31]. Recently, the genomes were carefully reassembled and re-annotated generating high confidence reference genomes for both, the *P. brasiliensis* complex and *P. lutzii*, providing support for genealogical concordance based on whole genome sequence typing [32]. Furthermore, a whole-genome sequence study including 36 *Paracoccidioides* isolates covering the five phylogenetic species of this genus was performed. Gene order was highly conserved across the major lineages of *Paracoccidioides* and few chromosomal rearrangements were found. The phylogenetic species *P. brasiliensis* S1 was split into two clades: S1a and S1b, which were deciphered by increasing the number of genomic loci [33].

With the increase of genomic information, new molecular markers specific for the *Paracoccidioides* genus were developed, enabling the identification of the fungus presence in the air and soil within the endemic regions [34, 35]. ITS sequence analysis of some *P. brasiliensis* S1a and PS2 samples recovered from soil and armadillos, showed that they cluster with clinical samples [21, 32]. However, ITS amplicon Sanger sequencing revealed additional genotypes of *Paracoccidioides* found in soil and aerosol that had not been previously reported in clinical samples [35]. These genotypes may represent noninfectious or low virulence strains that may be present in the environment. Once exposed to these low virulence strains, a mammalian host could be naturally vaccinated and acquire humoral response against other *Paracoccidioides* genotypes. Isolation of *Paracoccidioides* from the environment is laborious and has been mainly unsuccessful in many published attempts. Thus, molecular markers might be a better choice for environmental studies in the PCM field [35]. Moreover, the recently published molecular probe for *P. brasiliensis* and *P. lutzii* may be useful for diagnostics in clinical practices, since it presents a great specificity in in vitro assays [35].

Population genetics of the genus *Paracoccidioides* revealed different breed strategies [3]. For example, the species *P. brasiliensis* PS3 has little genetic variation and a genetic constitution compatible with a clonal microorganism [2]. Phylogeographic analysis suggests that PS3 species emerged from a migration event from *P. brasiliensis* S1b, which kept stable after the Andean uplift [21]. The remaining species (S1a, PS2 and *P. lutzii*) show much more variation and wider distribution. Moreover, Most Recent Common Ancestor (MRCA) estimations supported S1b as the early-diverging *P. brasiliensis* population, with the later emergence of PS2, S1a, and PS3 [33]. Population genetic studies reveals that those species are recombining and sexual reproduction could be the source of genetic and phenotypic variation of those populations [2, 21, 22]. The genus *Paracoccidioides* was considered for many years asexual, since no sexual structures had been reported. However, comparative genomic analysis revealed that the species from the genus *Paracoccidioides* harbor a genomic architecture shared with the close Ajellomycetacean species *H. capsulatum* and *B. dermatitidis* [36, 37], both with a defined sexual stage [38]. The ***MA***ting-***T***ype (*MAT*) locus that regulates the sexual cycle in many Ascomycetes is present in *Paracoccidioides* species in chromosomal synteny with the sexual counterparts of *H. capsulatum* and *B. dermatitidis* [38]. A ratio of 1:1 between both *MAT1-1* and *MAT1-2* idiomorphs was identified, which is compatible with sexual recombining

species [29, 39]. Moreover, a series of meiotic regulators were found in all *Paracoccidioides* genomes, which are conserved with the well characterized *Aspergillus* and *Saccharomyces* species [38, 40]. Finally, sexual structures were reported for both *P. brasiliensis* and *P. lutzii* by co-incubating different mating-type strains under soil extract agar. Those authors revealed the presence of young ascocarps (immature gymnothecia) by configuring arrays of intercoiling hyphae [38]. None mature gymnothecia or ascospores have been observed so far. Also, nuclear migration in intercoiling hyphae sections and parasexual cycle should not be discarded, since mature sexual structures were not observed. Aneuploidy was already reported for some *Paracoccidioides* isolates which also indicate mitotic recombination followed by haploidization [41].

9.3 Epidemiology

PCM is a systemic disease affecting Latin America, where it is diagnosed from Mexico (23°N) to Argentina (34° S), with Brazil accounting for more than 80% of all reported cases, followed by Venezuela, Colombia, Ecuador, Bolivia and Argentina. No cases have been reported in Chile, Surinam, Nicaragua or Belize, and with the exception of few isolated reports, the mycosis has neither been observed in Trinidad, Granada or Guadeloupe. Additionally, in endemic countries, the mycosis is distributed irregularly throughout the territory, preferring sites where humidity is high and forests abound [42–44]. Mention should be made of the non-autochthonous patients - less than 100 - reported in Europe, Japan or the United States. All those patients had previously resided or visited the Latin America endemic regions for a mean period of 14 years before the mycosis became manifested. These observations revealed the ability of the etiologic agents to become latent once in the host with no loss of viability such as exemplified by the patient diagnosed in Japan by Kurai et al. [45].

The mycosis is caused by two species of the genus *Paracoccidioides,* namely, *P. brasiliensis* and *P. lutzii*, with the former predominating within the endemic region (Fig. 9.1) [21, 46, 47].

Based on Brazilian statistics, the incidence of PCM was estimated in 10–30 cases per 100,000 inhabitants per year [48]. Nonetheless, as an example, variations were recorded in the state of Paraná where 102 cases were diagnosed in a period of 18 months [49]. Additionally, another place with high incidence of PCM appears to be the Brazilian state of Rondônia where 39.1 cases/100,000 inhabitants were informed from 1997 to 2012 [50].

As for mortality, a study in the Brazilian state of Paraná estimated the annual death toll to be 3.48 per million inhabitants, thus making PCM the fifth cause of death among the chronic infectious diseases of the low respiratory tract [51]. During the period 1998–2006, a Brazilian study was performed covering 35% of the country, which represented 27% of the 5560 Brazilian municipalities - including the larger part of the endemic PCM area. The study constituted a population estimated at

4.3 per one million inhabitants, and analyzed 6732 hospitalization events attributable to PCM (82% male). They reported an estimated global mortality of 1.4 per one million inhabitants with 60% of deaths being attributed to the mycosis itself and the remaining to the fibrotic sequelae [52]. Another retrospective Brazilian study analyzed 3,583 death certificates attributed to various systemic mycoses. It found that PCM was the most frequent cause of death with annual tolls of 53.9% and 44.6% at the beginning and end of the study, respectively. Additionally, the association with AIDS was found to be detrimental [53].

Studies in groups of patients have shown that work with soil and certain crops, common in rural Latin American fields (coffee, cotton, tobacco, sugar cane), predominated as the most common occupation (<60%). Other jobs mentioned were masonry and lumberjacking [44, 54–56].

Even though the precise micro-niche of the etiologic agent has not been fully recognized, it is accepted that PCM is acquired by inhalation of the fungus' conidia [57]. Most individuals exposed to the fungal propagules develop an asymptomatic pulmonary infection that disseminates to regional lymph nodes reaching, at times, deep organs [44, 55, 58].

In infected individuals *P. brasiliensis* may remain latent but viable for long periods of time, as demonstrated by patients diagnosed in non-endemic countries [45, 55]. Once revitalized, the fungus may spread to other organs and tissues through blood and lymphatic systems [55, 59]. If this occurs, reactivation of the disease process may take place with dissemination to lymph nodes, skin, mucosae, larynx, spleen, liver, bones, adrenals, central nervous system and other organs [58, 60].

A series of cases have revealed that, at time of diagnosis, 70% of the patients were older than 30 years of age, 20% were adolescents with the remaining 10% corresponding to children with most cases (58.2%) being observed in patients aged between 40 and 59 years [42, 43, 56, 58, 61]. There is predominance of the mycosis in males over females (13 to 1), although in certain countries (Argentina, Colombia) this relation can be as high as 70 males per 1 female [42, 55, 62] Among children and youngsters aged less than 14 years, no gender differences are noticed, probably because 17 beta-estradiol, the hormone known to hinder the transformation of the infectious air-borne, mycelial-derived conidia into the yeast tissue forms, has not yet been expressed as it occurs in adult patients [63].

No race predilection has been noticed, except that the European and Asiatic emigrants established in the PCM endemic regions, tend to develop a more severe disease [46, 56, 58].

Among the risk factors to develop PCM, agriculture, as mentioned before, has been most frequently mentioned with over 70% of the patients being farmers [56, 58, 60]. Construction work and masonry have also been mentioned [42, 58, 61]. As noted above, male gender is also a risk factor as men develop PCM much more frequently than females [55, 58, 62] even if infection - as shown by delayed type hypersensitivity to the paracoccidioidin test - is equal in both genders [64]. Young age is related to the severity of the mycosis but not with an increased number of cases [65].

9.4 Clinical Manifestations.

PCM has been divided into four categories—two devoid of clinical manifestations and another two directly related to disease processes and largely dependent on age and host immune status:

Subclinical infection: Considered to be a latent, clinically asymptomatic process lasting for 1 month to years after the initial fungal contact, and which might evolve towards a progressive, clinically manifested disease [66].

Residual form: Characterized by the presence of sequelae originating in the previously infected fibrous tissues, mainly in the lungs, but also reported in adrenal glands and mucosae.

Acute/subacute disease: An overt and severe process evidenced by involvement of multiple organs, with lymph node, liver, and spleen hypertrophy and manifestation also of skin lesions, the latter often multiple and widely distributed throughout the body. The lungs do not often reveal radiographic abnormalities, nor are the clinical manifestations obvious, even though *P. brasiliensis* is frequently detected in respiratory specimens. This form is usually diagnosed in children, young adults, and AIDS patients, as well as in those with other immune alterations.

Chronic progressive disease: The most common (90%) of the clinical presentations, diagnosed in older patients and characterized by important lung involvement and frequent lesions in the mucosae, skin, adrenal glands, and other sites.

In any of the clinically manifested forms, constitutional symptoms (fever, asthenia, general malaise, weight loss) are regularly observed [67].

9.4.1 Physiopathology

Lungs are the primary site of infection, whose clinical manifestations are cough, expectoration, hemoptoic sputum, chest pain, and some degree of dyspnea are regularly noticed. Auscultation reveals minimal abnormalities compared to radiographic findings, with a clear dissociation between symptoms and imaging studies [57, 68].

Chest radiograph images reveal mostly interstitial infiltrates (64%), followed by mixed lesions consisting of nodular and alveolar infiltrates that are occasionally confluent, frequently bilateral, symmetrical, and located preferentially in the central and lower fields [57, 68, 69]. High-resolution computed tomography demonstrates abnormal findings in more than 90% of the patients with chronic pulmonary disease, with the most common abnormalities being interlobular septal thickening (88%), peribronchovascular interstitial thickening (78%), centrilobular opacities (63%), intralobular lines (59%), ground-glass opacities (34%), cavities (17%), and airspace consolidation (12%) [70]. Gallium-67 scans reveal pulmonary lesions in practically all patients [71]. At diagnosis, one third of patients with long-lasting

disease have serious pulmonary sequelae fibrosis (32%), bullae (27%), as well as indirect signs of pulmonary hypertension with right ventricle enlargement and even cor pulmonale [57, 68, 69].

The oral mucosa is involved in 50% of the cases, with lesions regularly localized in the gingiva and the palate and, with extension to the neighboring skin on the lips and nose. The larynx, pharynx, and gastrointestinal mucosa are also affected. Lesions are infiltrative, edematous, ulcerated, and have a granulomatous base, mulberry-like in appearance. They are extremely painful and hinder the patient's food intake; upon healing, they produce fibrous scarring [72, 73]. This compromise is the principal difference with tuberculosis, although nowadays the oral presentation of TB is rare, with a prevalence ranging from 0.1 to 0.4% [74, 75].

Cutaneous lesions are observed in patients with either the adult form (25%) or the acute/subacute presentation (50%). Skin abnormalities may represent hematogenous dissemination from the primary pulmonary focus or secondary extension from contiguous mucocutaneous lesions. They tend to ulcerate and show a thick well-delimited external border with a granulomatous base, accompanied by infiltration of the neighboring tissues. In AIDS patients, lesions are multiple and rather small, flat, covered by crusts, and with no inflammation in the surrounding tissues [73, 75].

All **lymph node chains** may be involved, with predilection for cervical, axillary, mediastinal, and mesenteric nodes. If mucosal or skin lesions exist, enlargement of the corresponding draining lymph nodes is noted, and they may form fistulas. Lymphatic hypertrophy can cause complications by compression of contiguous structures [67, 68].

In endemic countries, this mycosis is one of the main causes of adrenal insufficiency. Involvement is evidenced by gland hypertrophy with damage to both the cortex and the medulla; in addition, 10% of the patients may present Addison's syndrome. When cortisol is measured at basal and post–adrenocorticotropic hormone stimulation times, diminished adrenal reserve is detected in 33–40% of the patients. Autopsy studies, however, have shown that adrenal involvement may occur in up to 85% of cases. Prompt initiation of antifungal therapy may restore adrenal function [76].

Other lesions: PCM also involves the central nervous system in 9.65–25.45%, with formation of cerebral and meningeal lesions and spine compromise with spondylodiscitis. Lesions are more frequent in the brain hemispheres (69%), in 65% there are multiple granuloma characterized by hypodense images with annular or nodular enhancing [77–79]. Other organs affected are the liver, spleen, and pancreas, simulating malignant neoplasia [80, 81].

The prevalence of PCM in immunocompromised patients by AIDS, is relatively low and occurs more frequently in advanced AIDS patients (CD4 counts <200 cells/mL, viral copies >100,000 mL). In the Brazilian State of São Paulo, the prevalence of this comorbid association was estimated to be 1.4% of HIV-infected patients [82]. In those patients, skin lesions are extremely common and 40% of

them had received trimethoprim-sulfamethoxazole treatment for pneumocystosis and toxoplasmosis prophylaxis. This finding suggests that prevention of PCM with this medication, had proved ineffective. In these cases, treatment follows the same schemes used for the non-HIV patients, except that maintenance therapy should be required up to the time when CD4 counts are consistently greater than 200/cells/mL and viral copies undetectable through administration of antiretroviral therapy [75, 83].

The most important differential diagnosis is tuberculosis, a disease that may coexist with the mycosis in 8–13% of patients [84]. Other diseases that should be considered are cancer and neoplastic disorders (including lymphoma), histoplasmosis, leishmaniasis, leprosy, and syphilis. Only laboratory testing can establish the correct diagnosis [45, 85].

9.5 Diagnosis Trends

Current diagnostic approaches include traditional techniques, namely direct examination, culture, histology, antigen and antibody detection, and nucleic acid-based methods such as polymerase chain reaction (PCR) assays [77, 86–88]. More recently, commercial platforms have also been developed such as MALDI-TOF MS (matrix-assisted laser desorption ionization time-of-flight mass spectroscopy), which is unique in that it does not depend on analysis of genetic information, but is instead based on analyses of the spectrum of organic biomolecules/proteins present in the isolate [89, 90]. The definitive diagnosis of PCM depends on microscopic examination and positive culture. Current culture-based diagnostic tools continue to be the "gold standard" for diagnosis; however, isolation and identification of *Paracoccidioides* spp. from clinical specimens may take up to 4 weeks. The best approach is typically a use of a combination of methods for diagnosis when possible.

9.5.1 Direct Examination and Histopathology

In clinical specimens, such as sputum, bronchoalveolar lavage fluid, oral, pharyngeal, adrenal glands, pus from draining lymph nodes, or biopsy materials from any other tissues, the characteristic translucent-thick refracting wall of yeast cells of *Paracoccidioides* with multiple peripheral buds (pilot wheel configuration) plus prominent intracytoplasmatic vacuoles can be found (Fig. 9.3a). Often, yeast cells appear in chains and have single buds and some large yeast cells can also be observed. Sensitivities vary from 85 to 100% depending on specimen, clinical manifestations, and treatment status on direct examination [67, 91, 92]. Several procedures are

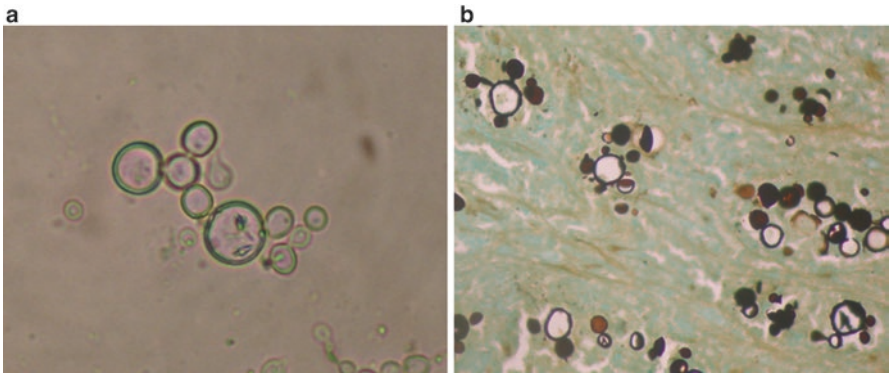

Fig. 9.3 Wet mount of (**a**) sputum in KOH showing blastoconidia of *P. brasiliensis*. Magnification ×200; (**b**) Gomori methenamine-silver stain of lung tissue showing blastoconidia of *P. brasiliensis*. Magnification ×100

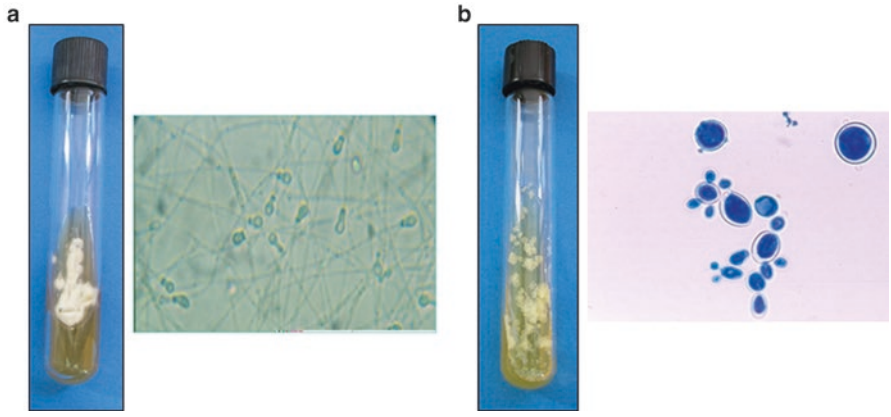

Fig. 9.4 (**a**) *P. brasiliensis* colonies of mycelial phase incubated at 25 °C and microscopic observation showing thin septate hyphae and microconidia (magnification ×100). (**b**) *P. brasiliensis* colonies of yeast phase incubated at 37 °C and microscopic observation showing round mother cell surrounded by spherical to elongated daughter cells (magnification ×40)

adequate for visualizing fungal elements, including fresh or wet KOH preparations, as well as calcofluor and immunofluorescence methods. Staining of preparations with lactophenol cotton blue and Gram staining can also be helpful. Histopathology preparations stained with hematoxylin and eosin (H&E), Gomori methenamine-silver (GMS; Fig. 9.3b), or periodic Acid-Schiff are also useful as they reveal the multiple budding yeast elements, especially within the granulomatous foci commonly observed. It is important to differentiate small yeast of *P. brasiliensis* from *Cryptococcus neoformans*, *Blastomyces dermatitidis*, and even from *Histoplasma capsulatum* [43, 67, 68, 86, 91–93].

9.5.2 Cultures

P. brasiliensis is a thermally dimorphic fungus. At room temperature (19–24 °C) it grows as a mold and requires a lengthy incubation, of up to 30 days (Fig. 9.4 a). Isolation of *P. brasiliensis* from clinical specimens requires a battery of culture media such as simple Sabouraud dextrose agar, more enriched media like brain-heart infusion (BHI) plus glucose, and a selective medium, Mycosel™, which includes antibacterial drugs (chloramphenicol or gentamicin) and mold inhibitors (cycloheximide) that provide a useful differential tool in the process of identifying the fungus [43, 67, 86]. When incubated at 25–30 °C, the isolate produces a variety of forms from flat to glabrous colonies, with a few tufts of aerial mycelium. Colonies can also look wrinkled, and a variation in color can be observed, which can go from white to beige-brownish. Microscopically, the mold shows thin septate hyphae and occasionally chlamydospores (15–30 μm) are present. Under conditions of nutritional deprivation, some isolates produce conidia, which vary in structure from arthroconidia to microconidia measuring less than 5 μm (Fig. 9.4a). The mycelial form is not distinctive and dimorphism must be demonstrated [43, 67, 86]. Conversion to the yeast phase is also very slow. Conidia respond to temperature changes, germinating into hyphae at 20–24 °C or converting into yeasts at 36 °C on appropriate media. When cultures are transferred to 37 °C on rich media such as BHI, Pine's, or Kelley's agar, the resulting yeast colonies are generally folded, with cerebriform appearance (Fig. 9.4b). Yeasts are 2–30 μm in diameter, are oval or irregular in shape, and display the characteristic "pilot's wheel" described previously (Fig. 9.4b). There is no commercial DNA probe test for *P. brasiliensis*. Biosafety level 2 practices and facilities are recommended for handling and processing clinical specimens [86], although procedures involving the manipulation of sporulating cultures should be handled under biosafety level 3 procedures.

9.5.3 Immunodiagnostic Tests

Immune-based methods for antibody and antigen detection are useful not only for diagnosis but also for monitoring the patient's course [86, 94–96]. The most popular methods are immunodiffusion (ID), double immunodiffusion (DID), complement fixation (CF), but other tests, such as ELISAs, counter-immunoelectrophoresis (CIE), dot blot, and immunoblotting have also been employed [86, 94]. The ID test is highly specific and is positive in 65–100% of cases of acute or chronic pulmonary infection or disseminated PCM. The CF test allows a more precise evaluation of the patient's response to treatment, but is less specific than the ID as cross-reactions with *H. capsulatum* occur. As it is simple to perform, the gel immunodiffusion or DID test is typically the most common method used in endemic countries. This test uses antigen preparations from either mycelial or yeast cell lysates. Cell wall antigens have proved less useful than culture filtrate antigens, largely because cell wall antigens are dominated by cross-reactive galactomannan [86, 94, 95]. While a series

of immunoreactive antigens are present in the yeast cell lysates (27 kDa, 43 kDa, 60 kDa, 70 kDa and 87 kDa), the predominant antigen is a 43-kDa glycoprotein, gp43 [67, 94]. Commercial mycelial-form culture filtrate antigen can be obtained for "in-house use in ID from IMMY (*Paracoccidioides* ID Antigen), but its sensitivity has not been widely studied [86]. Complement fixation (CF) test is performed with *P. brasiliensis* yeast-form culture-filtrate antigen but this reagent is not commercially available. CF titers of ≥1:8 are considered presumptive evidence of PCM and falling CF titers are often predictive of successful treatment [86]. No commercial kits or reagents are available. Several laboratories have developed their own "in house" immunodiagnostic tests based on methods such as ELISA, inhibition ELISA, competition ELISA, Western blot, or dot blot [94–96]. Improvements include the detection of antibodies against chemically characterized and/or recombinant *P. brasiliensis* antigens, notably gp43, pb27, and the 87-kDa heat-shock protein [97–99]. A combination of two recombinant products has resulted in increased sensitivity (92%) and specificity (88%) [100]. Antigen detection tests have some important advantages over antibody detection in the diagnosis of PCM, particularly in immunocompromised patients and in those previously exposed to *P. brasiliensis* who may have preexisting antibody titers [86, 94].

Many efforts are being made and are underway to search for new serological markers for the diagnosis and monitoring of PCM, especially because of the recent advances in characterizing the taxonomy of the genus, and this is certainly a new challenge [24, 94]. It has been found by DID that the serum from patients with PCM due to *P. brasiliensis* does not recognize antigen contained in the cell-free preparations of *P. lutzii*, unlike the serum from patients with PCM due to *P. lutzii*, which recognizes both antigens (*P. lutzii* and *P. brasiliensis*), suggesting that *P. lutzii* is antigenically more complex and has species-specific antigens but also shares common antigens with *P. brasiliensis* [24]. Many laboratories in Latin America have had difficulty obtaining antigens because the method requires many steps, and efforts have been made to simplify the methodologies. However, there is no standard way of obtaining the antigens, and the diagnosis techniques used in different mycology reference centers show large inter-laboratory variability in results when assessing the same clinical sample [95, 101]. Understanding the uses and limitations of the battery of serological methods is essential for the diagnosis of PCM [94, 95]. New technologies have also started to be applied, with the use of gold-nanoparticles functionalized with specific antibodies, or antigens for detecting circulating antigens of antibodies alone or in combination with PCR for the early diagnosis of PCM [102, 103].

9.5.4 Molecular Detection

A variety of molecular methods have been designed and tested over the years, and they now play a significant and growing role in clinical mycology and offer distinct advantages over conventional methods, as they provide faster, more sensitive and more specific identification. However, for the detection of *Paracoccidioides* in

human clinical samples the number of "in house" tests developed and evaluated so far is still very limited, they have no extensive validation across laboratories and they have not been made commercially available [87, 88]. Amplification and sequencing of conserved rDNA regions, especially 5.8 and 28S subunits, as well as intergenic spacer regions have been successfully developed and enabled discrimination between *Paracoccidioides* and other human pathogenic fungi by PCR using DNA extracted from cultures [87, 104]. Although in more recent studies, genes specific for *Paracoccidioides* have been chosen as PCR targets and have often had higher specificity. A real-time PCR that used as a target the ITS1 region of ribosomal DNA (rDNA) was developed to detect *P. brasiliensis* DNA in both cultures, and clinical specimens and, although the evaluation was carried out with a low number of clinical isolates ($n = 10$) and human samples ($n = 2$) the authors reported 100% sensitivity and specificity [105]. Some other attempts to develop a PCR with the same target have had very limited success.

Among the *Paracoccidioides*-specific targets, gp43 is considered the immunodominant antigen for diagnosis of PCM. A nested PCR assay to amplify the gp43 gene was evaluated using an experimental mouse model of PCM, and the test was positive in 91% of lung homogenates and did not cross-react with other pathogens [106]. The same technique was then evaluated and validated for use in a variety of clinical samples ($n = 25$ cases with proven PCM), and a 100% sensitivity and specificity was reported [107]. Another PCR assay based on the 5′ nuclease assay, using a fluorescent probe derived from the sequence of the gene coding for the gp43 antigen was developed, and the authors reported that the assay could detect at least 10 copies of this DNA sequence, providing sufficient accuracy to be useful for diagnosis of PCM [108]. A loop-mediated isothermal amplification (LAMP) assay was tested for its ability to detect the gp43 gene of *P. brasiliensis* [109], and positive results were reported for DNA extracted from formalin fixed paraffin embedded tissues from PCM patients [109]. In another study, respiratory samples were evaluated by LAMP, with reported sensitivity and specificity values of 61% and 100%, respectively [110]. LAMP methodology exhibits advantages in speed in developing the assay and in simplicity, when compared to the classic diagnostic methods, as it does not require sophisticated equipment. Molecular detection and discrimination of *P. brasiliensis* and *P. lutzii* has been reported by Single Nucleotide Polymorphism (SNP) genotyping [21]. Much work still needs to be done to reach a consensus for extraction protocols, amplification targets and best approaches and platforms for measurement. Extensive clinical validation of the techniques is also much needed.

9.5.5 MALDI-TOF Mass Spectroscopy

Isolates of *P. brasiliensis* and *P. lutzii* ($n = 22$), previously characterized by molecular techniques, including multilocus sequence typing, internal transcribed spacer (ITS) rRNA gene sequencing, and PCR of the hsp70 gene, were recently identified

for the first time by MALDI-TOF mass spectrometry [90]. The use of a commercial platform does not require extensive training, could be well adapted to reference routine laboratories and could become a method of choice for species differentiation within the genus *Paracoccidioides*, providing to epidemiologists and clinicians new tools to address unresolved issues of PCM [90].

9.6 Virulence Factors

9.6.1 Dimorphism

Once conidia or hyphal fragments are inhaled into the lung alveoli, *Paracoccidioides* spp., as well as other endemic dimorphic fungi, switch to a unicellular yeast form due to temperature, oxidative stress, changes in carbon dioxide tension, and hormones. This morphological switching, known as dimorphic transition, represents an essential step for the survival and maintenance of the fungus within the host. In dimorphic fungi, three signaling pathways have been identified that induce the dimorphic switching and yeast growth at 37 °C; the two-component signaling, the heteromeric G protein and Ras signaling, and calcium signaling [111]. The two-component signaling systems are regulated through DRK1 (dimorphism-regulating histidine kinase 1) [111]. In *B. dermatitidis* and *H. capsulatum*, *DRK1* mutants are avirulent in a murine model of infection, failing to convert to the pathogenic yeast form, and growing as mycelia at 37 °C [112]. The *DKR1* orthologue in *P. brasiliensis* is highly expressed in the virulent yeast phase, and is fundamental in the mycelial to yeast transition, suggesting a potential new drug target [113, 114].

9.6.2 Cell Wall Polysaccharides Composition

The dimorphic transition promotes changes in the cell wall composition and carbohydrate polymer structure. *Paracoccidioides* yeast cell walls' most remarkable change is the occurrence of α-(1,3)-glucan and the drastic reduction of β-(1,3)-glucan that correlates with pathogenicity [115]. The initial observation in *P. brasiliensis* that α-(1,3)-glucan might act as a protective shield against host defense was demonstrated 30 years later in *H. capsulatum* [116]. The presence of α-(1,3)-glucan in the outermost layer of the cell wall of *H. capsulatum* yeast masks β-(1,3)-glucan, avoiding its recognition from pattern recognition receptors (PRR) found on host phagocytic cells. Furthermore, disturbance of the α-(1,3)-glucan synthesis by deleting the *H. capsulatum* α-(1,4)-amylase (*AMY1*), involved in priming the oligosaccharide synthesis, reduces cell wall α-(1,3)-glucan content and fungal virulence [117, 118].

9.6.3 Adhesins

Multiple studies have characterized diverse molecules synthesized by *Paracoccidioides* spp., known as surface adhesins, including: gp43 [119, 120] HAD32 [121, 122], a 30-kDa adhesin [123], enolase (ENO) [124], malate synthase (MLS) [125], triosephosphate isomerase (TPI) [126], glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [127] and fructose-1,6-bisphosphate aldolase (ALD) [128]. These surface proteins interact with host cells to promote successful colonization and/or dissemination of the fungus into the host organism, by mediating fungal cell binding to host extracellular matrix components (mainly fibronectin, laminin, fibrinogen, type I and IV collagen, and plasminogen) and to epithelial lung cells. Adhesins play a critical role in the establishment of the infection; lately, it is also reported that their differential expression among *Paracoccidioides* spp. leads to different clinical manifestations [129, 130].

9.6.4 Defenses against Oxidative Stress

Macrophages produce abundant reactive oxygen species (ROS) and reactive nitrogen species (RNS), that along with the release of hydrolytic enzymes and toxic metabolites inside the phagolysosome aim to kill fungal pathogens. However, high throughput analyses [131, 132] and functional studies [133, 134] in *Paracoccidioides* revealed that upon macrophage phagocytosis, the fungus can cope with oxidative stress. In response to H_2O_2, *Paracoccidioides* spp. is able to resist highly oxidative stress not only by generating ROS scavenging enzymes, such as superoxide dismutases, thioredoxins, cytochrome C peroxidase (CCP), and catalases; but also through preventing the protein misfolding or unfolding under long term stress conditions [132]. These data correlate with the transcriptional upregulation of genes encoding peroxisomal catalase and Mn-superoxide dismutase in yeast cells derived from infected macrophages [131]. Moreover, it was shown that in *Paracoccidioides* spp. *ccp*-aRNA strains were more sensitive to mitochondrial-generated ROS stress, suggesting that CCP avoids cell damage caused by oxidative stress, thus playing a critical role in fungal survival inside macrophages. A *ccp* mutant in *C. neoformans* has been shown to reduce intracellular growth when cultured with macrophages [135] Other study have reported *Paracoccidioides* ability to reduce nitric oxide (NO) levels by secreting adhesin gp43, a mannose-rich glycoprotein, that prevents the release of NO from macrophages and stimulates release of IL-10, hence reducing the iNOS expression and its enzymatic activity [136].

9.6.5 Other Virulence Factors

In addition to the described proteins and enzymes, many other microbial determinants have been associated with *Paracoccidioides* spp. virulence, such as biofilm formation, secretion of vesicles and melanin production.

A recent study reported that *Paracoccidioides* spp. can colonize surfaces and form biofilms [137]. This biofilm consisted of a dense network of yeasts cells characterized by a tightly regulated expression of genes encoding adhesins (gp43, GAPDH) and hydrolytic enzymes (aspartyl proteinase), consistent with the adhesion, invasion, and tissue destruction also reported for *Candida albicans* biofilms [138]. Biofilm formation by *Paracoccidioides* spp. is a critical factor in the persistence of the fungal infection, since it hinders the action of antifungal drugs and may contribute to a chronic state of the disease.

Fungal extracellular vesicles, resembling mammalian exosomes have also been reported to act as "virulence bags" [139]. These extracellular compartments composed of lipid bilayers have the potential to regulate key pathogenic steps during fungal infections. In *Paracoccidioides* spp. yeast cells, pioneer studies demonstrated that extracellular vesicles carrying antigenic components bearing highly immunogenic α-linked galactosyl epitopes were recognized by both anti-α-Gal and MOA (*Marasmius oreades* agglutinin) lectin. Additionally, these secreted vesicles could stimulate cytokine expression of macrophages in vitro [140].

Melanin pigments are remarkable and unique substances present in all biological kingdoms, closely associated to virulence in fungal pathogenesis [141]. Fungal melanin is deposited in the cell wall and cytoplasm; particularly in *Paracoccidioides* spp. melanization contributes to virulence by acting as a scavenger of NO and ROS, and through the binding to antifungal drugs, thus changing their activities [142, 143].

9.7 Recognition by Immune Cells and Immunoevasion Strategies

The host cellular immunity, mediated by cells of the innate and adaptive systems, is fundamental to achieve a successful microbial clearance. Initially, dendritic cells and macrophages recognize and present fungal antigens (e.g. chitin, β-glucans, and mannans), known as pathogen-associated molecular patterns (PAMPs), to T-lymphocytes. An effective T-cell response leads the generation of Th1 cytokines, such as tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ), resulting in a classic activation of macrophages to produce ROS and RNS that kill fungi or inhibit their growth [144, 145].

Paracoccidioides spp. exploit several strategies to bypass host defense mechanisms, some of which are described in the following sections.

9.7.1 Shielding of Stimulatory PAMPs

An iconic survival strategy used by dimorphic fungi to avoid recognition by host phagocytic cells' pattern recognition receptors (PRRs) is their quick cell wall turnover during infection, as a consequence of the morphological switching. One PRR,

Dectin-1, present on host phagocytic cells recognizes fungal cell wall β-(1,3)-glucan, triggering phagocytosis, respiratory burst, and release of cytokines such as TNF-α, IL-12, and other interleukins; however, due to the spatial arrangement of the yeast cell wall polysaccharides in *Paracoccidioides* spp. and *H. capsulatum*, α-(1,3)-glucan can actively interfere with these events [115, 116]. Indeed, *H. capsulatum* mutants lacking α-(1,3)-glucan, induced an increased production of proinflammatory TNF-α by macrophages [116]. When *dectin-1*-silenced macrophages were in contact with the *H. capsulatum* mutant depleted of α-(1,3)-glucan, they failed to produce proinflammatory TNF-α, suggesting that α-(1,3)-glucan effectively shields β-(1,3)-glucan from innate immune recognition by the Dectin-1 receptor [116].

9.7.2 Intracellular Survival

Aside from *Paracoccidioides* spp. yeast cell adaptation to high temperatures within the host, the fungus manages to ease its invasion to pulmonary epithelial cells and keratinocytes by affecting host cells cytoskeleton structure, a process that is also promoted by gp43, which participates in the cytokeratin [146, 147]. On the other hand, Cdc42, a fungal Rho GTPase involved in regulating morphogenesis by controlling actin-mediated polarized growth, supports the large, multibudded state of *Paracoccidioides* spp. yeast cells, a morphology that inhibits phagocytosis. RNAi *cdc42* strains were more efficiently phagocytosed by macrophages and displayed decreased pathogenicity [148]. Similarly, studies have shown that *Paracoccidioides* gp43 inhibits the phagocytic and fungicidal capacity of macrophages through binding to mannose receptors and inducing IL-18 production [136, 149].

Host cells inhibit intracellular microbial proliferation by limiting available iron through its sequestration by high-affinity iron-binding proteins such as transferrin and ferritin. Recent proteomic analyses in *Paracoccidioides* spp. demonstrated that under iron deprivation and carbon starvation, proteins involved in alternative carbon metabolism, such as enzymes involved in gluconeogenesis, β-oxidation of fatty acids and amino acids catabolism, were positively regulated, whereas proteins related to glycolysis and protein synthesis were down-regulated [134, 150, 151]. This suggests that under stress conditions similar to that found in the macrophage, *Paracoccidioides* spp. shifts to a starvation mode prioritizing iron-independent metabolic pathways in order to persist in vivo microenvironmental conditions.

Paracoccidioides spp. must also tolerate and overcome stress conditions caused by low oxygen levels. It is well established that in inflamed tissues, oxygen supply is restricted by the high volume of host phagocytic cells or the microbe itself at blood vessels. Recent characterization of *Paracoccidioides* hypoxia response, revealed that members of the genus contain homologues of the key regulator of hypoxia adaptation in fungi, SrbA, a sterol regulatory element binding protein (SREBP) [150]. Functional complementation of an *Aspergillus fumigatus srbA* null mutant by *Paracoccidioides srbA* (*PbsrbA*) restored the null mutant hyphal growth under hypoxia, suggesting that

PbsrbA promotes adaptation to hypoxic microenvironments. Moreover, this study also showed that *Paracoccidioides* SrbA is likely involved in azole drug resistance responses by regulating ergosterol biosynthesis to compensate the effects on membrane fluidity due to low oxygen levels.

The induction or prevention of apoptosis can be a critical step in the development of the infectious processes. It is also reported that *P. brasiliensis* benefits its intracellular survival and further dissemination by modulating programmed cell death of macrophages and epithelial cells. Such strategy is accomplished by the expression of caspase-2, 3 and 8, strongly influenced by the 14-3-3 and gp43 adhesins [152, 153].

9.7.3 Altering T-Cell Repertoire

Proper differentiation and maturation of T-cells occurs in the thymus, thus integrity of the thymic microenvironment is crucial for the maturation of thymocytes. Experimental data have shown that acute infection with *Paracoccidioides* yeast cells promotes thymus invasion, causing its microenvironment atrophy, as consequence of epithelial cell spatial disarrangement and increased gene expression of inflammatory mediators [154, 155]. These studies suggested that impaired differentiation of pathogen-specific T-cells leads to host immunosuppression favoring the fungal ability to thrive and multiply in the thymic microenvironment.

Data summarized in this section intend to show *Paracoccidioides* species remarkable ability to become successful pathogens, which could also be partly explained by recalling the fungi soil-related environment. The precise ecological niche of these fungi remains undefined; however, there is close association with armadillos, disturbed vegetation, and shaded mildly acidic soil, in other words a diverse and harsh microenvironment that has stimulated the fungus evolution to enhance its adaptation and survival mechanisms. The emergence of CRISPR (Clustered regularly interspaced short palindromic repeats) technology might help to increase our understanding of the pathophysiology of these fungi, which could eventually be translated into benefits for patients.

9.8 Treatment

Because of the systemic nature of the mycosis, consideration should be given to patients' general status, target organs and systems, and to the form of presentation (acute/subacute, chronic). Existence of comorbidities, such as tuberculosis, AIDS, and cancer, should be determined and treated accordingly. Treatment should include supportive general measures, such as bed rest, anemia correction, and suspension of tobacco use and alcohol intake, as well as balanced nutrition. Associated clinical complications should also be treated [156, 157].

Antifungal therapy is the mainstay for patient improvement, with three groups of medications being available: sulfonamides, amphotericin B, and azole derivatives [158].

Trimethoprim/sulfamethoxazole (160–240 mg/days / 800–1,200 mg/days) with a high rate of response, but the treatment should be prolonged for 2 or more years, and the probability of relapse is high (20%) [159].

Amphotericin B administered intravenously, is reserved for patients with serious disease, for those who do not tolerate the oral route, for women in their first 12 weeks of pregnancy, and for patients with fourfold increase in hepatic enzymes. Cumulative total dosages vary from 1 to 2 g, with 0.6–1 mg/kg/days of daily doses. Amphotericin B is not fungicidal in vivo, and all patients thus treated should also receive maintenance sulfonamide or azole therapy to avoid relapses [158, 160].

Ketoconazole for oral administration has proven effective, but its hepatotoxicity and multiple interactions with other medications argue against its use.

Among the newly developed triazoles, **itraconazole** 200–400 mg/day for 6 months, is the choice for treatment of patients with both minor and moderate clinical manifestations. It is also recommended for maintenance therapy in severe cases, once the initial amphotericin B course has been concluded. Relapse rates are lower than with other medications (3–5%) [159].

Voriconazole has been used successfully in patients with neuroparacoccidioidomycosis because it has better penetration into the central nervous system than itraconazole and other drugs [161, 162].

Alternative triazoles have been incompletely evaluated in the treatment of paracoccidioidomycosis, although **posaconazole** and **isavuconazole** have exhibited efficacy [163].

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Chapter 10
Sporothrix and Sporotrichosis

Leila M. Lopes-Bezerra, Héctor M. Mora-Montes, and Alexandro Bonifaz

Abstract Sporotrichosis is the mycosis due to implantation of greatest global distribution. It is caused by a complex of thermodymorphic fungi of cryptic species, of which the most frequent and important are *Sporothrix schenckii* and *Sporothrix brasiliensis*. These fungi live in nature, matter in decomposition and several plants. The route of entry usually involves traumatic inoculation, but sometimes it could be by the respiratory route. In general, they produce a condition confined to the subcutaneous cellular tissue and, to a lesser extent, affect various viscera, such as the lungs and central nervous system. Clinically, it is a polymorphic condition with various manifestations depending mainly on the patient's immune status and strain virulence. In cases associated with immunosuppression, cutaneous and extracutaneous features may occur. The main clinical form is lymphangitic cutaneous sporotrichosis. In this chapter, the authors discuss the biological aspects of the main pathogenic species of the *Sporothrix schenckii* complex, including virulence factors, clinical-epidemiological aspects, diagnosis, and treatment.

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10.1 Definition

Sporotrichosis is a worldwide-distributed human and animal mycosis caused by implantation; however, cutaneous disseminated and extracutaneous forms also are reported [1]. The causative agent usually affects skin and subcutaneous tissues and less frequently the invasion of deep tissues, such as lungs and other viscera [1–4]. The disease often undergoes a subacute or chronic phase and is caused by dimorphic fungi belonging to the *Sporothrix schenckii* complex [1, 4, 5]. These organisms generally affect the skin and lymphatic ganglions and may cause problems in bones and joints or, in rare occasions, to the lungs and other deep organs. This mycosis has been traditionally named the rose picker's disease or the gardener's disease [1, 4, 6–10]. Zoonotic transmission is mainly observed in Latin America [10]. The zoonosis and sapronosis are currently related to the distinct species of *Sporothrix*.

10.2 Etiology

As mentioned, the causative agents are thermodymorphic organisms of the *Sporothrix* genus. Originally, the disease was related to infection with *S. schenckii*; however, molecular and phenotypical studies have revealed that this is in fact a group of cryptic, phylogenetically closely related cryptic species, named the *Sporothrix schenckii* complex [5]. The complex is composed of at least four pathogenic species, including *S. schenckii sensu stricto, Sporothrix globosa*, *Sporothrix brasiliensis*, and *Sporothrix lurei*. The rest of the complex members include *Sporothrix mexicana* and *Sporothrix pallida*, which are a less common cause of the disease; most of the isolates are considered a nonpathogenic environmental clade [1, 5, 6, 10–12]. No teleomorphic phase has been reported for any of the complex members, and molecular studies indicate that they are not related to the species of the genus *Ophiostoma*. The species *S. schenckii s. str.* is regarded as the most important of the complex, with a worldwide distribution and accounting for 80% of the cases reported. Infections caused by *S. brasiliensis* are restricted to Brazil, and *S. globosa* is mainly reported in Asia [1, 10, 13–15].

Members of the *S. schenckii* complex are thermo-dimorphic organisms and share most of the morphological and biochemical traits, making a complicated task the establishment of the species by traditional means [1, 4]. In *S. schenckii s. str*., the conidium size is bigger (5–10 μm) and generates big blastoconidia composed of pigmented, pseudoparenchymatous mycelia [1, 4, 7]. Based on molecular studies conducted by Japanese groups, the analysis of mitochondrial DNA allowed to propose two *S. schenckii* groups: A and B; the former is widely distributed in America and the latter is limited to Japan and other areas of Asia [16]. The use of the DNA sequence of the encoding genes for chitin synthase, ß-tubulin and calmodulin, helped to classify the species in five clades: *S. brasiliensis* in clade I, *S. schenckii s.*

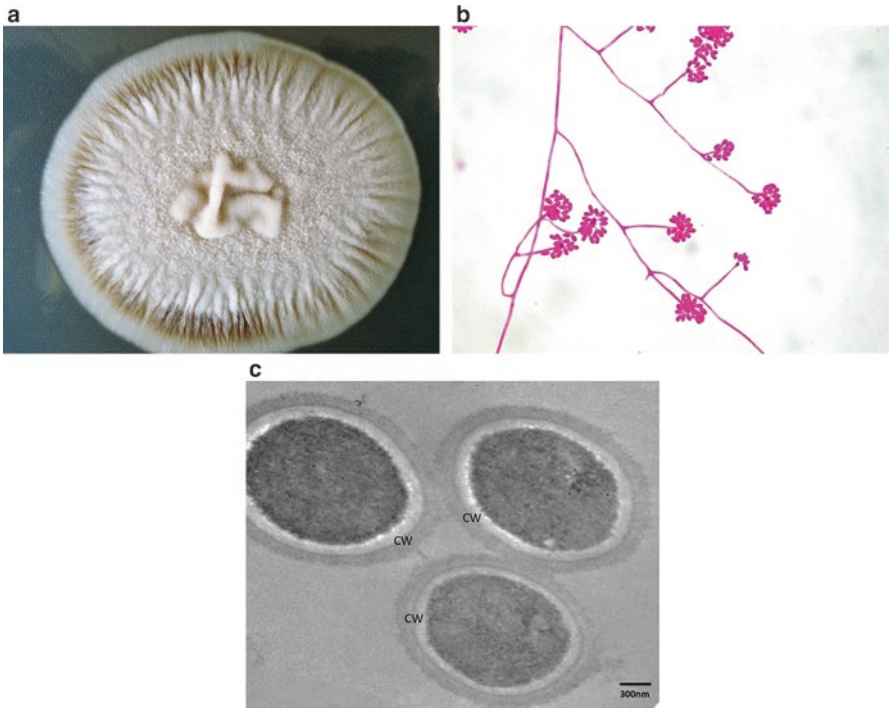

Fig. 10.1 (**a**) Culture of *S. schenckii*, on Saboraud dextrose agar, 10 days at 28 °C, (**b**) *S. schenckii*, sympodial microconidia (filamentous phase), like a "daisy flowers"; (**c**) Transmission electron microscopy showing the cell wall (CW) of *S. brasiliensis* yeast cells

str. in clade II, *S. globosa* in clade III, *S. mexicana* in clade IV, and *S. pallida* (formerly known as *S. albicans*) in clade V [1, 5, 10, 17–21].

The morphology of the different members of the complex is similar when cultured at 28 °C in Sabouraud-dextrose agar, YPD agar, or potato-dextrose agar (Fig. 10.1a). Under microscopic inspection, it is easy to observe thin, hyaline, and septate hyphae, 1–3 μm in diameter, that usually produce branches (Fig. 10.1b). The anamorphic phase generates microconidia that adopt an ovoid, round, elongated, or piriform shape (the predominant morphology may vary depending on the species of the complex). These microconidia can be generated by two ways: Ovoid or pyriform microconidia, derived from the denticle (sympudolic) emerge from a conidiophore of 10–30 μm in length, in an arrangement that resembles a "peach flower or daisy flower" (Fig. 10.1b); and sessile conidia that directly emerge from hypha, which are usually named microaleurioconidia or raduloconidia. After detachment, conidia become thicker and tend to be grouped, generating the melanic pigment of the fungal colony [1, 2, 4, 6, 22].

The dimorphism is usually triggered at 37 °C in rich media, such as blood agar, chocolate agar, and BHI agar. The generation of yeast-like cells (Fig. 10.1c) also can be stimulated by growing the fungus in presence of 5% CO_2. On plate,

white-yellowish, soft colonies that resemble bacterial colonies emerge after 3–5 days of incubation. They contain ovoid or enlarged yeast cells approximately 2–4 × 3–6 μm, along with fragmented mycelia [1, 2, 4, 6, 23].

10.3 Historic Aspects

The first case of sporotrichosis was reported in 1898 in the United States by Schenck who described a classic lymphangitic sporotrichosis. The organism rescued from lesions was classified by Smith as part of the fungal genus *Sporotrichum*. Other cases were documented across the country by Hektoen and Perkins, and they reclassified this fungus within the genus *Sporothrix* and described the new species *S. schenckii*. In France, de Beurmann isolated a pigmented strain, different from the etiological agent of the American cases already reported, and it was classified by Matruchot as *Sporotrichum* variety *beurmanni*. In 1962, Michael described the differences of the two genera and confirmed *Sporothrix schenckii* as the sole species of this genus [1, 2, 7, 23]. Simon documented the first epidemic in 1947, which took place in South Africa [2, 15]. The yeast parasitic phase was described in Brazil by Lutz and Spendore in 1947 [13].

Most recently, the work of Guarro's group (2006–2008) has reshaped the etiology of this disease, proposing the *Sporothrix schenckii* complex, a group of cryptic species, whereas the work by Sasaki et al. on the chromosomal polymorphisms has helped to explain the genetic diversity of members of the *Sporothrix* complex [1–4, 11, 69].

10.4 Epidemiology

Geographic distribution. The sporotrichosis is a worldwide-distributed mycosis caused by implantation [10, 15] and has been reported in all the continents, although the cases tend to be concentrated in specific areas. Example of this is the epidemic outbreak reported in 1947 in Transvaal, South Africa, where 3,000 cases with lesions in lungs and skin were documented; most of them were related to contaminated wood inside a mine [15, 24]. Other endemic areas with high burden of cases are China (northeast, Jillin province, south of Guangdong and across the Yangtze River banks) [17, 25], Japan (Sendai and Nagasaki provinces) [26, 27], India (sub-Himalayan and Kangra regions), and Australia (the southeast coast and the coast of New South Wales) [14, 28, 29]. However, the main number of cases are found in America (in the area limited by the tropics), with particular emphasis in Southeast Brazil (where the most important zoonotic outbreak of sporotrichosis transmitted by domestic cats (*Felis catus*) has been reported [1, 9, 15, 30–33]; Peru (Andes region and Abancay) [34–36], Mexico (Jalisco and Puebla) [2, 4, 6, 37–41], Colombia (Cundinamarca and Boyaca provinces) [42], Venezuela (Aragua and Miranda

provinces) [43], Uruguay [43, 44], and Guatemala [44, 45]. There are few reports of this infection in Europe, and most of them are immigrants or patients who previously visited endemic areas. France, Italy, and Spain are the countries that gather most of the reported cases of sporotrichosis [4, 14]. In United States, there also are reports of outbreaks related to working with sphagnum moss [13].

Source of infection. Sporotrichosis has been regarded as an occupational disease, in particular of farmers, housekeepers, children, people in close contact with flowers, veterinaries, hunters, miners, fishermen, etc. [1–4, 6, 22, 37].

Sporothrix spp. are usually found in Tropical and Subtropical areas with humid and template climates, temperatures between 20 °C and 30 °C, and a relative humidity over 80%; however, there are reports of strains showing thermotolerance to higher temperatures and capable of surviving in cold weathers, such as those isolated from Jilin, China (collected from regions with 2–6 °C of environmental temperature). *Sporothrix* spp. usually grows in a wide range of pH (3.5–9.6). Although the disease is found throughout the year, most of the affected population refers contact with the pathogen during the fall and winter seasons [1, 2, 4, 6, 17]. This interesting observation can be associated with the end of the rainy season (in Mexico, for example), where the optimum humidity and temperature for the fungal growth are reached [4, 6]. In addition, during this time of the year, the chances to have contact with plant biomass increases due to the harvesting of vegetables and flowers with ornamental purposes. *Sporothrix* has been even isolated from the roots and stalk of maize plant in China [17]. *Sporothrix* spp. as a saprophyte lives in soil with abundance in cellulose, associated with vegetal detritus and decaying wood. The fungus was isolated directly from soil and grass [1, 4, 46, 47]. In the United States, these organisms have been isolated from sphagnum moss used for gardening and to grow Bonsai trees [48].

The disease also has been documented after traumatic interactions with rodents, such as rats, mice, or squirrels, insects, reptiles, spiders, and bats [1, 2, 4, 6, 49]. It is important to stress that cats have a natural susceptibility to be infected by these organisms, which makes likely the establishment of disseminated cases with fatal outcomes [50–57]. The cat to human transmission of sporotrichosis is a zoonosis, and the fungus has been isolated from claws, lesions, and the animal habitat. Thus far, the biggest epidemic outbreak in animals has been reported in Rio de Janeiro and has spread to the southern regions of Brazil. Domestic cats and dogs have been affected since 1998, and it is estimated that approximately 10,000 cats and 200 dogs have been infected. As expected, this has been extended to humans, with more than 4,000 cases of sporotrichosis diagnosed only in one health center. The main etiologic agent associated with this zoonotic outbreak is *S. brasiliensis* [57]. However, all of these numbers are an underestimation of the problem, as sporotrichosis is not an infection of compulsory notification. In other regions of the globe, there also are reports of the disease transmitted by cats but associated with *S. schenckii s. str.*, which indicates that the feline is susceptible to the *Sporothrix* genus rather than to one species in particular.

Route of entry. The fungus usually penetrates tissues after a traumatic lesion, i.e., requires loss of continuity in the epithelial and mucosal tissues. Although rare, it

could be an airborne disease, establishing the primary infection in lungs. The incubation period varies but tends to be 3 weeks in the cutaneous cases. When affecting the lungs, the incubation period is difficult to establish, because it depends on the size of the inoculum and the patient's immune status [1–4, 6, 7–10]. The gender distribution of the disease is similar, with a ratio 1:1 in most studies, or with a slight bias towards being more associated with males. However, data from the epidemic outbreak in Brazil [57, 58] indicate an increased number of cases associated with females and elderly people. The most likely explanation is that this population is usually in closer contact with domestic cats. There is not a specific age period to acquire the disease; it is found from kids to elderly people, but the increased number of cases often is associated with occupational age: children attending school (age 5–15 years, represent 30% of cases) and young adults (age 16–35 years, representing 50% of cases) [40, 41, 59]. There are some exceptions; for example, in the mountains region in Peru and Guerrero (Mexico), there are hyperendemic areas where more than 60% of cases are children younger than 15 years old [35, 36, 60].

Predisposing factors. Besides those already mentioned and related to the occupational risk, other factors, such as diabetes mellitus, chronic alcohol abuse, cancer, and chronic treatment with steroids are usually associated with the disseminated disease, with a fatal outcome. The real incidence and prevalence of the disease is unknown; as mentioned, this is not an infection of compulsory notification worldwide. In Mexico, there are not real figures about the incidence of the disease, but Mayortga et al. [37] has reported more than 1,000 cases in Jalisco state, whereas an incidence of 25 cases per 1,000 habitants in the highlands in Puebla state has been documented. The 53.2% of this population has a positive reactivity to sporotrichin, which is similar to the hyperendemic region for sporotrichosis in Acambay, Peru [35, 60, 61].

10.5 Pathogenesis

The cutaneous disease begins with the traumatic inoculation of the causative agent, generating in approximately 10 days a cutaneous-lymphatic complex or sporotrichotic chancre in the infection site. It is possible to have auto-limitation and spontaneous resolution of the infection, or it can spread and generate nodular and progressive lesions localized in the regional lymphatic vessels that stop in the main lymphatic ganglion. Alternatively, it could be established as a sole lesion that grows slowly with a verrucous appearance. The disseminate disease (cutaneous and deep seated) is associated with temporal or permanent immunodeficiency [1–4, 6–10].

Some groups have tried to correlate the clinical form with the patient's immunological status; however, there is a study from Zhang et al. [62] where a strain from a disseminated cutaneous disease was compared with organisms from fixed cutaneous or cutaneous-lymphangitic diseases. The authors found that the strain from the disseminate disease showed genotype variations, with a deletion of 10 pb in the NTS ribosomal region, suggesting that the virulence of the strains can account for

the capability of causing the disseminate infection. Moreover, an increased virulence in *S. brasiliensis* strains isolated from chronic cases of disseminate sporotrichosis has been demonstrated [10, 63]. Also, reports indicate a relationship between the strain genotype and the clinical form, but no formal confirmation has been obtained [18]. The primary lung disease, acquired by aspirating the conidia, is asymptomatic in most cases. If the fungal load is big or the host's immune system is altered, it is likely to develop classic symptoms of pneumonia, similar to those found in tuberculosis [64, 65].

The three main pathogenic species, *S. schenckii s. str*, *S. brasiliensis*, and *S. globosa*, exhibit significant differences in virulence when assayed in animal models [66–68]. The virulence factors already known of *Sporothrix* spp. are the fungal dimorphism, thermotolerance, melanin, production of extracellular enzymes (proteases and phosphatases), adhesion to host tissues and antigenic molecules, such as the Gp60-Gp70 antigen, and also the peptidorhamnomannnan sugar epitopes [1, 2, 4, 18, 61, 64–73].

The cell-mediated immune response is the key to control sporotrichosis, participating lymphocytes T $CD4^+$, dendritic cells, macrophages, and neutrophils. These cells are responsible for establishing a granuloma, which often is suppurative and in rare occasions tuberculoid. Using immunohistochemistry, it has been possible to demonstrate that lymphocyte produce INF-γ, a classic marker of a Th1-polarized immune response that activates macrophages. Other studies suggest the recognition of the fungal cells via the receptor TLR4, stimulating the secretion of proinflammatory cytokines, such as IL-6 and TNF-α. In summary, for the establishment of a protective anti-*Sporothrix* immune response, both the Th1 and Th2 branches of the immune system are required [74–79]. Moreover, humanized antibodies raised against the Gp 60-70 antigen are protective to mice infected with either *S. schenckii* or *S. brasiliensis* [52, 53].

10.6 Clinical Aspects

Sporotrichosis is a subcutaneous infection with great clinical polymorphism. The main forms of the disease are:

10.6.1 Cutaneous-Lymphangitic Sporotrichosis

This is the most frequent form of the disease, accounting for 65–85% of all reports. It often affects upper limbs and, to a lesser extent, lower limbs, shoulders, and the face. It develops within the inoculation area, forming a cutaneous-lymphatic complex or sporotrichotic chancre (Fig. 10.2). The affected area usually shows swollen tissue, erythema, and the lesions are asymptomatic or with minimal erythema. After 2 to 3 weeks of the initial lesion, patients usually develop ulcerative nodules in

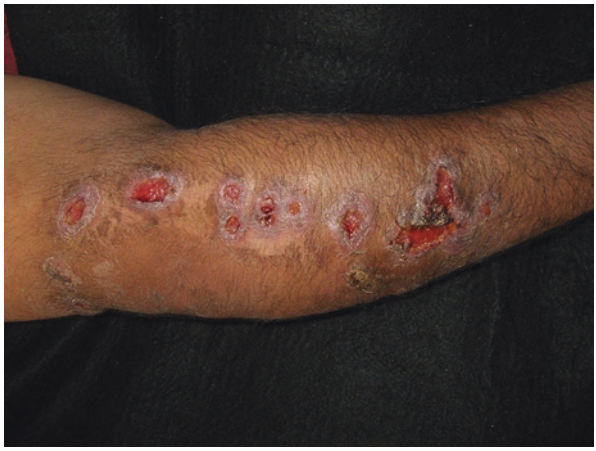

Fig. 10.2 Cutaneous lymphangitic sporotrichosis, nodular and ulcerative lesions

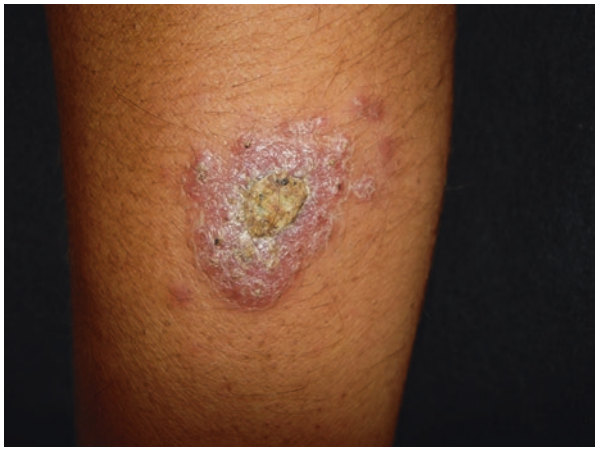

Fig. 10.3 Fixed cutaneous sporotrichosis, chronic verrucose plaque

lineal and progressive form, affecting regional lymphatic vessels and ganglions [2, 4, 6, 8, 22]. The nodules are covered with blood-containing crusts and develop an erythematosus-violaceous halo. In chronic situations, they could either progress to auto-resolution of the disease or develop more nodules. When inoculation occurs in multiple sites in the same area, it is possible to generate a mycetoma-like lesion with lymphostasis and elephantiasis [1, 2, 4, 8]. It is noteworthy that in children the facial topography of the disease occurs in approximately 40% of cases, either with unilateral or bilateral manifestations [4, 23, 31, 40, 41, 59, 79].

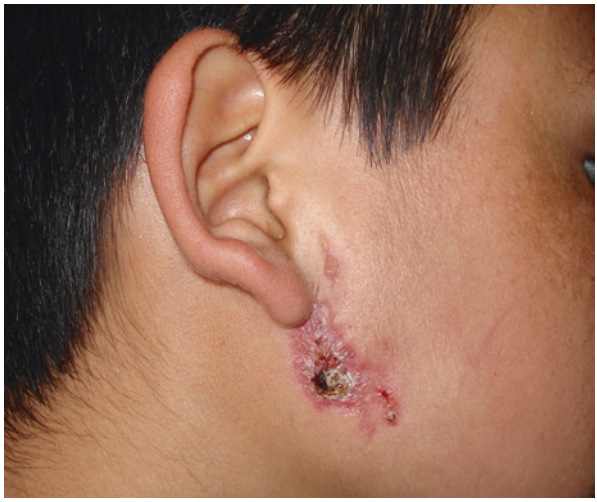

Fig. 10.4 Verrucous plaque of fixed facial cutaneous sporotrichosis (child)

10.6.2 Fixed Cutaneous Sporotrichosis

This also is named vegetative form and is the chronic manifestation of the disease. It is found in 25% of cases; however, in some countries, such as Japan, Korea, and Costa Rica, it is the most frequent manifestation of the infection and is present in approximately 60% of patients [1, 4, 6, 26, 27]. The lesion is limited to the inoculation area and looks like a sporotrichotic chancre that grows slowly, with a verrucous shape, well-delimited borders, and an erythematosus-violaceous color (Figs. 10.3 and 10.4). The lesion is usually covered with flakes and is asymptomatic. It is regarded as a disease in patients with good immunological constitution; therefore, it has good prognosis and in most cases is spontaneously resolved [4, 6, 23, 31, 41].

10.6.3 Disseminated Cutaneous Sporotrichosis

This is the third most prevalent form of the disease and is found in 1–5% of all cases. It could start as a lymphangitic form in immunosuppressed patients and is mostly reported in noncontrolled, diabetic patients, chronic alcohol abusers, patients with hematological malignances (lymphomas and leukemia), pregnancy, AIDS, and patients with prolonged exposure to systemic steroids. The lesions are ulcerated, necrotic, and tend to form verrucous plaques (Fig. 10.5). They can be found in any part of the skin and mucosal tissue, such as mouth, pharynx, nose, and genitals [2, 4, 6, 80–88].

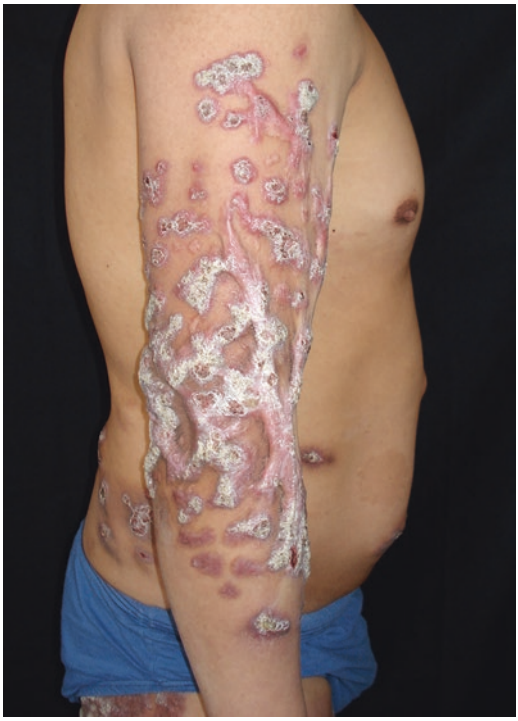

Fig. 10.5 Cutaneous disseminated sporotrichosis associated with chronic alcoholism

10.6.4 Pulmonary Sporotrichosis

This is a rare form, with only a few reports in the literature from hyperendemic areas, and is accompanied by immunosuppression. This form is divided in two groups: the most frequent is the chronic disease, which is asymptomatic (98% of cases), and similar to pulmonary tuberculosis, i.e., auto-limited with cavitary areas. Patients with the symptomatic form have pneumonia, with moderate coughing and expectoration, and the radiological inspection shows condensation areas and miliar infiltrates. The second group is the acute and progressive form that affects the hilar and tracheobronchial lymphangitic ganglions. These patients present massive adenopathy, symptomatology associated with weight loss, productive coughing, dyspnea, and cachexy. The radiological inspection is accompanied with hilar adenopathies and mediastinal widening [1, 2, 64, 65, 92, 93].

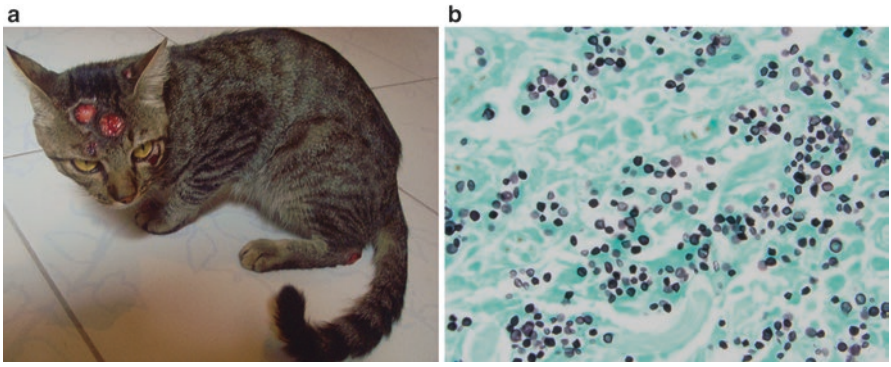

Fig. 10.6 (**a**) Feline sporotrichosis with multiple ulcers; (**b**) Multiple yeasts of *S. schenckii*, from feline sporotrichosis (Grocott, 40×) (Courtesy of Elisabete Aparecida da Silva and Hildebrando Montenegro, part of the Brazilian epidemic)

10.6.5 Extracutaneous Sporotrichosis

From these cutaneous lesions, the fungus can disseminate to other tissues or organs, which is associated with a bad prognosis. Lungs, bones, and joint are frequently affected (especially those found in the elbows and knees where osteomyelitis is generated), as well as the central nervous system, where meningitis caused by sporotrichosis is frequently fatal. The systemic form of the disease is rare and has been mainly associated with HIV infection (phase C) and chronic alcoholism [4, 8, 85, 88–91]. The disseminated or systemic cases can be found in immunosuppressed patients, which have fungaemia and fungal spreading to deep-seated organs, such as liver, spleen, testicles, and the central nervous system. The neurological infection has been exclusively associated with AIDS patients, with a fatal outcome [1, 2, 33, 85, 91–98]. In rare occasions, the infection can affect eyes and cardiac tissue [1, 99].

10.7 Veterinary Sporotrichosis

The most susceptible animals to *Sporothrix* spp. are cats (*Felis catus*) and to lesser degree dogs (ratio 30:1 for cats and dogs, respectively). Both animals are capable of transmitting the disease to humans. Most of the animal cases are caused by *S. brasiliensis* as discovered in the epidemic zoonotic outbreak in Brazil, as already commented [57]. *S. schenckii s. str.* also can infect these animals, and anecdotic cases of infection in horses, cows, goats, camels, pigs, birds, rats, mice, armadillo, and chimpanzee have been documented [1, 3, 50–58, 100, 101]. In cats and dogs, the clinical forms of the disease can be similar to those described in humans; the disseminated and systemic forms are the most frequent in cats [31]. Male felines that fight for breeding are mostly affected, because the bites and scratching can inoculate the

Table 10.1 Immunological classification and behavior of sporotrichosis [118]

	I. Hyperergic and normergic cases	II. Anergic and hypoergic cases
Clinical features	– Cutaneous lymphatic – Cutaneous fixed	– Hematogenous – Cutaneous-disseminated – Osteoarticular – Pulmonary/visceral
Yeast forms in direct examination and in tissues	Rare	Frequent
Histopathology	Suppurative granuloma	Unspecific granuloma
Sporotrichin (IDR)	Always positive	Frequently negative
Resistance	High	Low
Prognostic	Good	Bad
Spontaneous regression	Possible	Never
Treatment response	Good	Bad

fungal cells [3, 51, 55]. For this reason, most of the lesions are usually found in the head, nose, and ears and are ulcerative nodules with crust. They usually develop fistulas with production of purulent material (Fig. 10.6). Most cases have affectation of the mucosal tissue (nasal, oral, and genital), fever, anorexia, and cachexy [1, 3, 51–58, 100].

In the systemic or disseminated cases, the organs that often are affected include the liver and lungs, and to a lesser extent, the spleen, kidneys, testicles, eyes, and central nervous system. The laboratory examination of animals usually reports anemia, eosinophilia, and neutropenia [1, 3, 58, 100, 102] (Table 10.1).

10.8 Differential Diagnosis

Cutaneous-lymphangitic sporotrichosis: cutaneous tuberculosis, syphilis, mycetoma, tularemia, chromoblastomycosis, pyogenic infections, tuberculoid leprosy, and infections caused by nontuberculosis mycobacteria. The infiltrated lesions with keloid scars can be confused with lacaziosis (lobomycosis), whereas the acne-like forms can be confused with acne and cryptococcosis [2, 3, 4, 6–9].

Fixed cutaneous sporotrichosis: verrucous tuberculosis, chromoblastomycosis, leishmaniasis, squamous carcinoma, impetigo, infections caused by nontuberculosis mycobacteria, and Orf disease [4, 6, 7].

Disseminated cutaneous sporotrichosis: tuberculosis, syphilis, coccidioidomycosis, and infections caused by nontuberculosis mycobacteria [6–9, 103–106].

Pulmonary sporotrichosis: pneumonia, tuberculosis, aspergilloma, and other diseases caused by accumulation of fungal material [1, 62, 23].

Extracutaneous sporotrichosis: histoplasmosis, bacterial infections, and cryptococcosis [1, 4, 94, 95, 98].

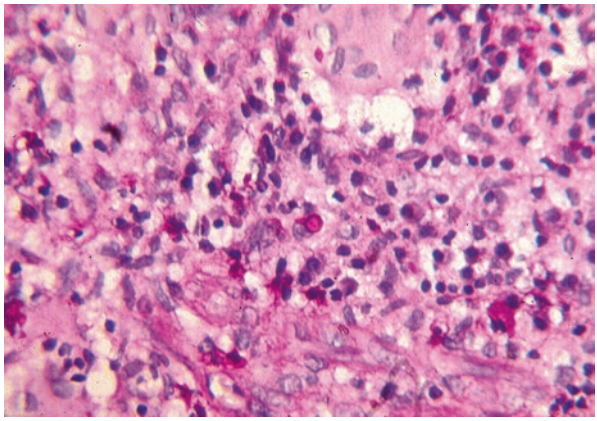

Fig. 10.7 Suppurative granuloma with *S. schenckii* yeast (PAS, 100×)

10.9 Laboratory Diagnosis

The direct examination using 10–20% KOH, the Gram, Giemsa, PAS, or Grocott straining have poor diagnostic value in cutaneous lymphangitic and fixed cutaneous sporotrichosis, because in only 5–35% of cases the yeast-like cells are observed. The staining with calcofluor white or immunofluorescence are more suitable to help in the diagnosis of the disease. The structures usually found during the inspection are elongated yeast-like cells that resemble a cigar or a ship, they are scare, and are visualized only in a handful of microscopic fields, being difficult to confirm the fungus. It is possible to observe asteroid bodies, which are clumps of yeast-like cells, forming structures similar to a crown (Splendore-Hoepli phenomenon) [1–4, 8, 9, 107]. It is of clinical value to determine IFN-γ by immunohistochemistry [108]. In cases of animal sporotrichosis or human cutaneous disseminated and systemic infections, the isolation of a significant number of yeast-like cells is common; and therefore, it is likely to confuse the infection with histoplasmosis [1, 58, 102, 105].

The current diagnostic tool available is the culturing of the organism from the lesion exudates, skin flakes, tissue samples, or sputum. The most useful culture media are Sabouraud-dextrose agar, Sabouraud-dextrose agar supplemented with antibiotics, and yeast-extract agar. When incubated at 28 °C, the fungal growth occurs after 5–8 days, with limited and membranous colonies of radial shape and whitish or beige-brown color. Then, the aerial mycelia are developed with the formation of *coremium.* Some species are unable to generate pigments, such as *S. pallida* [1–4, 7, 8]. It is important to keep in mind that the causative agents of sporotrichosis are dimorphic fungi; thus, if cultured in rich media, such as blood agar, chocolate agar, or BHI agar at 37 °C, the organisms will grow as yeast-like cells, easy to confound with bacterial colonies.

The histopathology is not specific of this disease. In lymphangitic and fixed cases, it is possible to observe asteroid bodies with budding cells, which are surrounded by eosinophilic material. In general, the fungal cells are well stained with

Grocott or PAS techniques. The histological inspection often shows a combination of a granulomatous and suppurative imagen with a pyogenic reaction, and this is composed of three zones: the central or chronic area with polymorphonuclear, histiocytes and lymphocytes microabscesses; the second zone surrounds the central one and shows a tuberculoid image composed of epithelioid cells and multinuclear giant cells similar to Langerhans cells; and the third zone contains lymphocytes, plasmocytes, and fibroblasts (Fig. 10.7). Quintella et al. [107], in a work with a significant number of cases, reported that 84% of cases showed suppurative granuloma, whereas the rest was tuberculoid, against a strange body, caseous and fibrinoid, with the confirmation of yeast-like cells or asteroid bodies only in 35% of the cases [2, 4, 109–114].

10.10 Cell Wall Antigens

The cell wall of *S. schenckii s. str.* is composed of beta-glucans, chitin, and a rare polysaccharide found only in a handful of fungal species, the peptidorhamnomannan. The latter is a potent immunogen, as infected patients have antibodies against it [13, 73]. Structural reports showed that the *N*-linked chain of rhamnomannan contains two antigenic epitopes: one found in both filament and yeast-like cells, and the second only on the surface of filament cells. These structures are a lateral chain with monorhamnosyl unit (Rha1 → 3 Man) and a lateral chain with dirhamnosyl unit (Rha 1 → 2 Rha 1 → 3 Man), respectively [13, 73]. Further analyses indicated the presence of other antigenic epitopes on the *O*-linked tetra- and pentasaccharides of peptido-rhamnomannans [73, 115]. These are in fact the main epitopes found within the *Sporothrix* cell wall and recognized by human IgG antibodies [115, 116].

Besides these antigenic polysaccharides, the cell wall also contains a peptide antigen, Gp70, a protein only found in the cell surface of pathogenic species of the *S. schenckii* complex, i.e., *S. schenckii s. str.*, *S. brasiliensis*, and *S. globosa*, but not in *S. mexicana* [73]. This antigen was sequenced and characterized using proteomic approaches [68], and interestingly, *S. brasiliensis* expresses an isoform of 60 kDa (Gp60) [117]. It is worth mentioning that a monoclonal antibody against Gp70 from *S. schenckii s. str.* is capable of conferring passive immunization in the mouse model of sporotrichosis [52, 53], which may lead to strategies to control both human and animal sporotrichosis.

10.11 New Molecular and Serological Diagnostic Tests

Most of the developed serological tests have not been efficient and are applicable only in systemic cases [2, 4, 118, 119]. However, in the past decade the use of ELISA-based methods, using specific epitopes from the *S. schenckii* cell wall have showed good specificity, sensitivity, and reliability [120, 121]. The ELISA test is a

fast and sensitive diagnostic method, which can be used to diagnose all clinical forms of sporotrichosis with a good specificity. This new serological tool is being successfully applied for therapeutic follow-up of patients, including those affected with *S. brasiliensis* [120, 121].

Another attractive approach that is currently being developed is the strain identification by molecular means, using biopsy samples and the PCR technique. The gene targets include those encoding for the chitin synthetase I, 26s rRNA, and topoisomerase II [1, 4, 122–127]. The tools already developed for the molecular identification of *Sporothrix spp.* can be used in parallel with the serological diagnosis.

10.12 Treatment

Fixed and Cutaneous-lymphangitic sporotrichosis: According to the North American Guidelines for the treatment of sporotrichosis, itraconazole (A-II) is the choice for these forms of the disease [128, 129]. The suggested posology is 200 mg/day for 3–6 months. There are reports indicating similar successful rates using intermittent doses or pulses, i.e., 400 mg/day during 1 week and then no treatment for the next 3 weeks [130, 131]. The advantage of the latter is the use of a lower number of doses. Nonresponsive patients to the indicated options are candidates for an increment in the dose to 300–400 mg/day; for children, the suggested dose is 6–10 mg/kg/day [4, 89, 129, 132–136]. Another therapeutic option is terbinafine (A-II), using doses of 250–500 mg/day or even 1 g/day in special cases. This drug is a good choice for children, and the recommendation is 125–250 mg/day [1, 4, 129, 136–140]. Of importance, most of the *S. schenckii* strains have increased *in vitro* sensitivity to terbinafine, but *in vivo*, itraconazole is more active [1, 141–143, 158].

Another good alternative for cutaneous cases is potassium iodide (A-II), which is the option in low-income countries [128, 129, 144]. It could be used as normal or saturate suspension. The posology of the former is 3–6 g/day, starting with 1 g/day to assess the patient tolerance. The administration route is oral, and it is recommended to prepare a suspension of 20 g in 300 mL of water in a dark container, having 1 g in 15 mL. In children, a dose of 1–3 g/day is recommended, starting with 500 mg. When the saturated suspension is selected, the treatment starts with 5 drops three times per day, gradually increasing the number of drops until reaching 40–50 drops three times per day. This treatment has to continue for an average of at least 3–5 months. The potassium iodide is usually well tolerated by patients, but it is possible to present secondary effects, such as gastritis and, rarely, rhinitis, bronchitis, rash, and nodosum erythema. It is important to assess the use of this drug in patients with diseases involving the heart or thyroid. This is not a patented drug; therefore, it has to be prepared by specialized personnel at the drugstore. In feline sporotrichosis, the potassium iodide has shown good results and is regarded as the treatment of choice [4, 136, 144–147].

If none of the above indicated drugs is available, fluconazole at 400–800 mg/day may be used [129, 147]. Another concomitant option is hyperthermia or heating the site of the lesion with warm baths at 45 °C per 15–20 min two or three times per day. This is because the members of the *S. schenckii* complex do not grow at temperatures higher than 40 °C [4, 129, 136, 148, 149]. This treatment is recommended for a limited number of sporotrichosis cases, only as a concomitant alternative. Pregnant patients or those incapable of receiving the mentioned drugs are candidates. It is likely that this approach will not kill the fungal cells but will help to stop dissemination to other tissues. After childbirth, the systemic treatment should be installed [129, 140, 150].

Disseminated cutaneous or extracutaneous (osteoarticular and pulmonary) sporotrichosis: The treatment of choice is the lipid formulation of amphotericin B (B-III) at 3–5 mg/kg/day or deoxycholate amphotericin B (B-III) at 0.7–1 mg/kg/day. Both must be applied within the hospital to assess the presence of collateral effects. In pregnant patients, it is the treatment of choice [1, 129, 151–153]. The second option for these forms of the infection is itraconazole (B-III) at 400 mg/day; it can be the primary treatment or the option after the use of amphotericin B. Alternatively, it could be a concomitant treatment for a period of 6–12 months, in particular in patients with AIDS, hematological cancer, and other immunosuppressive conditions [129, 153].

In particular cases of cutaneous-osteoarticular sporotrichosis, potassium iodide or itraconazole plus trimethoprim/sulfamethoxazole at 400 mg and 80 mg, respectively, during 3–4 months can be used as an option to control the infection [4, 154].

Posaconazole has been used recently in combination with amphotericin B in refractive cases to the conventional therapeutic schemes [155, 156].

It is important that, during the typification of isolates of the *S. schenckii* complex, the *in vitro* susceptibility to antifungal drugs will be part of the standard protocol of identification, because the resistance to antifungal drugs is significantly increasing [1, 142, 144, 157].

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Chapter 11
Fonsecaea and Chromoblastomycosis

Peiying Feng and G.S. de Hoog

Abstract Chromoblastomycosis is a chronic fungal infection of cutaneous and subcutaneous tissues caused by traumatic inoculation of a specific group of melanized fungi, with species of *Fonsecaea* and *Cladophialophora* as prevalent etiological agents. Chromoblastomycosis has a global distribution, particularly in tropical and subtropical rural areas. *Fonsecaea* spp. are prevalent in humid tropical climates, whereas *Cladophialophora* is found under arid conditions. The disease is difficult to treat due to its recalcitrant nature, which may lead to severe clinical forms with high morbidity, even leading to neoplastic transformation. In this review, we summarize current knowledge on *Fonsecaea* and chromoblastomycosis, including the taxonomy of *Fonsecaea,* pathogenic potentials of species, their epidemiology, and clinical manifestations. Notes on diagnostics and therapeutic options are provided.

11.1 Introduction

Chromoblastomycosis (CBM) is defined as a chronic cutaneous and subcutaneous infection caused by melanized fungi and characterized by verrucose lesions and dark-colored, thick-walled muriform cells in infected tissue; the latter provide a histopathological criterion for definite diagnosis. The disease has a global distribution but is prevalent in tropical and subtropical rural areas, such as Madagascar, Brazil, and China. The etiologic agents are hypothesized to gain entrance through the skin by traumatic implantation of contaminated materials, as the majority of lesions are observed on extremities of outdoor workers [1, 2]. Agents of CBM are members of the family *Herpotrichiellaceae* of the ascomycete order *Chaetothyriales*. By far three genera with five species have been proven as recurrent causative agents of the disease, i.e., *Cladopholophora carrionii, Fonsecaea monophora*, *F. nubica, F.*

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pedrosoi, and *Rhinocladiella aquaspersa*. Several *Exophiala, Cladophialophora,* and *Phialophora* species have been reported as occasional agents of the disease [3].

11.2 History

CBM was described for the first time by Dr. Maximilliano Willibaldo Rudolph, a German physician living in Brazil, who published the manuscript "Über die brasiliensische Figueira" (Brazilian fig tree) in a German Journal in 1914 [4, 5]. Rudolph noticed six patients with warty lesions on the lower limbs popularly known as fig tree. He was able to isolate black and velvety cultures in four patients, which today we notice that their microscopic features are quite similar to those of *F. pedrosoi*, and he successfully inoculated the disease in four white rats and two monkeys. Surprisingly, he did not describe the histologic aspects of the disease or the pathognomonic sclerotic cells, which Lane and Medlar described 1 year later in separated publications. Lane and Medlar [6, 7] reported the first CBM North American case observed in an Italian patient living in Boston. The patient presented a warty violet plaque lesion on the right buttock simulating verrucous tuberculosis. They found a number of spherical, pigmented, parasitic cells (Medlar bodies) in the tissue. Previously, Bruas in 1903 and Fontoynont 1909 in Madagascar, and Guiteras in 1904 in Cuba, respectively, referred to some possible CBM cases, but the isolation of the fungus by these authors to verify identification was not possible, neither the cases were published. In 1911, Alexandre Pedroso and Jose Maria Gomes in São Paulo, Brazil, began scientific research on four cases with verrucous dermatitis in Brazilian patients. They revealed the presence of spherical, brownish cells in skin biopsies, corresponding to the current muriform cell, the hallmark of CBM. The cultivation of the patient's skin lesions yielded dark fungal colonies and was classified as *Phialophora verrucosa*. They named the disease *blastomycose negra* at that time. However, Pedroso published their observations late only in 1920 [8]. Two years later, Brumpt [9] concluded that the agents isolated by Pedroso and Gomes could not be classified as *Phialophora* species, and he coined the denomination *Hormodendrum pedrosoi*, later renamed *Fonsecaea pedrosoi* by Negroni [10].

The term chromoblastomycosis was introduced by Terra et al. [11] to differentiate a cutaneous fungal disease that they were studying in Brazil from the confusing clinical entity known as "verrucous dermatitis". The name chromoblastomycosis suggests the disease is a fungal infection caused by pigmented "*Blastomyces*" fungus (Greek *chroma*, ~atos = complexion, skin color, + *Blastomyces* Greek *blastós* = sprout, bud + *mykes* = fungus + *osis* Greek suffix denoting the state of something/somebody). However, as the etiologic agents of the disease produce muriform cells but not budding yeast forms in tissue, Moore and Almeida [12] proposed a new term "chromomycosis" as a replacement of "chromoblastomycosis". With time, the name "chromomycosis" was used as an umbrella to encompass a heterogenic and diverse group of fungal infections caused by a wide spectrum of dark melanized fungi. With the introduction of the concept of phaeohyphomycosis

by Ajello [13] and McGinnis [14], differentiation among these diseases became more obvious. Nowadays, the term chromoblastomycosis is restricted to the cases in which muriform cells are present in tissue. Muriform cells, also known as sclerotic cells or Medlar bodies, are globe-shaped, cigar-colored, thick-walled structures that are 4–12 μm in diameter. In 1992, the International Society for Human and Animal Mycology (ISHAM) recommended that the best name to define the disease was chromoblastomycosis [15], which Terra et al. coined in 1922. Currently the disease is defined in the International Classification of Disease (ICD) as follows: ICD-9 no. 117.2, ICD 10-B43.

11.3 Taxonomy of *Fonsecaea*

Fonsecaea is an anamorph genus of black yeast and relatives in the family *Herpotrichiellaceae* (order *Chaetothyriales*). Colonies are restricted, dark olivaceous, velvety to cottony in texture and have an olivaceous-black reverse. Mophologically the genus *Fonsecaea* is characterized by absence of budding cells, and the presence of sympodial condidiogenesis with blunt, scattered denticles bearing conidia singly or in short chains (Figs. 11.1). *Cladophialophora* species such as the neurotropic fungus *C. bantiana* are located in the 'bantiana-clade' [16] together with *Fonsecaea.* These species are different by having very long conidial chains, but some species show intermediate morphology and are difficult to attribute to either one of the genera on a morphological basis. Note that *Cladophialophora carrionii*, frequent agent of CBM in arid climates, is located in another clade and to only distantly related to *C. bantiana* and *Fonsecaea*. A phialidic synanamorph may be produced on nutritionally poor media. All strains grow at 37 °C but not at 40 °C. Recent molecular investigation and characterization of environmental siblings of black agents of human CBM by multilocus sequence analysis has shown that the genus *Fonsecaea* contains five clinically relevant cryptic species i.e. *Fonsecaea pedrosoi*, *F. monophora*, *F. nubica*, *F. multimorphosa,* and *F. pugnacius* [17–19], and three environmental fungi i.e. *Fonsecaea minima*, *F. erecta*, and *F. brasiliensis* [20] (Fig. 11.2).

Fonsecaea pedrosoi was first isolated in 1914 as an etiological agent of CBM by Pedroso and later described and named by Brumpt [9] and Negroni [10]. Traditionally *F. compacta* was another species in the genus with reduced denticles and barrel-shaped conidia, which was described by Carrión from a CBM case in Puerto Rico. This taxon is now known to be a morphological variant of *F. pedrosoi*. In 2004, a new species, *F. monophora* was described using ITS rDNA and RAPD data. The type stain of *F. monophora* CBS 269.37 was isolated from a human CBM case in South America [22]. Morphologically, *F. pedrosoi* and *F. monophora* are very similar. *Fonsecaea monophora* on average has slightly longer conidial chains and slightly shorter denticles than that of *F. pedrosoi*. In 2010, *Fonsecaea nubica* was firstly described based on AFLP profiles and on sequences of ITS, *CDC42*, *BT2,* and *ACT1* genes in a retrospective study [23, 24]. However, detailed clinical

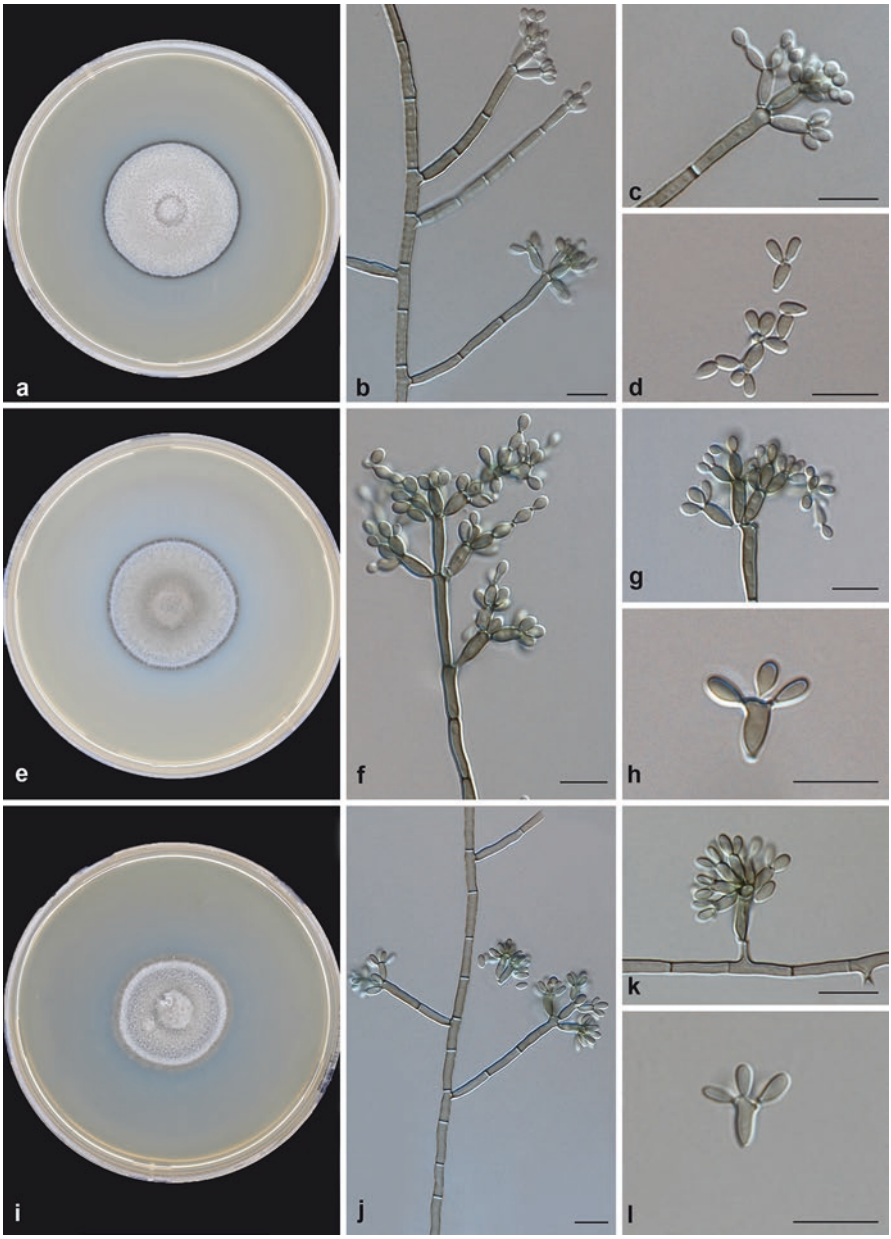

Fig. 11.1 *Fonsecaea monophora*, CBS 117238, (**a**) Colony on MEA after 4 weeks of incubation at 30 °C; (**b**, **c**) Conidiophores and conidia; (**d**) conidia. *Fonsecaea nubica*, CBS 269.64, (**e**) Colony on MEA after 4 weeks of incubation at 30 °C; (**f**, **g**) Conidiophores and conidia; (**h**) conidia. *Fonsecaea pedrosoi*, CBS 273.66. (**i**) Colony on MEA after 4 weeks of incubation at 30 °C; (**j**, **k**) Conidiophores and conidia; (**l**) conidia

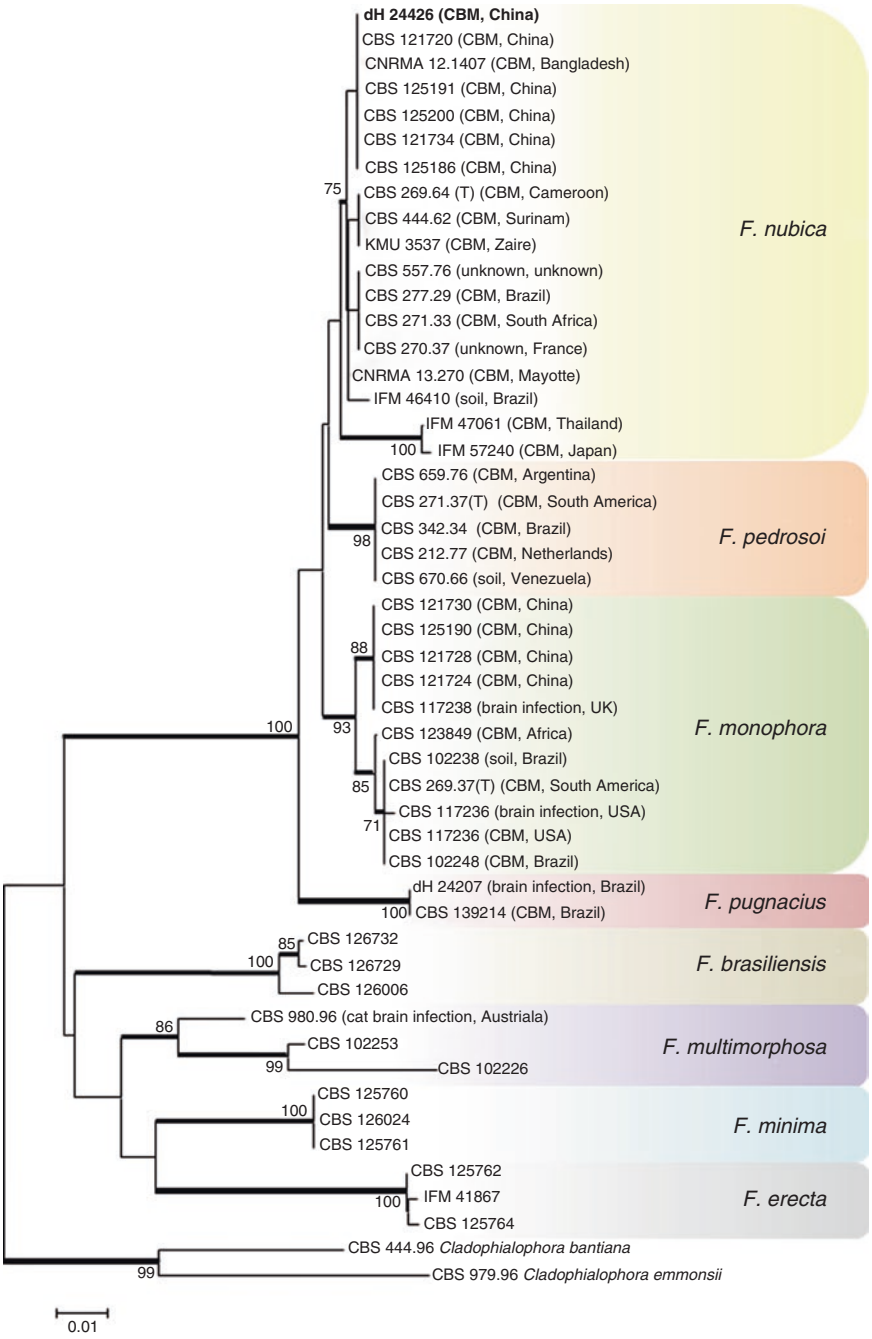

Fig. 11.2 Neighbor Joining tree based on ITS of the genus Fonsecaea. (Reprint from reference [21])

information about *F. nubica* scant, including on the type strain, CBS 269.64. It was originally isolated from a human CBM case with a dull appearance of lesions in West Cameroon. In 2015, another species was added to *Fonsecaea*, *F. pugnacius*, confirmed by sequence data. The case, from central Brazil, showed chronic skin lesions but disseminated to the brain and was fatal [17].

11.4 Putative Virulence Factors and Pathogenesis

Agents of CBM that can be isolated from the environment, but seem to have an advantage of causing human infection. However, relatively little is known regarding virulence factors and pathogenic mechanisms. Putative virulence factors include the presence of melanin, formation of muriform cells, thermotolerance, adherence, and hydrophobicity. The host immune responses to above virulence factors may play an important role in the pathogenesis of CBM.

Melanin is an insoluble compound which is mainly deposited in the cell walls of black fungi and plays an important role in virulence and pathogenicity. Melanin is resistant to a variety of physicochemical agents, including free radical compounds, toxic metals, desiccation, and even ionizing radiation [25, 26], and it is believed to contribute to the organism's ability to elude host immune responses through blocking of the effects of hydrolytic enzymes on the cell wall and scavenging of free radicals liberated by phagocytic cells during the oxidative burst [27]. *Fonsecaea pedrosoi* produces not only cell wall-associated but also extracellular melanin which is mainly produced in melanosomes associated with Fe^{2+} and Ca^{2+} [28]. The presence of melanin has an inhibitory effect on receptor-mediated phagocytosis, interfering with nitric oxide production [29]. Inhibition of phagocytosis was observed in *F. pedrosoi* studies even after stimulation with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) or after in vitro incubation with the whole melanized cell wall.

All etiological agents of CBM belong to chaetothyrialean fungi which are obligatorily melanized, and thus melanin does not explain these predilections. Host body temperatures can be correlated with maximum growth temperatures of invasive species in each of the chaetothyrialean fungal clades [16]. Roughly speaking, clades with species able to grow at temperatures well over 37 °C or higher (the bantiana-, dermatitidis- and jeanselmei-clades) may cause systemic or disseminated infections in humans, while those with a maximum around 36–37 °C (the carrionii- and europaea-clades) cause (sub)cutaneous and superficial infections. Species of the salmonis clade have maxima at 27–33 °C, exceptionally at 36 °C, and cause infection restricted to cold-blooded animals, and rarely in humans, and then mostly on extremities with decreased blood circulation. The clinically relevant *Fonsecaea* species are located in the bantiana-clade with relatively well growth at 36–37 °C which could partly explain they cause not only CBM but also brain phaeophyphomycosis.

It is not known how and under what conditions muriform cells are formed in tissue. This special cellular plasticity is regarded as an important virulence factor for black yeasts and relatives. The presence of the meristematic growth forms in gen-

eral is thought to be an adaptation to harsh environmental conditions, such as extreme temperatures, low water availability, acidity, nutrient deficiency, high UV exposure, or high salt concentrations. In addition, microbial adherence and hydrophobicity are two of the most important determinants of fungal pathogenesis [30]. For CBM, infectious propagules adhere to epithelial cells inside the host leading to differentiation of muriform cells that effectively resist destruction by host effector cells and allow the establishment of chronic disease. This phenomenon may be enhanced by relative cellular hydrophobicity due to the presence of hydrophilic extracellular polysaccharides. Other compounds such as peptidases, glycosphingolipids, and sialidase may be involved in pathogenesis as well [31].

The immunological mechanisms involved in prevention and control of CBM are still not very well understood. However, they probably include innate and adaptive immunity consisting of cellular humoral responses, which has been well reviewed by Seyedmousavi et al. [32]. The innate immune response functions as the first line of defense against invading pathogens. Macrophages are crucial cell in the immunological response in CBM. Rozental et al. [33] showed that activated macrophages were fungistatic to *F. pedrosoi*, delaying germ tube and hypha formation. Bocca et al. [34] showed that during infection with *F. pedrosoi*, macrophages were unable to produce NO, even after stimulation with lipopolysaccharide (LPS) and IFN-γ. Dendritic cells (DCs) are an important line of defense against black fungi when the infection starts with cutaneous or subcutaneous inoculation into tissues. Sousa et al. [35] reported that DCs are the first line of defense against inoculation of black yeasts and relatives into subcutaneous tissues. In their study, the interaction between *F. pedrosoi* and DCs obtained from patients with CBM was investigated. Monocyte-derived DCs from patients with severe forms of CBM induced $CD4^+$ T-cell activation and increased the expression of human leukocyte antigen D-related (HLA-DR) and co-stimulatory molecules (such as CD86, TNF-α, IL-10, and IL-12) in vitro. Notably, in the presence of conidia, the expression of HLA-DR and CD86 was upregulated by DCs from both patients and controls.

In cell-mediated immunity, it was demonstrated that $CD4^+$ T-helper cells and B-lymphocytes have a major role in defense against black yeasts [36]. The severity of diseases caused by black fungi is dependent on Th1/Th2 activation [32, 37]. Patients with chronic forms of infection exhibit increased IL-10 and low levels of IFN-γ. The T-helper 2 profile has been linked to extensive, severe verrucous forms. However, patients with erythematous atrophic plaques presented with a T-helper 1 profile. Recent studies showed that $CD4^+$ lymphocytes are potential key cells for the control of chromoblastomycosis. The role of Th17 lymphocytes in the host defense against CBM remains to be established. Recently, meristematic cells of *F. pedrosoi* were demonstrated to have a chitin-like component that inhibits dectin-1 mediated Th17 development, impairing immune response and contributing for CBM chronicity [38–40]. The host defense mechanism against CBM has not been fully understood yet and the role of humoral response in controlling the disease still remains unknown. In serum of patients with CBM antimicrobial antibodies are detected, however, these antibodies do not seem to play a role in CBM pathogenesis [41].

11.5 Update on Epidemiology

Chromoblastomycosis is not a reportable disease in most countries, thus the incidence of this disorder is not known, and epidemiological estimates are derived from surveys and case series. Furthermore, most of the species were identified by morphology; only during the last decade part of these reports have been well confirmed with molecular methods.

Chromoblastomycosis occurs worldwide, but the incidence is higher in the tropical and subtropical climates within a zone between 30° latitude North and 30° latitude South. Most cases are reported from Latin America, Africa, Asia and Australia countries like Brazil, Mexico, Venezuela, Madagascar, India and China account for the highest frequencies [2, 4]. In Brazil the estimate incidence rate of CBM is 3/100,000 [42]. Silva et al. [43] reported 325 cases in the Amazon region of Brazil, 98% of which had *Fonsecaea* spp. as the etiologic agent. In Mexico, more than 600 cases had been reported during the last seven decades, 90% of which was also caused by *Fonsecaea* spp. [44]. In Africa, Esterre et al. [45] reported 1343 cases of CBM from Madagascar, of which 98.5% were confirmed by histopathology, and *Fonsecaea* spp. were identified from 61.8% of the positive cultivation. Kombila et al. [46] reported 64 cases in Gabon, all caused by *Fonsecaea* spp. In China, Jiajun You first reported chromoblastomycosis in 1951. Since that time, more than 600 cases have been described in the Chinese and English literature, with the highest prevalence being reported in Guangdong (84/196) and Shandong Provinces (38/196). In this review, *Cladophialophora carrionii* is shown to be the predominant species in northern China, and *Fonsecaea* spp. in southern China [47]. In Japan, Kano et al. [48] described the first case of CBM. Later investigation showed Japan had the highest incidence of CBM among populations in Asia (1/416,000). Fukushiro [49] described approximately 296 cases of CBM reported in Japan from 1955 to 1982, and Kondo et al. [50] analyzed 212 additional cases reported from 1982 to 2001, using the database of the "Japan Centra Revuo Medicina". Recently molecular studies confirm *F. monophora* as the most prevalent species in Japan [51]. In Sri Lanka, 94% of 71 CBM cases were caused by *Fonsecaea* spp. [52]. Chromoblastomycosis is rarely seen in temperate Europe. A recent molecular confirmed case and review of the literature revealed a total of 31 cases in Europe since the first European case in Leningrad in 1929 [53]. In Australia, to date approximately 200 cases were published which is mainly due to *C. carrionii*, occurring in the dry bush county area [54]. In general, *Fonsecaea* spp. are prevalent in humid tropical climates, whereas *C. carrionii* infection is normally found in dry countries and desert regions.

In many published CBM case reports, etiologic agents were identified with histopathologic features in the tissue or phenotypic features of the culture pending precise molecular verification. More recently, Najafzadeh et al. [18, 19] assessed the geographic distribution of 81 *Fonsecaea* strains using amplified fragment length polymorphism (AFLP) technology and multilocus sequence typing. Judging from AFLP fingerprinting, *F. pedrosoi* is restricted to Central and South America, whereas

F. monophora and *F. nubica* have global geographical distributions and have been recovered from clinical specimens in Asia (Southern China, Japan), Western Hemisphere (Brazil, Mexico), Africa and Europe (France, England). It is noteworthy that both *F. monophora* and *F. nubica* clusters could be subdivided into two groups, and population 4 of *F. monophora* together with population 1 of *F. nubica* were prevalently confined to Southern China. Considering that the taxonomy of *Fonsecaea* species has been reevaluated, it becomes necessary to identify clinical isolates at the species level in order to study their epidemiology distribution, and to determine if different clinical presentation and response to antifungal therapy are associated with each of the taxa, which would be clinically critical for appropriate patient management.

The majority of the patients with CBM are between 30 and 50 years of age, with a male-to-female ratio of 4:1 to 9:1 [4, 44, 47, 55]. Most lesions are observed on the extremities of farmers and outdoor rural workers. The preponderance of males in most reported cases classically has been attributed to their higher exposure risk than gender susceptibility, but given the male preponderance in the neurotrope *Cladophialophora bantiana* and many other fungi, a hormonal cause seems more likely.

The ecological niches of *Fonsecaea* spp. and the mode of transmission of CBM remain unclear, though traumatic implantation is regarded as the main mode of infection of this disorder. For example, a Bangladesh CBM case recalled a trauma with a knife, while another remembered an injury with scrap wood [56, 57]. Thus far all known *F. nubica* and *F. pugnacius* strains were clinical CBM strains, however, a few *F. pedrosoi* and *F. monophora* strains were isolated from plant debris [20]. Occasional cases of CBM were acquired through different route, such as animal bite and insect sting [4]. Recently Slesak et al. [58] reported a CBM case associated with a leech bite. Other CBM cases associated with insect bites were reported in Thailand, due to *F. pedrosoi* [59] and in Venezuela, by a possibly undescribed species [60].

Numerous reports of epizootics of CBM-like infections in animals have been published [16, 22, 61]. In the animal model using immunosuppressed mice, *F. pedrosoi* and *F. monophora* replicate the infection with intracutaneous or intercutaneous lesions, but as yet there is no confirmed case of animal CBM.

11.6 Clinical Manifestations

Chromoblastomycosis is a chronic fungal infection of the skin and subcutaneous tissue. The infection usually appears after minor trauma preceding the lesions, however, only few patients recall any history of trauma as it is usually innocuous, occurs months or decades earlier, and is mostly forgotten. The exposed body parts, particularly the extremities, are involved most frequently, especially feet and shins [1, 2, 4]. Cases of auricle, face, trunk, and buttock infection have also been reported [21, 53,

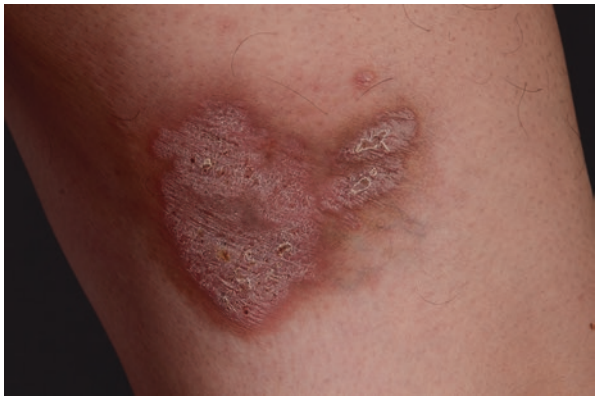

Fig. 11.3 Different types of lesions of chromoblastomycosis

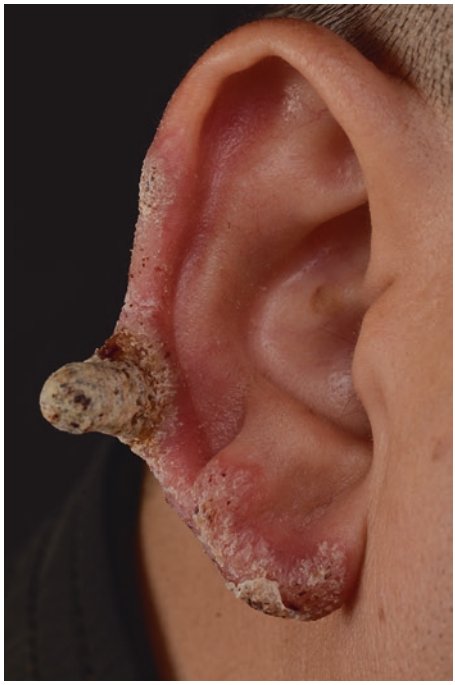

Fig. 11.4 Different types of lesions of chromoblastomycosis

62–64]. The primary lesion may start as a solitary erythematous macule, then progresses to a papular shape with pink smooth-surface [1] which gradually enlarges from the site of infection to become polymorphic over extended periods of time. In general, there are five different types of lesions. Nodular, tumoral (cauliflower-like), verrucous, plaque and cicatricial [1, 2, 4]. Recently pseudo-vacuolar, eczematous and horn-like lesions were also observed [21, 47, 65]. In advanced and severe cases,

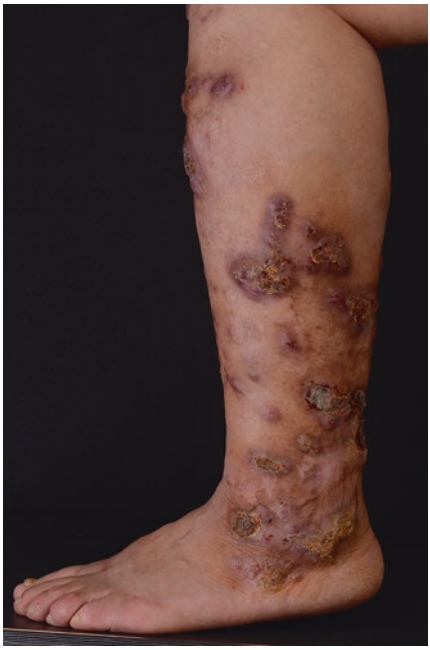

Fig. 11.5 Different types of lesions of chromoblastomycosis

Table 11.1 Differential diagnosis of chromoblastomycosis lesions

Infectious diseases	Fungi	Blastomycosis
	Fungi	Coccidiodomycosis
	Fungi	Phaeohyphomycosis
	Fungi	Sporotrichosis
	Protozoa	Leishmaniasis
	Protozoa	Rhinosporidiosis
	Bacteria	Ecthyma
	Bacteria	Nocardiosis
	Bacteria	Tuberculosis
	Virus	Warts
Non-infectious diseases		Carcinoma
		Lupus
		Psoriasis
		Sarcoidosis

more than one type of lesion can be observed in the same patient (Figs 11.3, 11.4, and 11.5; Table 11.1).

Apart from the division presented above, the lesions in CBM may be classified according to severity [2, 65]. Three levels are distinguished: (1) Mild form – a solitary plaque or nodules measuring <5 cm in diameter; (2) Moderate form – solitary or multiple lesions of plaque, verrucous or nodular type, isolated or conjoined, covering one or two adjacent body areas measuring <15 cm in diameter; (3) Severe

form – any type of single or multiple lesions covering extensive skin areas, whether adjacent or non-adjacent. Severe lesions tend to poorly respond to treatment or become treatment-resistant.

The initial and mild skin lesions are usually asymptomatic, may pruritic or become painful. Satellite lesions can develop from autoinoculation through scratching and from lymphatic dissemination. Common complications of CBM include ulceration and secondary bacterial infection. In severe cases, chronic lymphedema and ankyloses may develop, rarely, malignant transformation into squamous cell carcinoma may arise, which may lead to long term disability of the patient. Extracutaneous infection due to contiguous spread, hematogenic or lymphatic dissemination involving bone, lymph nodes, lungs and brain have been rarely reported [50, 66–70], but should be noted that these results are tentative pending precise identification of the etiologic agents by molecular methodology. Recently confirmed cases of central nervous system and invasive tissue infections show that the etiological agents are mostly attributed to *F. monophora* [70–76] and once to *F. pugnacius* [17]. An experimental murine model of disseminated infection with *F. monophora* was also documented [77]. In brain tissue histopathology is with pigmented hyphae rather than muriform cells, probably due to lower levels of immunity in the brain.

Clinical presentations of human CBM may vary according to the immunological status of the host, the time of evolution, the load of the inoculum, involved site, patient's hygienic habits, compliance to therapy and the pathogenicity of the strain, among other factors. In order to better plan dose and duration of therapy as well as the patient's prognosis, the CBM lesions must be also classified according to the predominant clinical type and severity grade.

11.7 Classical and Novel Diagnostics

Chromoblastomycosis is diagnosed through a combination of clinical and laboratory findings, i.e. direct examination of skin scrapings or biopsy tissue and/or histopathology. Direct examination of specimens is conducted with 10–40% potassium hydroxide (KOH) which displays muriform cells unambiguously. Thick-walled, chestnut brown, rounded cells (4–12 μm in diam) with transversal and longitudinal septation are readily seen, also known as sclerotic cells, Medlar bodies, or copper pennies; these are a pathognomonic criterion for diagnosis. Muriform cells are easily found in black dots at the lesion's surface which are small hematic crusts with cellular debris and fungal structures resulting from transepithelial elimination. Occasionally, pigmented hyphae can be also observed, especially when the patient has decreased immunity, muriform cells undergoing morphological transformation for germination (Fig. 11.6.).

Muriform cells are not informative for species identification of the agent. As CBM could be caused by a diverse group of melanized fungi, and the knowledge of fungal identification down to species level contributes to our understanding of their

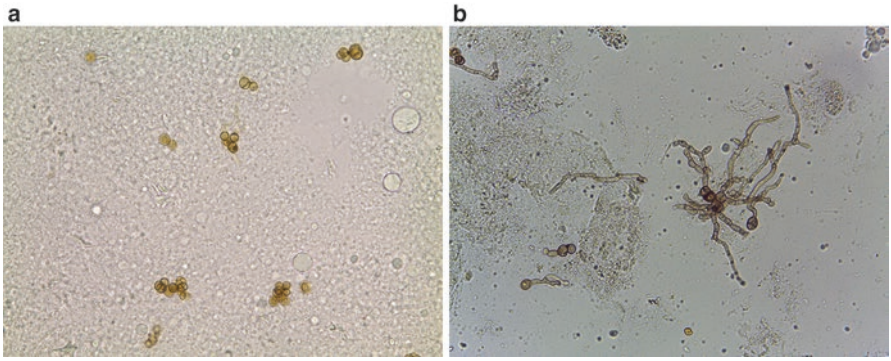

Fig. 11.6. Muriform cells (**a**) and germinating muriform cells (**b**)

epidemiological, biodiversity and clinical features. In addition, the causative agents of CBM could also cause other clinical syndromes, such as phaeohyphomycosis. For definitive CBM diagnosis isolation and identification of the etiological agent it is recommended to use the term "chromoblastomycosis" [27, 78]. Culturing is done on selective media supplemented with cycloheximide and chloramphenicol avoiding fast growing fungi and bacteria. Most agents of chromoblastomycosis have a maximum growth temperature around 36–37 °C [16] and require 2–3 weeks before small colonies are obtained. Colonies are flat to heaped and folded, velvety to cottony, olivaceous to black with black reverse. Conidiophores are short, branched, with short, sympodial, chains of conidia on denticles. Budding cells are absent. Phialides with collarettes may be present in low abundance on poor media. rDNA ITS is recommend as a genetic barcoding marker for *Fonsecaea* species. Shared traits between species are absence of growth with inulin as sole carbon source, absence of fermentation, tolerance of cycloheximide and intolerance of 10% NaCl. Members of the order *Dothideales*, including *Aureobasidium* and *Hortaea*, mostly grow with 10% NaCl. Urease production varies with the strain [22].

Muriform cells seen in CBM are frequently detected by direct microscopy, however, tissue invasion is best visualized and documented by permanent histopathological preparations. Although muriform cells may be easily seen in tissue with the routinely used hematoxylin and eosin (H&E) stain, other special stains such as Gomori methenamine silver (GMS) stain and periodic acid-Schiff (PAS) stain could be employed to enhance fungal detection, especially where fungal burden are low or with secondary infections. In skin, a characteristic pseudo-epitheliomatous hyperplasia with hyperkeratosis and parakeratosis occurs in the epidermis, and mixed granulomatous suppuration with fungal elements with irregular acanthosis alternating with areas of atrophy are seen in the dermis. Muriform cells are found in the stratum corneum and the following layers of the epidermis, and in the granulomatous processes and in giant cells (Fig. 11.7.).

Several serological tests have been developed to aid CBM diagnosis, though they are not routinely used because of relatively low sensitivity and specificity, and only

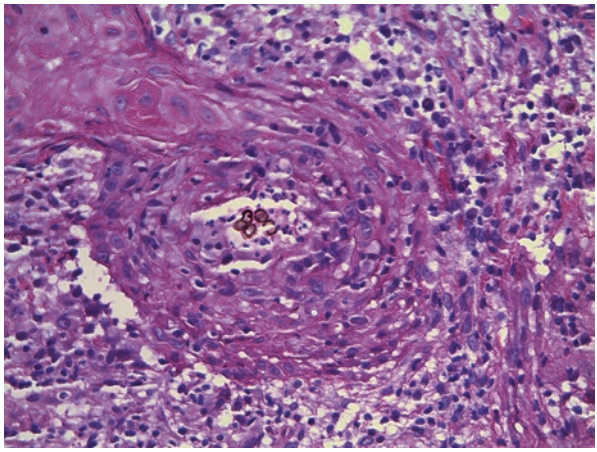

Fig. 11.7. Muriform cells in a granulomatous process

small numbers of patients have been studied. *Fonsecaea pedrosoi* was analyzed using immunoblotting, a 54-KD antigen with high sensitivity and specificity was found [79]. Double immunodiffusion (DID), counterimmunoelectrophoresis (CIE) and immunoenzymatic test (ELISA) have also been employed to evaluate humoral immune responses. Metabolic antigen (chromomycin) was used for immunoprecipitation tests (DID and CIE), while somatic antigens for ELISA were found. Results show variable sensitivity for all techniques, with relatively high specificity ranging from 83 to 90% [80]. Chromomycin was effective in detecting delayed hypersensitivity in patients with *F. pedrosoi* infection, with sensitivity and specificity of 90.0% and 98.8%, respectively. Recently, Bruña-Romero and Marques [81] evaluated the association between IgG antibody levels and the severity of CBM and therapeutic response of patients to itraconazole. The levels of antibodies in 55 CBM patients expressed in optical density and titration showed that, before treatment, patients with severe disease had higher levels of IgG, IgG1, IgG2, and IgG3 when compared with moderate or mild disease. Main antibody titers of IgG, IgG1, and IgG2 were reduced after treatment. However, the anti-*F. pedrosoi* antibody did not exhibit a protective role against infection.

Currently, nonculture methods are in use to improve sensitivity and specificity of mycological diagnosis. Molecular identification of individual *Fonsecaea* species is done with the rDNA ITS region, which is currently a universal DNA barcode marker for fungi. For distinction of closely related *Fonsecaea* species causing CBM, an additional gene such as partial β-tubulin (*BT2*) or translation elongation factor 1-α (*TEF1*) may be recommended. In addition, a quality-controlled reference databases should be used in the comparison. For example, Slesak et al. [58] recently reported a CBM case associated with a leech bite, identified by ITS rDNA sequencing, yielding 100% identity with *F. pedrosoi*, *F. monophora*, and *F. nubica* in GenBank. However, comparing HQ616145 with the CBS database (http://www.westerdijkinstitute.nl/) 100% similarity with *F. monophora* and 99.8% with *F. pedrosoi* was obtained, leading to final identification as *F. monophora*. The ITS database of the

International Society of Human and Animal Mycology (http://www.isham.org/ and http://its.mycologylab.org/) provides a quality-controlled standard tool for routine identification [82, 83].

Molecular identification may be hampered by sequence variability in the ITS domain caused by difficult-to-sequence homopolymeric regions and by poor taxonomic attribution of sequences deposited in GenBank. Heinrichs et al. [84] proposed short barcode identifiers (27 to 50 bp) based on sufficiently conserved ITS2 for the common clinical *Fonsecaea* species. Using the proposed barcode identifiers, a 4.1-fold increase of 100% matches in GenBank was achieved.

Isothermal amplification methods, such as Loop-mediated isothermal amplification (LAMP) and rolling circle amplification (RCA) have been developed for rapid detection and identification of *Fonsecaea* stains [18, 19, 85]. LAMP proved to be a fast and sensitive method for direct amplification of fungal DNA from environmental samples but could not distinguish individual *Fonsecaea* species, whereas for RCA ITS amplicons are needed but is species-specific.

11.8 Treatment

Despite in vitro susceptibility of agents to most antifungals, CBM is often recalcitrant and difficulty to cure. Except for the initial lesions, which can be surgically removed, CBM lesions constitute a true therapeutic challenge. A clinical guideline for diagnosis and management of infections by melanized fungi was published by ESCMID/ECMM [86], in which CBM is treated with itraconazole (ITZ) plus surgery (recommendation AII) or terbinafine (TBF) monotherapy plus surgery (recommendation BIII). Recommendations for refractory cases are combined antifungal therapy and cryotherapy or surgery (recommendation BIII). Laser, heat and potassium iodide therapies have also been used with successful outcome (recommendation BIII). Based on experimental and in vitro studies the new triazole drug posaconazole (PCZ) is promising and could be useful when other therapies have failed (recommendation BIII). However, this guideline did not distinguish between etiologic agents. In general, *Fonsecaea* infections are more difficult to treat than those by *C. carrionii* [65].

Up to now, breakpoints have not been established for melanized fungi, including *Fonsecaea* species. Several studies have documented that *F. pedrosoi* isolates exhibit high in vitro susceptibility to triazoles such as ITZ, voriconazole (VCZ), PCZ and isavuconazole (ISA), but limited in vitro susceptibility to amphotericin B (AMB), fluconazole (FCZ) and 5-flucytosine (5-FC). Najafzadeh et al. [23, 24] tested a total of 55 strains of *Fonsecaea* species using the CLSI document M38-A2. All isolates exhibited low MIC values for ITZ, VCZ, PCZ, ITZ and ISA. In contrast, higher MIC values were documented against AMB, FCZ, and echinocandins including caspofungin (CAS) and anidulafungin (AND). Although there were no significant differences in the in vitro activities of the new triazoles against *F. pedrosoi*, *F. monophora*

and *F. nubica*, it was observed that the MICs to VCZ and ISA were 1 or 2 dilutions higher in strains of *F. pedrosoi* compared to *F. monophora* and/or *F. nubica*.

Itraconazole is a fungistatic first generation triazole which is metabolized by the cytochrome P450. It is the most widely used drug in the treatment of CBM with satisfactory results and good tolerance, even with long-term treatment. The dose varies from 200–400 mg/day, and the duration of therapy ranges from months to years, with most of the cases showing improvement in 8–10 months. In an early study, safety and efficacy of ITZ were evaluated in 15 CBM patients caused by *F. pedrosoi* [87]. Patients with mild to severe disease were cured with ITZ after a mean duration of 7.2 months and 25.1 months, respectively. No significant side effects nor biochemical alteration during this trial were important enough to interrupt the treatment. However, it has been suggested that ITZ resistance might be acquired in patients under chronic use of this drug. Andrade et al. [88] found that sequential isolates from four patients presented higher ITZ MIC values than initial strains; for two of these patients, no response to therapy was observed.

Terbinafine (TBF) is an allylamine derivative causing a fungicidal and fungistatic effect through lanosterol synthesis. It has less drug-drug interactions than ITZ since TBF does not interfere with P450 cytochrome. Several reports have demonstrated efficacy and tolerability of TBF 250–500 mg/day obtaining cure CBM due to *Fonsecaea* species [89–91]. In an open trial, long courses of terbinafine at a dosage of 500 mg/day were administered orally to 43 CBM patients. Mycological cure was observed in 82.5% of patients infected with *F. pedrosoi* after 12 months of therapy. The efficacy of terbinafine in *C. carrionii*-infected patients seemed higher than in *Fonsecaea*-infected patients [90].

Combination therapy is an option for refractory or severe CBM cases, and its clinical effect in vivo has been clearly demonstrated. Zhang et al. [92] evaluated the in vitro effects of a combination of TBF plus ITZ on 18 clinical *F. monophora* isolates. The fractional inhibitory concentration index (FICI) analysis demonstrated that 12 (67%) were synergistic, 4 (22%) were additive, and 2 (11%) were indifferent, with no antagonism being observed. The minimal inhibitory concentrations (MICs) obtained with the terbinafine-itraconazole combination were within levels that can be achieved in plasma at clinically relevant doses. Gupta et al. [93] used ITZ and TBF in an alternate week or combination therapy on four patients with longstanding CBM (8–23 years) caused by *F. pedrosoi* with poor response to standard oral antifungal monotherapy. The combination therapy was considered effective in all four patients.

Posaconazole (PCZ) is a new, extended-spectrum triazole antifungal agent that has demonstrated in vitro activity against many melanized fungi including *Fonsecaea* species. Two clinical reports suggested that PCZ 800 mg/day was associated with success rates of more than 80% in refractory CBM patients [94, 95]. The efficacy of PCZ was also evaluated in a murine model of disseminated infection by *F. monophora*. The results suggested that PCZ may have a clinical role in the treatment of disseminated infections by *F. monophora*, because it prolonged survival significantly and reduced the fungal load in most of the organs tested [77].

In most cases, several modalities of physical methods may be used but as an adjuvant therapy, always in combination with antifungal drugs. Physical therapies that can be applied include surgery, thermotherapy, laser and photodynamic therapy. Surgical excision can be used for initial lesions only; unfortunately CBM initial lesions are scarcely observed in clinic. Thermotherapy encompasses heat and cold therapy. The use of pocket warmers or electric bed warmer has proven successful in the treatment of a limited number of cases, because the increase in skin temperature somehow impairs fungal development [96]. However, heat therapy as single treatment shows insufficient result, combination with other treatment modalities being required [97]. Cryotherapy is convenient and cost-effective, especially for localized lesions. Dissemination of lesions to adjacent areas after cryotherapy has been noted and therefore it is important to combine cryotherapy with ITZ or TBF. Photodynamic therapy (PDT) using 5-aminolevulinic acid (ALA) irradiation may be adjunctive in combination with antifungal medication [98, 99]. Hu et al. [100] combined oral terbinafine with weekly ALA-PDT in a case of CBM and with this combination, apparent clinical improvement was achieved within less than a year and no recurrence was observed.

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Chapter 12
Mycetoma

Ahmed Hassan Fahal

Abstract Mycetoma is a unique neglected disease, endemic in many tropical and subtropical regions, characterized by devastating deformities, disability, high morbidity and serious negative socioeconomic impacts on patients, families and community. It enjoys meager attention by health and social sectors across the world, as it is a non-glorious disease. Mycetoma commonly affects young adults and children of low socioeconomic status from rote rural areas in poor countries and it is an excellent example of a neglected tropical disease.

To date, its actual incidence, prevalence, and route of infection are not well characterized, as is its susceptibility, resistance, and response to medical treatment. This data scarcity is reflected on the available diagnostic and treatment modalities, which proved to be ineffective, have serious side effects, and are expensive for patients and health authorities in endemic areas. Early-stage detection of mycetoma and subsequent medical intervention lead to more favorable prognosis and minimization of disease recurrence, its sequelae, and economic burden.

12.1 Background

Mycetoma is a common health problem, endemic in many tropical and subtropical regions characterized by devastating deformities, disability and high morbidity (Figs. 12.1 and 12.2) [1, 2]. It is a debilitating disease, which progresses relatively silently. Mycetoma has serious negative medical and socioeconomic impacts on patients, families, communities, and health authorities. Yet, it enjoys meager attention across the globe which is translated in massive knowledge gaps in various aspects of mycetoma [1, 2].

Mycetoma is a chronic granulomatous subcutaneous inflammatory disease caused by either true fungi (eumycetoma) or certain bacteria (actinomycetoma). It affects the poorest in poor populations in the poor and most remote areas. Typically, young adult male farmers, laborers, and students between 15 and 30 years old of low socioeconomic status are mostly affected [3–6].

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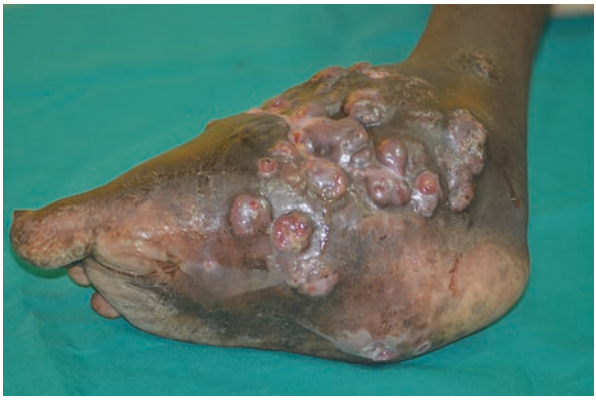

Fig. 12.1 Showing massive foot actinomycetoma

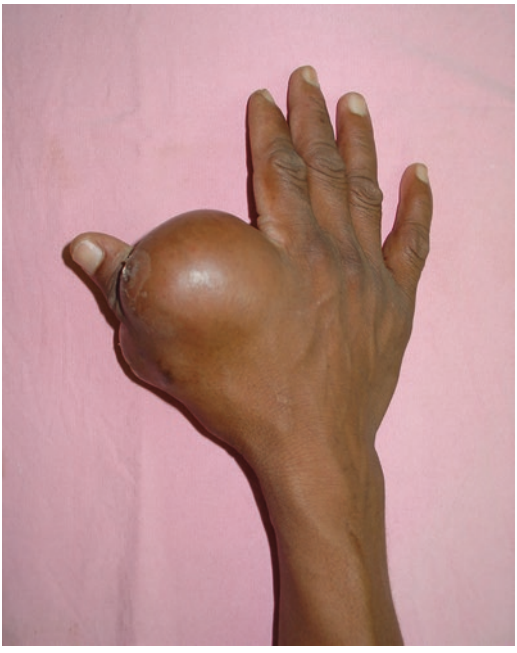

Fig. 12.2 Showing massive eumycetoma with deformity

12.2 Historical Note

The first reports on mycetoma appeared in 1694 by the German physician Engelbert Kaempfer. In 1842, Gill reported the disease in Madurai, Southern India, hence the name Madura foot, a term that is no longer used. Van Dyke Carter (1860) named the disease mycetoma (mykes = fungus and oma denotes tumor) after isolating a fungus for the first time. Although the first reports on mycetoma originated from India, by the beginning of the twentieth century, interest in mycetoma research had shifted to Africa. Gemy and Vincent reported the first African mycetoma case from Tunisia in

1894. Since then, many reports on the disease were published and have formed the foundation of the current knowledge and research on mycetoma [7].

12.3 Epidemiology

12.3.1 Incidence and Prevalence

For various understandable reasons, the true incidence and prevalence of mycetoma throughout the world are not exactly known. These reasons include the nature of the disease, which is usually painless, slowly progressive, and the late presentation of the majority of patients due to the lack of health education and financial constraints. In many centers, amputation is still the sole treatment for advanced cases, which may contribute to the fear of many mycetoma patients to seek medical advice. The medical records and statistical information, in most of the mycetoma endemic health facilities are deficient, which contributes to the inadequate data on mycetoma incidence. In many rural communities, mycetoma is a social stigma, particularly among females [8, 9].

In mycetoma endemic areas, due to several reasons, many individuals pursue medical help from local native healers and that is another cause for the late admission of patients in clinics or hospitals. Therefore, most of the data on mycetoma are related to hospital cases with advanced disease [1, 2, 9].

Although Sudan is considered the mycetoma homeland, data on its prevalence and incidence are meager and scanty. Since no real prevalence studies are performed, prevalence can only be roughly estimated based on epidemiological studies. Two large epidemiological studies have been performed on which an artificial and largely underestimated prevalence can be calculated. These are the studies performed by Abbott in Sudan in 1952–1955, and that reported by Lopez Martinez and colleagues from Mexico between 1956 and 1985 [7, 8, 10].

In Abbott's study, 1231 mycetoma cases were admitted to hospitals throughout the country in a period of 2.5 years. By dividing this number of cases seen in hospitals by the total population of Sudan in those years, a prevalence of 4.6 cases per 100,000 inhabitants would be obtained. No incidence data can be calculated [10].

Lopez Martinez reported on 2105 mycetoma cases from 14 dermatological centers throughout Mexico in a period of 30 years. Again, by applying the same formula an average prevalence of 0.6 cases per 100,000 inhabitants would be obtained. These grossly underestimated incidences are comparable to those of other neglected tropical infections such as Buruli ulcer, African trypanosomiasis, dracunculiasis, and leprosy [10].

More recent data were reported from the Mycetoma Research Centre, WHO Collaborating Centre on mycetoma, Khartoum, Sudan, the only one of its kind in the country. This center reported on 6792 patients with mycetoma and an incidence of 370 new cases per year in the period 1991–2015 [10].

12.3.2 Geographical Distribution

Mycetoma has a worldwide distribution but this is extremely uneven. It is endemic in many tropical and subtropical regions. The African continent seems to be the area of the highest prevalence. It prevails in the mycetoma belt, which includes Sudan, Somalia, Senegal, India, Yemen, Mexico, Venezuela, Columbia, Argentina, and others [2, 3, 7].

In Africa, mycetoma is most frequently seen in Sudan, Senegal, Mauritania, Kenya, Niger, Nigeria, Ethiopia, Chad, Cameroon, Djibouti, and Somalia. In Asia, it has been extensively reported from India and Yemen [2, 3, 7]. However, mycetoma has been reported in many temperate regions as well. Cases were reported from the United Kingdom, most of those afflicted were immigrants, who probably contracted the infection overseas, with the pale grain eumycetoma as the most common type. There are few reports on mycetoma from the United States, Ceylon, Germany, Egypt, Turkey, Philippines, Japan, Lebanon, Thailand, Saudi Arabia, Tunisia, and Iran [2, 3, 7].

Areas where mycetoma prevails are relatively arid zones with a short rainy season of 4–6 months, with the rainfall of 50–1000 mm per year, a relative humidity of 60–80% and constant temperatures of 30–37 °C day and night. This is followed by a dry season of 6–8 months with a relative humidity of 12–18%, day temperatures of 45–60 °C and night temperatures of 15–18 °C [2, 3, 7].

The geographical distribution of the individual mycetoma organism shows considerable variations, which can be convincingly explained by climatic factors, including the temperature, humidity and the rainfall; having the latter as the most relevant factor [1, 7].

Madurella mycetomatis, which is the commonest cause of eumycetoma, predominates in the tropical areas of Africa and India with a rainfall of 250–500 mm per year. Whereas, *Streptomyces somaliensis* is seen more often in the Middle East, Central, and West Africa and the arid region adjacent to the Sahara Desert, with a rainfall of 50–100 mm per year. However, it is sometimes seen in areas with higher rainfall as in Mexico [1, 7].

Actinomadura pelletierii is more prevalent in the relatively humid areas where the rainfall ranges from 250 to 1000 mm per year. *Nocardiae* are usually the causative organisms of mycetoma in temperate regions [1, 7].

The mycetoma belt encases an area of forest trees and savannah. The dominant plants usually are various species of Acacia and other thorny bushes and trees, like *Balanytesa egyptica,* which are armed with strong thorns.

There is an association between mycetoma pathogenesis and thorns. The penetrating thorns facilitate the entry of the organism into the subcutaneous tissue as it lives saprophytically on or inside the thorn. This was documented in serial sections of thorns removed from two mycetoma patients. In these patients, the thorns cells contained many fungal elements in different stages of development were reported. From one of them, *Leptosphaeria senegaliensis* was isolated and *Pyrenochaetaromeroifrom* in the other [7]. The presence of thorns in the vicinity of the mycetoma granuloma

had been observed histologically and during surgery. Thorns may have the ability to produce the granuloma, and probably they provide an appropriate nucleus for the development of the mycetoma organism. The fact that the disease is common among rural people who go barefooted is in line with these observations. However, in many patients there is no history of local trauma at the mycetoma site.

12.3.3 Susceptibility and Resistance

Mycetoma is reported in both males and females of all age groups, but some groups are affected most. It occurred more frequently in the young adult males, with ages ranging from 15 to 30 years, but no age is exempted, especially in endemic areas. Patients at this age group represent the most active and the earning members of the society, particularly in developing societies where the life expectancy is short [8, 11].

Not all occupations are equally encountered in the patient population. Most of the mycetoma patients appear to earn their living by working on the land, peasant and herdsmen and the farmers are affected more often. However, in a recent study, 30% of reported patients appeared to be young students. This may due to the fact that, mycetoma is commonly seen in this age group, increased educational facilities in the endemic areas and the children commonly share the various outdoor activities with their families. In endemic areas, other professionals such as workers, clerks and health workers may also be affected [2, 8, 11].

In all previous reports, male predominance is a constant finding in mycetoma. In general, the gender ratio in eumycetoma is (3:1) which is higher than that encountered in actinomycetoma (2.7:1) and the explanation behind this observation is unclear. The male predominance is commonly attributed to the greater risk of exposure to organisms in the soil during the outdoor activities. However, in areas where mycetoma is prevalent in the Sudan, both sexes go barefooted, and in Western Sudan women are more committed to outdoor activities than males and yet, the incidence of the disease is the same as that observed in other parts of the country. These findings are in line with reports from Kenya, Congo, and others [2, 8, 11].

It may be that women are inherently less susceptible to infection with mycetoma due to differences in sex-related hormone levels, but this seems not to be the case. Women have much higher levels of 17 β-estradiol than men, but in male mycetoma patients higher 17 β-estradiol levels were reported than in non-infected males [12]. However, it is interesting to note, during pregnancy the mycetoma lesions become more aggressive, active with formation of new discharging sinuses (Personal observation), and the depressed immunity during pregnancy may partially explain this observation; however, other causes must be considered.

Another difference between men and women, which could be of influence in developing mycetoma, is the make-up of the skin. Men have a thicker dermal compartment of the skin, less subcutaneous fat, higher basal blood flow in the skin, generate more sebum and sweat, have larger pores, and most importantly, have a

slower wound healing. All these differences can be important in a subcutaneous infection [10].

In general, mycetoma patients are considered to have a normal and responsive immune system, although there are some reports, which showed partial impairment of the cell-mediated immune (CMI) response in patients severely infected or not responding to medical treatment [7]. This finding was supported by animal studies, since mycetoma was more successfully induced in athymic mice than in immunocompetent animals [13]. In another report, some impairment in the innate immune response was noted. Overall, still no convincing evidence exists that showed mycetoma patients have particular immune defects.

12.4 Mycetoma Entry Route

Many believe that, the causative organisms may be present in the soil in the form of grains. After they are moistened by rain, they form conidia or other fungal forms capable of infecting the host. This infecting agent is then implanted into the host tissue through a breach in the skin produced by local trauma caused by sharp objects such as thorn pricks, stone, or splinters [2, 3, 7].

In areas where mycetoma is frequent, the habit of going barefooted is common and thorns are plentiful. As a result, natural infection is expected to be more frequent than it actually is, if this hypothesis of route of infection is true. Many workers in the field believe that, there is an intermediate host for the infection to develop but it is not known [2, 3, 7].

It is interesting to note, that it was not possible to isolate *Madurella mycetomatis*, the main causative agent of human eumycetoma in Sudan from different soils and thorn tree samples from different endemic areas in the country. However, PCR-mediated detection followed by restriction fragment length polymorphism (RFLP) analysis for the identification of *Madurella mycetomatis* DNA from the environmental samples was done, and it was positive in 23% of soil and in 5% of the thorn samples. These observations support the hypothesis that eumycetoma is primarily environmentally acquired, and suggest that *Madurella mycetomatis* needs special conditions for growth, as direct isolation from the environment seems to be impossible [14].

The Central Sudan is endemic area for eumycetoma due to *M. mycetomatis*. The majority of villagers in that area are farmers and live on animal husbandry. They have many cattle, goats, sheep, dogs, chickens, and donkeys, and they are kept in cages surrounded by walls made of mud or dry thorny bushes. The floors of the cages are covered with dry animal dung, thorns, and trash. The villagers' homes are in close proximity to these cages. As the frequency of *M. mycetomatis* on thorns is low as documented previously, and the common habit of going barefooted, contaminated animal dung and its role as an adjuvant may have important roles in contracting mycetoma [15].

The disease can occur naturally in several animals including goats, horses, donkeys, dogs, and cats. However, the disease is not contagious from animal to human or from one person to another and there is no report on hospital cross- infection [2, 3, 7].

12.5 The Incubation Period

The incubation period in mycetoma is unknown due to the difficulty in establishing the time of initial infection; however, in experimental animals the formation of the mycetoma lesion was noted after a period of 3 weeks from the inoculation of the organism [2, 7].

12.5.1 Mycetoma Pathogenesis

Unlike the life-threatening systemic fungal infections, there is no clear immune defect or deficiency known that could explain why people develop mycetoma. Serological studies with the *M. mycetomatis* specific antigens showed that Translationally Controlled Tumour Protein, fructose bisphosphate aldolase, and pyruvate kinase antibodies are elevated in both mycetoma patients and endemic controls. However, only the patients developed mycetoma. This suggested that all people from the endemic area have contacted this fungus at some stage but only a small portion developed the disease [16].

Mahgoub and his colleagues in 1973 studied the cell-mediated immunity of a group of mycetoma patients by means of the tuberculin test, 2,4-dinitrochlorobenzene sensitization and lymphocyte proliferation induced by phytohemagglutinin, and found defective T cell-mediated responses especially in severely infected patients and in those patients who did not respond well to treatment [17]. These findings were supported by animal studies, since mycetoma was more successfully induced in athymic mice than in immunocompetent mice [13]. However, on the other hand, Bendl and his colleagues had studied 31 patients with mycetoma but no immune defects were detected in that cohort.

Since it was observed in endemic areas that mycetoma is seen more frequent in certain families, either environmental or genetic factors seem to be important in the development of mycetoma. Van de Sande and colleagues therefore addressed the genetic susceptibility towards mycetoma by determining differences in allele frequencies for several single nucleotide polymorphisms (SNPs) in patients and healthy endemic controls. Two of those genetic studies addressed the role of single nucleotide polymorphisms (SNPs) in genes involved in the function of the immune system [18]. In the first study, the role of SNPs in genes neutrophil function was addressed. In that study, 11 SNPs in eight genes involved in neutrophil function were studied. Significant differences were found in genes encoding for interleukin

8 (CXCL8), its receptor CXCR2, thrombospondin-4 (TSP4), nitric oxide synthase 2 (NOS2) and complement receptor 1. The genotypes more commonly found in mycetoma patients for CXCL8, its receptor CXCR2 and TSP4 all were associated with a higher CXCL8 production in other studies. Therefore, this finding was confirmed by the measurement of high levels of CXCL8 in serum of mycetoma patients. In contrast, the NOS2 genotype obtained from the patients was associated with a lower Nitric oxide production. This finding was also confirmed by lower nitrite and nitrate levels in mycetoma patients.

Mhmoud and colleagues determined the role of interleukin 10 (IL-10) and CC chemokine ligand 5 (CCL5) in the granuloma formation of mycetoma [19]. Two SNPS in the promoter region of IL-10 and three SNPs in the promoter region of CCL5 were determined. Significant differences in allele distribution were demonstrated for one of the SNPs in the IL-10 promoter and two of the SNPs in the CCL5 promoter between mycetoma patients and healthy controls. Both IL-10 and CCL5 were found to be present in the mycetoma granuloma and secreted in the serum of patients. Since both, CCL5 and IL-10, play important roles in granuloma formation in general, it looks like the granuloma formation itself also plays an important role in the pathology of mycetoma.

Based on the results of these three genetic association studies performed in mycetoma patients, it can be concluded that, there are indeed subtle genetic differences between the Sudanese people who develop mycetoma and the people who don't. The exact role of these genetic differences should be further studied.

The role of sex-related hormone synthesis was studied because a clear male predominance was seen in mycetoma. In one study, 5 SNPs in 5 genes involved in sex hormone synthesis were studied. Significant differences in allele frequencies were found for catechol-O-methyltransferase (COMT) and cytochrome p450 subfamily 19 (CYP19). The genotypes more often obtained in mycetoma patients for COMT and CYP19 were previously described as influencing 17β-estradiol production. Also in the male mycetoma patients, significantly elevated serum levels of 17β-estradiol were found. In contrast, in these patients, also lowered levels of dehydroepiandrosteron (DHEA) were found. The differences in hormone levels noted between mycetoma patients and healthy controls did not directly affect the fungus itself. Indirect effects on the patients' hormone regulated immune states are the more likely explanations for mycetoma susceptibility [12].

As described above, SNPs in genes involved in the pathology of mycetoma can contribute to the risk of developing mycetoma once the causative agent is introduced into the subcutaneous tissue, but next to that it can also contribute to the extent of the disease. In the mentioned studies, it was noted that two of the SNPs found to be associated with the size of the mycetoma lesion, namely NOS2 and COMT.

12.6 Clinical Presentation

Male predominance is a constant finding in mycetoma with a sex ratio of 3:1. This is commonly attributed to the greater risk of exposure to organisms in the soil during the outdoor activities but many reports are questioning this. No age is exempted but mycetoma commonly affects adults between 15 and 30 years of age and these are the earning members of the society, especially in under developed countries. However, in endemic regions children and elderly people may also be affected [2, 4].

The clinical presentation of mycetoma is almost identical irrespective of the causal organism. However, the rate of progress is more rapid with actinomycetoma than with eumycetoma. In eumycetoma, the lesion grows slowly with clear defined margins and remains encapsulated for a long period, whereas, in actinomycetoma the lesion is not encapsulated, more inflammatory, more destructive and invades the bone at an earlier period [2, 4].

The characteristic triad, of a painless subcutaneous mass, sinuses and the presence of discharge containing grains are pathognomic of mycetoma. It presents as a slowly progressive painless subcutaneous swelling sometimes at the site of previous trauma. The swelling is usually firm and rounded but it may be soft, lobulated, rarely cystic and it is often mobile.

Multiple secondary nodules then evolve as well, the nodules may suppurate and drain through multiple sinuses tracts and these sinuses may close transiently after discharge during the active phase of the disease. Fresh adjacent sinuses may open while some of the old ones may heal completely. They are connected with each other, with deep abscesses and with the skin surface [2, 4].

The discharge is usually serous, serosanguinous or purulent. During the active phase of the disease, the sinuses discharge grains, the color of which depends on the causative organism. The grains can be black, yellow, white or red and they are of variable size and consistency. The black grains are usually due to *M. mycetomatis,* the red ones are due to *A. pelletierii*, the yellow are due to *Streptomyces somaliensis* and the white grains can be due to *A. madurae*. Pus, exudate, the dressing gauze and biopsy material should be examined for the presence of the grains.

Mycetoma is usually painless in nature, and it was suggested that produces substances that have an anesthetic action. At a late stage of the disease, the pain may become negligible due to nerve damage by the tense fibrous tissue reaction. Pain may be produced due to a secondary bacterial infection.

As the mycetoma granuloma increases in size, the skin over it becomes attached and stretched. The skin may become smooth, shiny and areas of hypo or hyperpigmentation may develop.

Local hyperhidrosis in the skin overlying the lesion is commonly seen in mycetoma. This corresponds with the hyperplasia and hypertrophy of sweat glands found in surgical biopsies. This can be explained by the increase in local temperature that is the result of increased blood flow to the area induced by the chronic inflammatory process. The increased local blood flow in mycetoma was confirmed by an

angiographic study, which showed dilated and tortuous terminal arterial branches, vascular blush and dilated veins proximal to the lesion.

Mycetoma eventually invades the subcutaneous tissue, fat, muscles and bone. This is usually gradual and delayed in eumycetoma while in actinomycetoma it is earlier and extensive especially in infections induced by *A. pelletierii.*

For unknown reasons, the tendons and the nerves are curiously spared until very late in the disease process, this may explain the rarity of neurological and trophic changes even in patients with long-standing mycetoma. The absence of trophic changes may also be explained by the adequate blood supply in the mycetoma area [2, 4].

In most patients, the regional lymph nodes are small and shotty. An enlarged regional lymph node is not uncommon and this may be due to secondary bacterial infection, genuine lymphatic spread of mycetoma or it may be due to immune complex deposition as part of a local immune response to mycetoma infection.

The infection remains localized and the constitutional disturbances are rare but when they do occur, they are generally due to secondary bacterial infection of the open sinuses tracts, fistula formation in some patients or generalized immunosuppression [20–35].

Cachexia and anemia may be seen in late mycetoma. This is often due to malnutrition, sepsis, and mental depression. Mycetoma can produce many disabilities, distortion and deformity. It can be fatal especially with cranial mycetoma [20–35].

Secondary bacterial infection commonly occurs in mycetoma. In material obtained by deep swabs and fine needle aspiration, 65% of patients have concomitant superficial bacterial infection; of these 56% are attributable to *Staphylococcus aureus*, 34% to *Streptococcus pyogenes* and 10% to *Proteus mirabilis.* The coexisting bacterial infections disturb the local environment and reduce the responses to various antifungal and antimicrobial agents. Elimination of coexisting infection results in better clinical response. [36].

It is interesting to note that in a recent study from a mycetoma endemic village in the Sudan, the villagers' knowledge of the disease was poor in 96.3% of them, 70% had appropriate attitudes and beliefs towards interaction with mycetoma patients and treatment methods, and 49% used satisfactory or good practices in the management of mycetoma. This indicates the need for objective and effective health education to improve awareness among affected communities [15].

12.7 Mycetoma Spread

In mycetoma, spread occurs locally and through the lymphatics thereby resembling sporotrichosis. Metastatic lesions may occur at various lymph node stations in which new satellites may develop. This phenomenon is more common in actinomycetoma, especially in cases with repeated surgery.

Hematological spread has also been described; *M. mycetomatis* and *A. pelletierii* grains have been detected in an intact blood vessel. In many spinal mycetoma, these

lesions occurred without involvement of the skin or surrounding tissues suggesting hematogenous spread [20–35].

12.7.1 Site of Mycetoma

The commonest site for mycetoma is the foot (79.2%), most of the lesions are seen on the dorsal aspect of the forefoot. Both feet are equally affected. The hand ranks as the second commonest site (6.6%), the right hand is more affected. This may imply a traumatic basis of the infection in this site [20–35].

In endemic areas, other parts of the body may be involved but less frequently and these include the knee, arm, leg, head and neck, thigh, and the perineum. Rare sites such as the chest and abdominal walls, fascial bones, mandible, paranasal sinuses, eyelid, vulva, orbit, scrotum and surgical incisions may be affected [20–35].

12.7.2 Differential Diagnosis

In endemic areas, subcutaneous masses should be considered as mycetoma until it proved otherwise. The clinical presentation, the etiological agent, and the prevalence of infectious diseases that can mimic mycetoma influence the differential diagnosis of mycetoma.

The differential diagnosis of mycetoma includes many of the soft tissue tumors such as Kaposi's sarcoma, fibroma, malignant melanoma, or fibrolipoma, as well as thorn granuloma. The radiological features of advanced mycetoma may be comparable to osteogenic sarcoma and bone tuberculosis. Primary osseous mycetoma is to be differentiated from chronic osteomyelitis, osteoclastoma, bone cysts, and from syphilitic osteitis [2, 3].

12.8 Mycetoma Diagnosis

In many endemic areas, due to diagnostic facilities scarcity, the disease diagnosis is often made clinically only, a practice, which may lead to the administration of inappropriate management.

The proper diagnosis of mycetoma needs the accurate identification of the causative organism and the disease extent. Various diagnostic tools are available for the precise identification of the organism and that includes the classical grain culture, histopathological and cytopathological techniques, serodiagnosis, as ELISA and CIE, and molecular techniques such as PCR. For the disease, extent numerous imaging techniques such as radiography, ultrasonography, CT, MRI are in use. It is

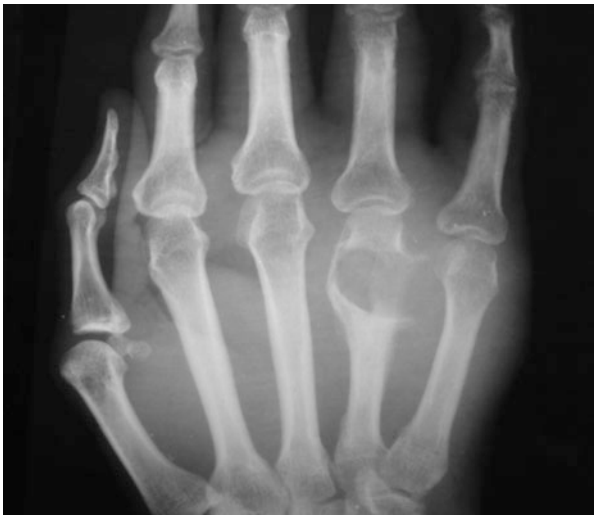

Fig. 12.3 X-ray of right hand showing massive soft tissue mass, periosteal reaction, and bone cavities in line with eumycetoma appearance

interesting to note most of these techniques are not available in majority of mycetoma endemic regions [37–42].

Presently, in endemic areas, it is recommended for diagnosis to start with lesion ultrasound examination and fine needle aspiration for cytology and cell blocks, to identify the causative organism; in cases where this is not successful, deep surgical biopsy is carried out for histological and mycological identification. For massive lesions, radiography and MRI are mandatory to determine the disease extent. Molecular and phenotyping assays are only performed in highly specialized laboratories.

12.9 Mycetoma Imaging

12.9.1 Radiology

A series of radiological changes are seen in mycetoma. This is due to the fact that all mycetoma agents are osteophilic and it may be due to the effect of the granuloma on both, the affected bone and its blood supply (Fig. 12.3).

In early disease, the radiography is essentially normal. Eventually with the development of the granuloma in size, a soft tissue mass with obliteration of fascial planes may be seen. The granuloma, is shown as a dense shadow or as scattered multiple soft tissue shadows. Calcification and obliteration of the fascial planes may sometimes be the disease progresses, the cortex may be compressed from outside by the granuloma leading to bone scalloping, this is followed by a variable amount of periosteal reaction. Periosteal new bone spicules are laid down at right angle to the

cortex to create a sun-ray appearance and Codman triangle, an appearance that may be undistinguishable from that due to osteogenic sarcoma [37–39].

Late in the disease, there may be multiple punched out cavities through the normal density of the bone. These cavities are large in size, few in number with well-defined margins in eumycetoma. Whereas, the bone cavities in actinomycetoma are usually smaller in size, numerous and have no definite margins. The cavities are produced by the replacement of the osseous tissue by the grains. Their size is due to the size of the grains of the causative organism. The cavities are usually filled with solid masses of grains and fibrous tissue, which provides bone support. This may explain the rarity of pathological fractures in mycetoma. The bony changes in the skull are unique: they are purely sclerotic with dense bone formation and loss of trabeculation [2, 3, 37–39].

Osteoporosis at, and distal to, the affected part is well observed in mycetoma and this may be due to disuse atrophy or due to the compression of the bone and its blood supply by the mycetoma granuloma.

Chemotherapy causes radiological improvement consisting of remoulding, absorption of the sclerotic bone and reappearance of the normal trabecular pattern.

In a retrospective study of 516 patients seen at the Mycetoma Research Centre, Sudan, a tertiary center, only 3% had a normal radiograph. The most common abnormalities were soft tissue swelling (93%), bone sclerosis (56%), and bone invasion (46%). Other abnormalities included bone cavities (32%) and osteoporosis (19%) [39].

12.9.2 Ultrasonic Imaging of Mycetoma

The mycetoma lesions, its capsule and the accompanying inflammatory granuloma have characteristic ultrasonic appearances (Fig. 12.4). Ultrasound imaging can differentiate between eumycetoma and actinomycetoma and between mycetoma and other non-mycetoma lesions. In eumycetoma lesions, the grains produce numerous sharp bright hyperreflective echoes, which are consistent with the black grains. The grain cement substance is most probably the origin of these sharp echoes. In addition, there are multiple thick-walled cavities with absent acoustic enhancement. In actinomycetoma lesions, the findings are similar but the grains are less distinct. This may be due to their smaller size and consistency, individual embedding of the grains or the absence of the cement substances in few of them [40].

The ultrasonic diagnosis of mycetoma is more precise and accurate in lesions with no sinuses. The extent of the lesion can be accurately determined ultrasonically and this is useful in planning surgical incisions and procedures.

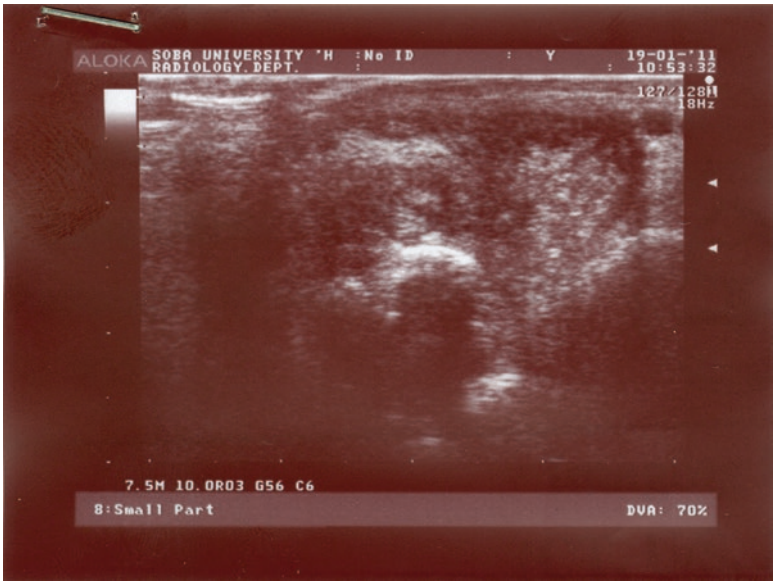

Fig. 12.4 Ultrasound showing typical eumycetoma lesion; multiple thick-walled cavities with sharp hyper-reflective echoes denoting grains without acoustic enhancement

12.9.3 Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) is useful for determining the extent of the lesions and the invasion of structures (Fig. 12.5). [41] It has greater sensitivity than radiographs, ultrasound, and CT.

MRI usually shows multiple 2–5 mm lesions of high signal intensity that indicates the granuloma, interspersed within a low-intensity matrix denoting the fibrous tissue. The 'dot in circle' sign which indicates the presence of grains, is highly characteristic.

A MRI grading system; the Mycetoma Skin, Muscle, Bone Grading System (MSMBS), was recently proposed, it grades disease severity, compares patients and helps to manage them. The MRI appearance should be differentiated from chronic osteomyelitis, granulomas, soft tissue tumors, bone tuberculosis, and cold abscesses. This procedure, however, is only possible in well-equipped hospitals.

In general, for small early lesions, ultrasound examination is recommended, meanwhile, radiography and MRI are recommended for large lesions [41].

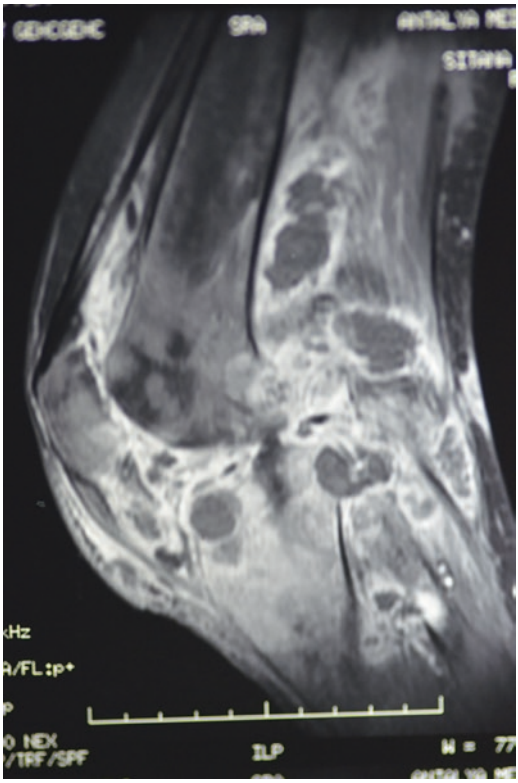

Fig. 12.5 MRI showing involvement of soft tissue and bone by mycetoma and the *dot-in-circle* sign which is characteristic of mycetoma

12.9.4 CT Scan

CT findings in mycetoma are not specific but are helpful to detect early bone involvement. Helical computed tomography is superior to plain CT scanning as it allows three-dimensional reconstruction and more precise information on the degree of organ involvement. It also allows vascular involvement to be visualized.

12.9.5 Organism Identification

Identification of the agent is important to guide treatment. Goodfellow and colleagues have described a stepwise procedure from the collection of specimens through to the isolation and identification of established causal agents of actinomycetoma [44].

To identify the causative agent, grains need to be isolated from the lesion. Grain material can be obtained by surgical excision or by extraction with a cotton swab from the sinuses or by fine needle aspiration (FNA). Deep-seated grains obtained by

surgical excision are preferred over those extruded through sinuses because the latter are often inviable and contaminated [44].

A first indication of the causative agent is obtained by looking at the grain itself: the size, shape, color and consistency help to identify the organism; however, causal agents must be isolated for definitive identification. In direct examination, grains are mounted on a slide and crushed under a cover glass. The size of the filaments, septation, morphological characteristics and pigment formation can be used to differentiate between actinomycetoma and eumycetoma.

In actinomycetoma, fine filaments are seen and can be stained with Gram staining. In eumycetoma, the filaments stain with Periodic Acid Schiff (PAS). Grains are plated onto appropriate culture media and incubated for at least 6 weeks. Identification based on colony morphology can be difficult, due to a large variety of colony types, and to the fact that many species resemble each other. Lack of conidiation can further complicate matters.

12.9.6 Histopathological Identification

Stained sections usually show the grain morphology and the tissue reaction to the organisms. Since it does not require aseptic procedure or the rigid time schedule required for culture, this technique is attractive; however, it lacks the precision of culture [45–49]. Histopathological identification requires a deep biopsy with the risk of disease spread. It is less reliable than culture and does not allow differentiation between most species. This tool has been used as the sole identification tool in many centers in the past decades.

Various staining procedures are in use and that include H&E, PAS and Gram. Still, there are many difficulties in identifying causative agents based on histology alone, particularly for *Nocardia* spp. It is known that, it is practically impossible to define *Nocardiae* to the species level due to their similar appearance [50].

In eumycetoma, differentiation between *Acremoniumspp, Fusariumspp* and *Pseudallescheriaboydii,* and between *Exophialajeanselmei, Leptosphaeria senegalensis, Leptosphaeriatompkinsii, Nigrogranaromeroi,* and *Madurella griseais* is also difficult. Furthermore, other fungi, such as *M. mycetomatis*, can present multiple grain types in histological slides, complicating the identification process even more.

The inflammation reaction around the grain is variable. Three types of tissue reaction can be seen. In type I tissue reaction, there is a zone of neutrophils in the vicinity of the grain. Some histiocytes may also be seen among the neutrophils, but they are more numerous outside the neutrophil zone [45–49].

In type II tissue reaction, the neutrophil zone is absent, instead a layer of histiocytes and multinucleated giant cells is seen. Some of these giant cells contain fragments of grain or pigmented cement substance. At this stage, the grain is usually small and fragmented [45–49].

The most uncommon reaction is the type III tissue reaction, in which the grain material has largely or completely disappeared. This leaves a compact epithelioid granuloma with or without Langerhans giant cells.

12.9.7 Fine-Needle Aspiration Cytology of Mycetoma

Mycetoma can be accurately diagnosed in smears obtained by fine-needle aspiration (FNA) [42, 43]. Mycetoma lesion has a distinct appearance in a cytology smear characterized by the presence of polymorphous inflammatory cells consisting of an admixture of neutrophils, lymphocytes, plasma cells, histiocytes, macrophages, and foreign body giant cells and grains.

In sections, the grain is closely surrounded by, and occasionally infiltrated by, neutrophils causing its fragmentation. Outside the neutrophil zone, monocytic cells and giant cells are seen. This is surrounded by granulation tissue rich in fibroblasts and blood vessels [42, 43].

The technique allows morphological identification of mycetoma and its classification into eumycetoma and actinomycetoma, this is important as the treatment depends mainly on the etiological agents.

The technique is simple, cheap, rapid, sensitive, and can be tolerated by patients. It can be used not only in routine diagnosis, but can be used as an effective mean in collection of material for culture and immunological studies. Due to the simplicity of the technique, it can be used in epidemiological survey of mycetoma and for detection of early cases, in which the radiological and serological techniques may not be helpful [42, 43].

12.10 Culture

A large variety of microorganisms are capable of producing mycetoma. They can be identified by their textural description, morphological and biological activities in pure culture [44, 50].

The grains are the source of the culture and they should be alive and free of contaminants. For successful culture, a deep-seated surgical biopsy specimen is preferred to collect grains discharged with pus through the sinuses. The specimen obtained is divided into two portions. One portion is used for direct microscopy, the other for culture [44, 50].

Direct microscopy can be performed by examination of crushed grains in 10% KOH. Black grains usually are composed of hyphae of approximately 15 μm in width.

For culture, grains are washed in saline buffer containing chloramphenicol, grown on blood agar and Sabouraud dextrose agar and incubated at 37 °C and 26 °C

for 6–8 weeks. Identification of isolates is achieved by observing the rate of growth, colony morphology, production of conidia, and assimilation patterns.

Culturing of fungi remains troublesome, often no growth is obtained or cultures are contaminated with bacteria. Identification of the various fungi responsible for black-grain mycetoma remains difficult with standard mycological procedures, since these fungi rarely produce conidia. Misidentification, therefore, often occurs. Furthermore, most of these fungi have low growth rates, which may delay the identification until up to 12 weeks.

Many culture media are in use, e.g., Sabouraud, blood agar, and Malt extract agar.

The culture technique is cumbersome, time-consuming, and chance contamination may give a false positive result. It also requires experience to identify the causative organisms [44, 50].

12.11 Serodiagnosis in Mycetoma

In the absence of the classical triad of mycetoma, the demonstration of significant antibodies titers against the causative organism may be of diagnostic value. Serodiagnosis is of a great help in identification and classification of the various organisms, which is an essential prerequisite for medical treatment, and is mandatory for the follow-up of these patients [51–54].

It has many advantages over the culture and histopathological techniques, as both require surgical biopsy, which may enhance the spread of the organism. The common serodiagnostic tests for mycetoma are the counter-immuno-electrophoresis (CIE) and Enzyme-linked immunosorbent assay (ELISA). CIE distinguishes between eumycetoma and actinomycetoma, and between Actinomycetes themselves; but cross reactivity between *A. madurae* and *A. pelletierii* is very common. However, it is time-consuming and the preparation of the antigens take a considerable time [51–54].

ELISA was used in the diagnosis of mycetoma. It appeared to be a sensitive test for the detection of antibodies; however, the high sensitivity of the test makes the cross reactivity unavoidable. ELISA may be a useful tool in community studies, as sero-epidemiological surveys could give valuable information on the distribution and prevalence of exposure to mycetoma.

Recombinant based ELISA performed with pure antigens of *M. mycetomatis,* had detected translationally controlled tumor protein (TCTP) and luminex assays based on TCTP, fructose-bisphosphatealdolase (FBA) and pyruvate kinase (PK). Although patients had higher antibody levels, antibodies were detected in healthy controls, making these techniques unsuitable as a diagnostic tool.

It is clear that these serodiagnostic tests have many limitations that include tedious and lengthy preparation of antigens, the antigens are crude and not standardized hence cross reactivity between different mycetoma causative organisms is found.

12.12 Organism Biological Activity Identification

The causative organism biological activity may include acid fastness, optimal temperature, proteolytic activity, utilization of sugars, and nitrogenous compounds.

These biological activities, while effective in distinguishing between genera of actinomycetes, are laborious and time-consuming and are being complemented and replaced by molecular systematic procedures, notably by 16S rRNA gene sequencing studies [44, 55, 56].

12.12.1 Molecular Diagnosis

Polymerase Chain Reaction (PCR) can be used for molecular typing of the causative agents. Different techniques are in use, which include restriction endonuclease analyses (REA), random amplified polymorphic DNA (RAPD), and amplification fragment length polymorphism (AFLP) [57–60].

For *M. mycetomatis*, a species-specific PCR procedure was developed. This PCR-RFLP analysis showed strict homogeneity between *M. mycetomatis* isolates and can be used to identify the causative agent in clinical material and also from soil and thorn samples [57].

Although results with RAPD are variable, REA and AFLP were able to differentiate between *M. mycetomatis* isolates from different countries, or even within a country.

Certain AFLP types were associated with the origin of the strain or the size of the lesion.

For eumycetoma, various molecular techniques have been used to identify causative agents and all of them are based on the identification of the internal transcribed spacer (ITS). To identify all fungal mycetoma causative agents, the ITS regions are usually amplified with pan-fungal primers and sequenced. Identification is based on comparing the resulting sequence with sequences previously submitted to Genbank [57, 61].

Using this approach, multiple studies have shown that a number of causal agents for eumycetoma are underspeciated, as exemplified by the identification of three new *Madurella* species; *Madurella fahalii, Madurella pseudomycetomatis* and *Madurella tropicana*, and *Pleurostomophoraochracea* [62, 63].

All of these tests are expensive, are not field friendly, nor are available in endemic areas.

12.13 Management

The management of this distressing and devastating disease is disappointing. The treatment depends mainly on its etiological agent, the site and extent of the disease. Until recently, the only available treatment for mycetoma was amputation or multiple mutilating, disfiguring surgical excisions. Combined medical treatment in the form of antifungals for the eumycetoma, and antibiotics and antimicrobial agents for actinomycetoma, and various surgical excisions is the gold standard in mycetoma [64–70].

Reports on medical treatment in eumycetoma are scarce and inadequate. Over the years and till now, the treatment of eumycetoma was based on personal clinical experience, and on the results of sporadic case reports, rather than controlled clinical trials. In general, massive surgical excisions or amputation of affected part is the treatment of choice.

Various antifungal agents have been tried with little success. This is perhaps surprising, as the eumycetoma causative agents are low-grade infective organisms and their eradication should be readily achieved by the administration of safe systemically antifungal drugs. Local administration of mycetoma chemotherapeutic agents was used, but the results were not impressive with a high rate of failure and complications.

For eumycetoma, surgery is always needed. The aim of surgery is complete excision of the lesion. This is only possible when the mycetoma lesion is small and well encapsulated. In larger lesions, only reduction of the amount of infected tissue is possible, and occasionally, multiple surgeries are needed to excise most of the lesion. Especially in large lesions, effective surgery needs aggressive excision or debridement under general anesthesia, which usually cripples the limb or leads to permanent disability due to mandatory amputation. To prevent recurrent infections, surgery is always combined with antifungal treatment, both before and after the procedure. Antifungal therapy for eumycetoma still depends mainly on the azole antifungals, and itraconazole is the drug of choice. The duration of treatment varies with the severity of the infection and the general health status of individual patients. Treatment may need to be continued for 18–24 months or more, and the liver function of the patients' needs regular monitoring, especially with long-term itraconazole treatment in cases of advanced mycetoma. However, long-term treatment may lead to antifungal resistance, which may in turn complicate patient management. Itraconazole is implicated in encapsulating the mycetoma lesion. This renders final surgical treatment a likely option, but in advanced lesions, especially when the bone tissue is involved, the response to chemotherapeutic treatment still remains very poor. In the search for new, alternative antifungal strategies in the treatment of mycetoma, terbinafine has been tried in the treatment of eumycetoma. Recently, the effect of posaconazole was studied and it showed some success [64–67].

Generally, actinomycetoma is amenable to medical treatment with antibiotics and other chemotherapeutic agents. Combined drug therapy is always preferred to a single drug, to avoid drug resistance and for disease eradication. Cure is possible,

although a prolonged period of treatment is needed. Combined medical and surgical treatments facilitate surgery, accelerate healing and reduce the chance of relapse. However, a good number of patients respond to medical treatment alone [67–70].

There is more information on the treatment of actinomycetoma, although well-controlled comparative studies are lacking. In the 1960s, trimethoprim-sulfamethoxazole (TMP-SMX) became the gold standard for treatment, replacing sulphonamides. It was used as therapy in early lesions and has a cure rate of 60%. In the early 70s, for extensive lesions, streptomycin was added as a second drug, achieving a cure rate of 63% and a varying degree of improvement in the remaining patients [71].

Streptomycin was later replaced by amikacin, an aminoglycoside that is effective in vitro, in vivo, and clinically against *N. brasiliensis* mycetoma. In extensive and unresponsive cases, TMP-SMX 8/40 mg/kg/day in cycles for 5 weeks and amikacin 15 mg/kg/day in a divided dose every 12 h for 3 weeks are administered. The two-week interval of amikacin in the five-week cycle is used for renal and audiometric monitoring. This is currently the first line of treatment in many centers [71]. This regime has been proven effective (90%) in the cure of severe cases, if adverse effects do not occur. It is [71] important to maintain treatment if there are no adverse effects until cure or remission of the infection is achieved, which is generally obtained after 10–25 weeks. In case of resistance or allergy to TMP-SMX and amikacin, amoxicillin-clavulanic acid and netilmicin, respectively, may be used as alternatives. Amoxicillin-clavulanic acid has been used as rescue therapy with good results in some cases [71]. It can also be used alone during pregnancy; however, acquired resistance may develop, and it is generally not effective against *A. madurae*. Amikacin combined with a carbapenem, such as imipenem or meropenem, may also be used in refractory cases. Surgery is rarely required. It is recommended to determine the MICs to carbapenems because of resistant strains and the cost of these drugs [71].

12.13.1 Surgery for Mycetoma

Surgery in mycetoma is indicated when the disease process is limited and localized, cases resistant to medical treatment, or as a lifesaving procedure for advanced disease complicated by secondary bacterial infection, massive bone involvement, and poor general condition.

The goal of surgery is the complete removal of the lesion or the reduction of size, followed by medical treatment. Eumycetoma is well encapsulated and great care must be exercised not to rupture the capsule, which may lead to recurrence by transferring the fungal element into other parts of the operative field [72].

Actinomycetoma has an ill-defined border; therefore, a margin of healthy tissue should always be excised with the lesion. Simple bone curettage and soft tissue excision is recommended for localized bony lesions.

A bloodless operative field using a tourniquet is mandatory to identify margins of the lesion to avoid rupturing it. It is advisable to flood the wound at the end of surgery with tincture of iodine and hydrogen peroxide to clean the surgical field from left out grains, especially if there is doubt about the completeness of the excision or contamination of the field [72].

The wound can be closed primarily or by delayed primary sutures and in many cases skin grafts may be required. The open postoperative wounds are usually dressed with iodine and hydrogen peroxide.

In general, in mycetoma, advanced cases of mycetoma not responding to medical treatment for a prolonged period nothing short of amputation is likely to succeed. The amputation rate ranges from 25 to 50%. Extensive repeated excisions of the diseased tissue, including bone may be carried out several times to avoid the social consequences of amputation. This debulking procedure must be coupled with chemotherapy. In less advanced cases, less mutilating surgery is advised, for example, toe, mid tarsal, or Syme's amputation. However, in many cases of inadequate surgery, recurrence is inevitable and radical amputation is the only treatment.

Recurrence is more common after an incomplete or irregular course of medical treatment. With drug incompliance, there is a good chance for the organism to develop drug resistance [72].

Medical treatment for both types of mycetoma must continue until the patient is clinically, radiologically, ultrasonically, and cytologically cured. Cure is considered when the skin becomes normal, the mass disappears, the sinuses heal and the organisms are eliminated from the tissue. Clinical improvement is judged by reduction in the size of the mass and healing of most of the sinuses.

Radiological examination is an essential tool for follow-up of patients on medical treatment. It usually shows reappearance of normal bone pattern and the disappearance of the soft-tissue mass. Absent grains cytologically with type III tissue reaction and the disappearance of the grains and cavities ultrasonically are reliable evidences for cure.

Mycetoma has many serious medical and socioeconomic impacts on patients, community and health authorities. The postoperative recurrence rate varies from 25 to 50%. The available treatment although not very effective, has many serious side effects, they are expensive and it may amount to more than US$3000 per year per patient with eumycetoma. Most of the patients cannot afford to have that and the drugs are not always available in endemic regions. The treatment is of a variable duration and may lapse for 2–3 years with a mean of 18 months [72].

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Chapter 13
Dermatophytes and Dermatophytosis

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Abstract Dermatophytosis, are the most common fungal infection worldwide. Transmission is mostly by direct contact with infected animals, humans or contact with fomites. Clinical features vary according to the etiological agent. Dermatophytes belong to the genera Microsporum, Trichophyton, Epidermophyton (anamorphic state), and Arthroderma (teleomorphic state). The main etiological agents in humans are T. rubrum, T. tonsurans, T. mentagrophytes complex, and M. canis, M. gypseum, and E. floccosum. Recent phylogenetic studies indicate the existence of a fourth genus, Chrysosporium. In this chapter we analyze epidemiology, biology, clinical forms, pathogenesis, diagnosis, detection methods and treatment. Also molecular taxonomy, molecular epidemiology, molecular techniques, molecular identification and treatment are widely analyzed.

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13.1 Introduction

Dermatophytosis, also known as *tinea* or ringworm, has become the most common fungal infection worldwide. It is caused by a group of keratinophilic fungi that affect the skin, hair, and nails, and is of both public and veterinary health concern [1]. These fungal infections are rarely associated with a fatal outcome; regardless, they are associated with high morbidity [2]. The transmission of dermatophytosis is mostly by direct contact with infected animals or humans or by indirect contact with fomites. Clinical cases vary according to the etiological agent (species) and anatomical location. Symptoms can be mild or severe based on the immunological status of the host; subcutaneous or internal organ infections are usually rare. The typical lesions resulting from skin infection are presented in the form of an erythematous, flaky, itchy, circular plaque as a direct result of fungal infection, indicating a hypersensitive reaction to the fungi per se or to their metabolites [3]. This condition affects a disproportionate number of infants, young adults, and people of low economic status; likewise, the distribution of these fungi can vary considerably, depending on the geographical area of origin and other epidemiological factors such as age, gender, and seasonality [4, 5].

Dermatophytes belong mostly to the genera *Microsporum*, *Trichophyton*, *Epidermophyton* (in the anamorphic state), and *Arthroderma* (in the teleomorphic state), and so far they include 40 known species responsible of infections in humans and other mammals [6]. The fungal species *T. rubrum*, *T. tonsurans*, and the *T. mentagrophytes* complex, as well as *M. canis*, *M. gypseum*, and *E. floccosum*, are considered the main etiological agents of dermatophytosis in humans [5]. Based on their ecology, dermatophytes have been classified into three groups: anthropophilic, zoophilic, and geophilic. Anthropophilic dermatophytes are associated with humans, mainly causing *tinea capitis*, *tinea corporis*, *tinea pedis,* and *tinea unguium,* and they rarely infect animals. Zoophilic species are common pathogens in animals [5, 7, 8], although they can occasionally infect humans too. Geophilic dermatophytes are associated with keratinized materials (hair, feathers, nails, and horns) scattered throughout the environment [5]. These fungi, which are able to degrade the keratin from hair, nails, and feathers, can also produce superficial infections (dermatophytosis) in the skin of human and animal hosts and penetrate into deeper tissues in immunocompromised hosts [9].

13.2 Classification

The sexual stages of these organisms are the basis of the taxonomy and nomenclature of these fungi. To date, the anamorphic states of dermatophytes (*genera Epidermophyton, Microsporum*, and *Trichophyton*) pertain to the *class* Hyphomycetes, *phylum* Deuteromycota, and teleomorphs (most zoophilic and geophilic species of *Microsporum* and *Trichophyton*) are classified within the teleomorphic *genus Arthroderma*, *order* Onygenales, *phylum* Ascomycota [10].

Based on their ecology, dermatophytes have been divided into three groups: anthropophilic, zoophilic, and geophilic; their members are included within the *genera Trichophyton*, *Microsporum, Epidermophyton*, and *Arthroderma*. Based on the morphology of anamorphic states, Simpanya [11] considers two species within the *genus Epidermophyton*, approximately 18 species in *Microsporum*, and 25 species in *Trichophyton.* Dermatophytes included in these *genera* are closely related phylogenetically and taxonomically, and therefore, their phylogeny is not yet clear. Since the morphological characteristics of these fungi are rather limited to facilitate their identification, Gräser et al. [8] recommend a polyphasic approach involving, in addition to phenotypic identification, other characteristics such as physiological and biochemical traits and the production of secondary metabolites, among others, along with universally applied molecular methods, to achieve an accurate identification.

Thus, a report by Molina de Diego [10] considers two species within the *genus Epidermophyton*: *E. floccosum* and *E. stockdaleae*, among which only the former is pathogenic for humans; 16 species within the *genus Microsporum* (*M. amazonicum, M. audouinii, M. boullardii, M. canis* complex, *M. cookei, M. ferrugineum, M. equinum, M. fulvum, M. gypseum, M. gallinae, M. nanum, M. praecox, M. persicolor, M. racemosum, M. vanbreyseghemii*, and *M. ripariae*), of which only 10 are pathogenic for men; and 31 within the *genus Trichophyton* (*T. ajelloi, T. concentricum, T. equinum, T. eboreum, T. erinacei, T. fischeri, T. flavescens, T. fluviomuniense, T. gallinae, T. gloriae, T. gourvilii, T. interdigitale, T. kanei, T. krajdenii, T. longifusum, T. megninii, T. mentagrophytes* complex, *T. phaseoliforme, T. quinckeanum, T. raubitschekii, T. rubrum, T. sarkisorii, T. schoenleinii, T. simii, T. soudanense, T. terrestre, T. tonsurans* complex, *T. vanbreuseghemii, T. verrucosum, T. violaceum,* and *T. yaoundei*), with 10 species that are pathogenic for humans.

However, molecular methods have provided a large contribution toward improving knowledge regarding the biological diversity of dermatophytes, although it has also raised challenges in terms of their taxonomic classification. Cafarchia et al. [12] mention that most phylogenetic reconstructions from DNA sequences support the separation of geophilic species from the remaining members of the *family* arthrodermataceae, known as the "true" dermatophytes. Moreover, the scarce available information on the mating of some species have underscored the importance of phylogenetic studies for defining *species*, which have led to a reduction in the number of species or varieties of dermatophytes. Based on the classification proposed by Gräser et al. [8], Cafarchia et al. [12] consider five species in the *Arthroderma otae* complex (*M. canis, M. equinum, M. distortum, M. audouinii*, and *M. ferrugineum*); 10 species in the *A. vanbreuseghemii* complex (*T. tonsurans, T. equinum, T. interdigitale, T. mentagrophytes* var. *mentagrophytes*, *T. mentagrophytes* var. *granulosum*, *T. verrucosum* var. *autotrophicum, T. mentagrophytes* var. *goetzii*, *T. mentagrophytes* var. *interdigitale* and var. *nodulare*, and *T. krajdenii*); six species in the *A. simii* complex (*T. simii, T. mentagrophytes, T. mentagrophytes* var. *quinckeanum*, *T. langeronii*, *T. sarkisovii*, and *T. schoenleinii*); six species in the *A. benhamiae* complex (*T. erinacei, T. mentagrophytes* var. *granulosum, T. verrucosum, T. concentricum, T. bullous*, and *T. eriotrephon*); and 11 species in the *T. rubrum* complex

(*T. rubrum, T. raubitschekii, T. soudanense*, *T. gourvilii*, *T. megninii, T. fischeri*, *T. kanei*, *T. raubitschekii, T. violaceum, T. yaoundei*, and *T. glabrum*).

However, recent phylogenetic studies indicate the existence of a fourth *genus*, *Chrysosporium*, which is congruent with the ecological groups based on studies of mitochondrial DNA or ribosomal RNA-coding DNA (mtDNA or rDNA) [13].

13.3 Epidemiology

Simpanya [11] mentions that some dermatophyte species evolved from their natural habitat in soil and developed an affinity toward a specific host, with the latter mostly attributed to differences in the keratin of each host.

Likewise, the severity of an infection caused by different species of the *genera Epidermophyton*, *Microsporum*, and *Trichophyton* depends largely on the pathogenic potential of the invading species, socioeconomic factors, and the local environmental conditions. Nevertheless, it is generally acknowledged that between 15 and 20% of the global population is infected [14].

The epidemiology of dermatophytes has been modified during the last 70 years; a review by Seebacher et al. [15] mentions that before the Second World War, in Germany, *M. audouinii* and *E. floccosum* were the main causative agents of dermatophytosis, whereas from the 1950s onwards, *T. rubrum* has become the most common dermatophyte, representing 80–90% of the cases of infection, followed by *T. mentagrophytes*. This switch also occurred in Central America and Northern Europe in association with the incidence of *tinea pedis*. In contrast, in Southern Europe and Arab countries, zoophilic dermatophytes such as *M. canis* or *T. verrucosum* are more frequent. Moreover, this review also mentions that the infection incidence of *M. canis* in Mediterranean countries has increased dramatically during the past few years; this dermatophyte is commonly associated with *tinea capitis* in infants. Table 13.1 shows the most recent epidemiological data (2013–2016) on dermatophytosis worldwide.

In addition, an extensive review by Sigurgeirsson and Baran [41] on the global incidence of onychomycosis reveals that the average prevalence of this ailment in Europe and North America was 4.3% in studies based on the general population and 8.9% in studies based on nosocomial data. Both studies demonstrated that onychomycosis is more common in toenails and more frequent in males, and the main causative agent was *T. rubrum* (65.0%).

Furthermore, *tinea pedis*, which can be a chronic or recurrent infection that involves interdigital spaces or other regions of the feet, is often caused by anthropophilic etiological agents, including *T. rubrum sensu stricto*, the most common agent, followed by *T. interdigitale* and *E. floccosum*. Notwithstanding, Ilkit and Durdu [42] consider the current situation and the changing patterns of *tinea pedis* to necessitate revision beginning from the second half of the twentieth century, during which a global increment in this mycosis and clonal propagation of *T. rubrum* were observed. They mention that these phenomena are probably due to increased urbanization, the use of sport and physical conditioning facilities, the growing prevalence of obesity, and aging.

Table 13.1 Epidemiological data on dermatophytosis worldwide

Continent	Clinical form Frequency (%)	Etiological agents Frequency (%)	Age	Sex Frequency (%)	References
America					
Brazil	*Tinea corporis* (31.81) *Tinea capitis* (31.81) *Tinea unguium* (15.15) *Tinea pedis* (12.12) *Tinea cruris* (9.09)	*T. mentagrophytes* (40.0) *T. rubrum* (30.0) *T. tonsurans* (10.0) *T. verrucosum* (5.0) *M. gypseum* (5.0) *M. canis* (5.0) *E. floccosum* (5.0)	1–20	Male (42.42) Female (57.57)	Silveira-Gomes et al. [16]
Brazil	*Tinea unguium* (62.26) *Tinea pedis* (25.42) *Tinea corporis* (6.26) *Tinea cruris* (3.42) *Tinea manuum* (1.65) *Tinea capitis* (0.99)	*T. rubrum* (96.9) *T. mentagrophytes* (3.0) *M. gypseum* (0.9) *T. tonsurans* (0.6) *E. floccosum* (0.5) *M. canis* (0.4)	0 > 60	Male (29.0) Female (71.0)	Di Chiacchio et al. [17]
Brazil	*Tinea unguium* (100)	*T. rubrum* (68.6) *T. mentagrophytes* (6.8) *Trichophyton* spp. (3.2) *T. tonsurans* (2.5) *T. raubistscheckii* (0.1) *M. gypseum* (0.2) *E. floccosum* (0.1)	1–100	Male (34.0) Female (66.0)	de Carvalho Ribeiro et al. [18]
Chile	*Tinea unguium* (95.24) *Tinea pedis* (4.76)	*T. rubrum* (59.45) *T. mentagrophytes* (40.54)	ND	Male (40.32) Female (59.68)	Díaz Jarabrán et al. [19]
Brazil	*Tinea unguium* (48.5) *Tinea pedis* (33.1)	*T. rubrum* (59.6) *T. interdigitale* (34) *T. tonsurans* (0.9) *M. canis* (2.6) *E. floccosum* (2.5) *M. gypseum* (1.3)	1–98	Male (79.3) Female (53.9)	Heidrich et al. [20]

(continued)

Table 13.1 (continued)

Continent	Clinical form Frequency (%)	Etiological agents Frequency (%)	Age	Sex Frequency (%)	References
Europe					
Greece	*Tinea unguium pedum* (42.2) *Tinea pedis* (41.4) *Tinea corporis* (5.6) *Tinea manuum* (4.1) *Tinea unguium manuum* (3.0) *Tinea capitis* (2.4) *Tinea cruris* (1.3)	*T. rubrum* (54.2) *T. mentagrophytes* (37.9) *E. floccosum* (3.1) *M. canis* (3.0)	20–78	Male (54.91) Female (45.08)	Budak et al. [21]
Italy	*Tinea capitis* (67.92) *Tinea corporis* (28.30 *Tinea unguium* (3.77)	*T. violaceum* (43.39) *T. soudanense* (56.69)	2–43	Male (44.23) Female (55.76)	Farina et al. [22]
Italy	*Tinea unguium* (14.2)	Dermatophytes	1–60	Male (41.09) Female (53.83)	Papini et al. [23]
France	*Tinea unguium* (37.1) *Tinea pedis* (21.6) *Tinea corporis* (27) *Tinea manuum* (2.4) *Tinea unguium manuum* (7.36)	*T. rubrum* (78.6) *T. interdigitale* (9.5) *T. tonsurans* (1.8) *T. soudanense* (1.4) *M. audouinii* (0.8) *T. violaceum* (0.6) *E. floccosum* (0.4)	15–70	Male (52.7) Female (47.3)	Faure-Cognet et al. [24]
Greece	*Tinea corporis* (16.1) *Tinea capitis* (3.6) *Tinea unguium manuum* (16.7) *Tinea unguium pedum* (63.6)	*T. rubrum* (53.9) *T. mentagrophytes* (6.9) *T. interdigitale* (10.8) *M. canis* (22.5)	2–85	ND	Nasr et al. [25]

Africa					
Cameroon	*Tinea capitis* (3.6)	*T. soudannense* (56.8) *T. schoenleinii* (2.7) *T. violaceum* (2.7) *T. tonsurans* (2.7) *M. canis* (2.7)	4–15	Male (63.7) Female (36.3)	Kechia et al. [26]
Senegal	*Tinea unguium* (37.92)	*T. rubrum* (53.6) *T. interdigitale* (26.1)	0–82	Male (28.2) Female (71.8)	Seck et al. [27]
Senegal	*Tinea capitis* (34.51)	*T. soudanense* (56.18) *T. rubrum* (18.37) *M. langeronii* (12.72) *M. canis* (6.36) *T. mentagrophytes* (4.60).	1–83	Male (17.13) Female (82.86)	Ndiaye et al. [28]
Kenya	*Tinea capitis* (81.3)	*Trichophyton* spp. (61.3) *Microsporum* spp. (13.3) *Epidermophyton* spp. (7.3)	3–14	Male (59.3) Female (40.7)	Ndunge Moto et al. [29]
Mali	*Tinea capitis* (39.3)	*M. audouinii* (ND) *T. soudanense* (ND)	6–15	Male (48.47) Female (51.52)	Coulibaly et al. [30]
Morocco	*Tinea capitis* (64.67)	*M. canis* (63.26) *T. violaceum* (26.51) *T. mentographytes* (4.81) *M. langeronii* (3.01) *T. verrucosum* (1.8) *T. schoenleinii* (0.61)	0–32	Male (63.88) Female (36.12)	El Mezouari et al. [31]
Asia					
Iran	*Tinea unguium* (44.2)	*T. mentagrophytes* (17.7) *T. rubrum* (1.7) *E. floccosum* (0.7) *T. violaceum* (0.2) *T. verrucosum* (0.2) *T. tonsourans* (0.2) *M. gypseum* (0.2%)	0–70	Male (37.1) Female (62.9)	Afshar et al. [32]

(continued)

Table 13.1 (continued)

Continent	Clinical form Frequency (%)	Etiological agents Frequency (%)	Age	Sex Frequency (%)	References
Israel	*Tinea unguium pedum* (89.6) *Tinea unguium manuum* (33.4)	*T. rubrum* (38.2) *T. mentagrophytes* (5) *T. tonsourans* (1) *T. violaceum* (ND) *T. terrestre* (ND)	0–60	Toenails: Male (57.8) Female (42.2) Fingernails: Male (74.0) Female (26.0)	Segal et al. [33]
Iran	*Tinea unguium* (56.4)	*T. mentagrophytes* var. *interdigitale* (75) *T. rubrum* (25)	0–60	Male (29.3) Female (70.7)	Soltani et al. [34]
India	*Tinea corporis* (78.0) *Tinea cruris* (10.0) *Tinea manuum* (2.5) *Tinea faciei* (1.8) *Tinea pedis*(0.7) *Tinea unguium* (6.7)	*T. rubrum* (79.0) *T. mentagrophytes* (14.5) *M. canis* (3.2) *M. gypseum* (3.2)	22–45	Male (56.0) Female (443.0)	Lakshmanan et al. [35]
China	*Tinea unguium* (28.55) *Tinea capitis* (15.64) *Tinea pedis* (15.06) *Tinea cruris* (10.33) *Tinea corporis* (8.75) *Tinea manuum* (5)	*T. rubrum* (56.24) *T. mentagrophytes* (13.35) *M. canis* (10.19) *T. violaceum* (ND) *T. tonsurans* (ND) *M. gypseum* (ND) *E. floccosum* (ND)		*Tinea unguium* Male (23.8) Female (34.73) *Tinea capitis* Male (15.58) Female(15.76) *Tinea pedis* Male (14.54) Female (15.76) *Tinea cruris* Male (16.88) Female (2.25) *Tinea corporis* Male (10.65) Female (6.43) *Tinea manuum* Male (2.08) Female (3.86)	Cai et al. [36]

Iran	*Tinea corporis* (32.0) *Tinea cruris* (22.0) *Tinea capitis* (12.0) *Tinea manuum* (11.2) *Tinea pedis* (2.9) *Tinea unguium* (6.9) *Tinea faciei* (5.2) *Tinea barbae* (0.4)	*T. interdigitale* (58.7) *E. floccosum* (35.4) *M. canis* (3) *T. rubrum* (1.5) *A. benhamiae* (0.5) *T. tonsurans* (0.3) *T. violaceum* (0.3)	1–89	ND	Rezaei-Matehkolaei et al. [37]
Palestine	*Tinea pedis* (ND) *Tinea capitis* (ND) *Tinea cruris* (ND) *Tinea corporis* (ND) *Tinea unguium* (ND)	*M. canis* (75.4) *T. rubrum* (22.5) *T. mentagrophytes* (2.2)	2–70	Male (61.36) Female (38.63)	Ali-Shtayeh et al. [38]
Oceania					
New Zealand	*Tinea unguium* (ND) *Tinea corporis* (ND) *Tinea capitis* (ND)	*T. rubrum* (69.0) *T. mentagrophytes* (19.0) *M canis* (6.0) *E. floccosum* (3.0) *T. tonsurans* (1.0) *T. violaceum* (1.0)	0–99	ND	Singh et al. [39]
New Zealand	*Tinea capitis*	*T. soudanense* (47.82) *M. audouinii* (26.08) *T. violaceum* (17.39)	6–12	Male (56.0) Female (44.0)	McPherson et al. [40]

ND Non-determined

13.4 Biology

In general, each *genus* of dermatophytes is described based on the colony morphology, conidia, and its formation; the following paragraphs describe the distinctive morphology of each pathogenic *genus*.

Epidermophyton **spp.** usually shows grainy, folded colonies with a suede-like texture and a yellowish-green color; these colonies are easily bleached and become floccose and sterile. It forms abundant, typically smooth, clavate macroconidia with thin or moderately thick walls, 8–59 septa, and a size of 20–60 by 4–13 μm. Microconidia are typically absent [10].

Microsporum **spp.** forms dusty, grainy colonies with a cottony surface and yellowish-orange color. It generates macroconidia with a fusiform, pyriform, or cylindro-fusiform shape; with echinulate or verrucous walls, and containing 1–15 septa, moderately thick to thick, and have a size of 6–160 by 6–25 μm. It presents sessile or clavate microconidia that are borne laterally directly on the hyphae or in clusters [10].

Trichophyton **spp.** has variable macroscopic features, presenting differences between species; the colonies can be grainy, cottony, cerebriform, and hairy, among others. The underside of the colonies can have reddish or brownish pigmentation. When present, macroconidia have smooth, usually thin, walls with 1–12 septa, either individual or clustered, clavate to fusiform. They have a variable size ranging from 8 to 86 by 4 to 14 μm. They also present microconidia, which might be more abundant than macroconidia, with a spherical, clavate, or fusiform shape, sessile, and borne laterally directly on the hyphae or in pedicels [10].

13.5 Clinical forms of Dermatophytosis

Dermatophytoses, also known as *tineas* or ringworm, result in the development of clinical characteristics such as inflammation, erythematous flaky skin, vesicles, and pustules, although there are also specific clinical characteristics for each case of *tinea* or dermatophytosis. The characteristics are described individually in the following paragraphs [43–46].

Tinea capitis. An infection that occurs mainly in infants and teenagers, affecting the hair and scalp. It has a broad spectrum of clinical manifestations that range from mild peeling lesions to inflammatory lesions such as vesicles and pustules. According to the type of lesion, it is classified as either dry or inflammatory; however, some authors have reported the existence of a less frequent form of *tinea capitis* in adults older than 70 years that presents a loss of sebum production in perimenopausal women, as well as in patients presenting some degree of immunosuppresion (leukemia, diabetes, treatment with corticosteroids, transplants, among others) [47–51]. In agreement with this classification, dry *tinea capitis* is divided

into two varieties: microsporic and trichophytic. The microsporic variety is associated with the *genus Microsporum,* displaying one or several big plaques with short hair that can become confluent and form a single, extended plaque; major apparent symptoms include an erythematous area with irregular alopecia and dry scales. If the infection is not treated soon and persists without spontaneous healing, it may result in severe peeling of the scalp, rupture of the cuticle, and fragile and brittle hair. *M. canis* is the main etiological agent of this clinical form, followed by *M. gypseum* and *M. audouinii* [52, 53]. The trichophytic variety of dry *tinea*, associated with the *genus Trichophyton*, is commonly observed in African-American infants. The lesion starts as an erythematous flaky plaque in the scalp, and although multiple lesions are often present, single lesions are seen sporadically. Hair is usually broken at the shaft, and detritus can be observed within the follicle aperture, showing the appearance of black grains similar to gunpowder. The main causal agent of this condition is *T. tonsuras*, followed by *T. mentagrophytes* and *T. rubrum* [52, 53]. Inflammatory *tinea*, also known as Celsus' kerion, is associated with immunological mechanisms of the host, and lesions can be secondary to bacterial infections [54]. This *tinea* has evident clinical characteristics, such as an exudative plaque with pustules, adjacent edematous and erythematous areas, extensive scabbed areas, and alopecia. It is usually accompanied by fever, clearly delimited and localized adenopathies, malaise, dull and gray tinted mangy hair, and a mucopurulent exudate. The most common agents are *M. canis* and *T. tonsurans* [55, 5]. Adults suffering from *tinea capitis* have infections in the scalp similar to seborrheic dermatitis and erythematosus discoid lupus, as well as large, slightly erythematous plaques with alopecia. This type of ringworm can also appear in either dry or inflammatory forms, and the causal agents vary according to the geographical location. Several authors advise against minimizing the importance of this infection based on reports of adult patients with nonhealing scalp affectations [56, 49, 50, 57, 58].

Tinea favosa. Also known as favid, *favus* is characterized as a chronic infection, mainly of the scalp; however, it can also be observed sporadically in glabrous and hairy skin, and in nail roots. Scalp infection represents the most severe form of *tinea capitis*, presenting fibrillar clusters with the aspect of honey called scutulum, and a mousy odor. Severe alopecia and scabbing are frequent [59–61].

Some varieties can be identified using Sabouraud: (a) favus pityroides, with large, peeling plates are predominant; the hair takes on a gray coloration, and scutulae are visible after scraping, creating contusions with seborrheic dermatitis; (b) favus papyroides, observed in outbreaks and characterized by pliable, adherent, and erythematous scabs with a humid consistency; erosion is observed after scab removal; (c) favus impetigoides, characterized by suppurating scabs; lesions with scabs resembling impetigo are usually rare; no scutulae are present [61]. It must be mentioned that although *tinea favosa* is rare in adults, it can affect immunosuppressed or immunocompetent people. Common etiological agents, from high to low relevance, are *T. schoenleinii*, *M. gypseum*, *T. violaceum*, and *T. mentagrophytes* var. *quinckeanun* [60–62]. It is important to consider that in reports from industrialized countries, the causal agents are mostly related to the *genus Trichophyton*, whereas

in developing countries, they are associated with species of the *genus Microsporum*; *E. floccosum*, in contrast, is rare [48, 52].

Tinea barbae. This condition is mostly exclusive to adolescent or adult males; it is limited to the beard, chin, and neck areas [63–65], and is caused by dermatophytes that infect and colonize keratinized tissues, including *T. metagrophytes*, *T. interdigitale*, *T. verrucosum*, *T. rubrum*, and more rarely *T. violaceum*, *T. erinacei*, *M. canis*, and *A. benhamiae,* the latter being a zoophilic dermatophyte of the *T. mentagrophytes* complex [63, 66–68, 65, 69]. The clinical manifestations can vary according to the pathogenic agent responsible for the disease, and clinical variants can be diagnosed as inflammatory and noninflammatory [63, 67].

The inflammatory variety is mostly related to zoophilic dermatophytes such as *T. mentagrophytes*, *T. verrucosum, M. canis,* and *T. erinacei*, and it is capable of inducing severe inflammatory damage ranging from inflamed nodules to multiple pustules. Broken and loose hair can also be found, as well as vesiculopustular lesions and exudate. This variety is also observed in regional adenopathies, such as fever and malaise [63, 65, 66, 68, 69].

The noninflammatory variety has a diverse range of manifestations, from flaky plates with papules, pustules, or scabs, to erythematous annular eruptions and facial edema. Broken and loose hair near the skin potentially obstructs the follicle, resulting in folliculitis. It is common to find patients lacking regional adenopathies who are not feverish. The dermatophytes associated with this variety are mostly anthropophilic, including *T. rubrum*, *T. violaceum*, and *T. tonsurans* Pruritus is present in both varieties [63, 64, 67].

Tinea pedis. *Tinea pedis* is also known as foot tinea or athlete's foot, with the latter name related to its high prevalence in athletes, consisting of mainly adult males. The main causal agents are *T. rubrum*, *T. mentagrophytes* var. *mentagrophytes* or *interdigitale* and, at a lower frequency, *E. floccosum* and *M. gypseum.* These fungi can be isolated from the floors of pools and showers, as well as from closed footwear, explaining the occurrence of this condition in females and infants, although only sporadically [70–73]. The causal agents of this infection often invade the plantar aspect, interdigital spaces, and lateral foot, which are commonly affected in at-risk populations (immunocompetent or immunosuppressed). It may present recurrence and resistance to treatment. Although it is not caused by them directly, bacteria are often implicated in the infection onset, and the condition is in fact exacerbated and worsened by their presence, augmenting the bad odor, maceration, inflammation, and other symptoms [74, 75, 72].

To date, *tinea pedis* has been classified in four varieties according to their clinical characteristics: (a) chronic or interdigital intertrigo, the most frequent form of the infection, onset is characterized by erosion and peeling, followed by maceration, particularly between the fourth and fifth interdigital space, which may extend to the plantar aspect and in turn develop into a bacterial infection. It may or may not present erythema and fissures between the fourth and fifth toes, and also presents hyperhidrosis, pruritus, and bad odor. Studies investigating this variety have reported that the most common causal agents are of anthropophilic origin: *T. rubrum* and *E. floccosum*

[72, 74–76]; (b) vesiculose, generally subacute, presents small vesicles or vesiculopustules over an erythematous background in the lateral foot, with or without fissures; it extends from the interdigital space toward the midfoot and plantar aspect, although the lateral foot is more commonly affected. It can be painful and exhibit pruritus; it evolves quickly and is exclusively associated with zoophilic dermatophytes, such as species of the *T. mentagrophytes* complex [72, 74–77]; (c) moccasin, also called chronic hyperkeratosis; at the onset of infection, peeling and hyperkeratosis can be observed in the plantar aspect, heel, and lateral foot, which can be extended in a superior direction along the frontiers (moccasin distribution). It also presents fissures over one or both feet. This variety is observed in patients with atopic dermatitis, and although *tinea manuum* is frequently found in these infections, *T. rubrum*, a pathogen of anthropophilic origin, is most commonly associated with this disease (two feet-one hand syndrome) [74–76] (Porche 2015); (d) acute ulcerated, is the rarest of all four varieties, and can present ulcers, pustules, erosions, and rapid extension to the interdigital spaces; it is an exacerbated form of the interdigital or chronic intertrigo varieties. Patients may acquire a secondary bacterial infection that can be severe, developing cellulite, lymphangitis, and fever. This *tinea pedis* variety is common in patients with immunosuppression, such as AIDS, diabetes, or hypertension (HT). It is associated with the zoophilic dermatophytes *T. interdigitale*, *T. mentagrophytes*, and *M. canis* [74–76] (Porche 2015).

Tinea manuum. Also known as *hand tinea*, it is not a common affliction and usually affects the interdigital regions, back, and palms of the hands; often, only one hand is affected, although rarely both hands can be infected. It is more common in adult males and rare in infants, and it can present pruritus with variable and recalcitrant lesions [78–80]. A hyperkeratosis variant has also been classified that produces eruptions with dry margins in a white or yellow color and flaky erythematous plaques; annular manifestations occasionally occur, and verrucous lesions are rare. This *tinea* is associated with the pathogen *T. rubrum* [81, 82]. The inflammatory form is manifested as an acute inflammatory plaque with suppurative folliculitis, dyshidrosis, and vesicle eruptions. The pathogenic agent associated with this type of *tinea*, *T. mentagrophytes*, is a zoophilic dermatophyte. Although some studies have reported the involvement of *T. erinacei*, *M. canis*, *T. rubrum*, and *E. floccosum*, the latter two are of anthropophilic origin [82, 83]. This infection has been observed along with the presence of two feet-one hand syndrome. Physical examination reveals a papular erythematous affection, flaky skin and superficial erosion, erythematous margins between fingers and toes, and onychomycosis [84, 78, 82].

Tinea cruris. Also known as inguinal *tinea*, *tinea cruris* is a superficial infection caused by *T. rubrum*, *T. mentagrophytes*, and *E. floccosum* that mainly affects adult males due to the occlusive function of the scrotum that promotes a humid and warm environment favoring fungal growth; although it can also affect females and infants, it is most prevalent in male teenagers [85, 78, 86–89]. The inguinal, perineum, and gluteus areas are most commonly affected, and, rarely, the scrotum or penis [85, 86]. At onset, the infection is usually, and briefly, unilateral due to predisposing factors such as contaminated underwear [85, 78, 90, 88, 89]. The acute forms present

one or several well-delimited erythematous and flaky plaques that range in color from red to intense brown. These plaques present maceration and are exudative and eczematiform, which is why they have been previously known as thread-like margin eczema [85, 3, 78, 86, 87, 89]. Pruritus is common and can present inflammation, vesicles, and pustules when the causal agent is *T. mentagrophytes* [85].

Tinea imbricata. Tinea imbricata is a type of chronic *tinea* characterized as the most superficial and dry tinea. It has a limited distribution and is usually endemic to isolated rural communities where poverty and lack of hygiene are the norm; some of the most affected locations are in Oceania (Polynesia and Melanesia), where it is known as *tokelau*, and with less frequency, India, Central, and South America, where it is known as *chimbere* in Brazil [91, 92]. The onset of this *tinea* occurs during infancy, but it can be observed in all age ranges and affects both genders equally. It is thought that a genetic predisposition could play an important role in this condition and could be of autosomal recessive or dominant heredity [93, 91]. Lesions can be manifested throughout the body, although the torso and extremities are usually affected. Nails, palms, and plantar aspects are rarely infected, and hair is never affected. Large, flaky concentric or annular plaques are observed at onset, with or without erythema. These plaques are overlapping and exhibit the appearance of lamellar structures that might be hypo- or hyperpigmented with posterior detachment [93–95]. It is common to observe macules or papules in recent lesions, which are often itchy. The responsible pathogen is *T. concentricum* [93, 91, 94, 95].

Tinea corporis. This infection is present in exposed glabrous skin, often in the neck, torso, shoulders, and extremities [77, 86, 87, 96], sporadically in legs [78, 96, 89], and never in the scalp, beard, feet, or hands, although examination of the latter could reveal the primary source of infection [86]. The responsible agents are *M. canis, T. rubrum, T. mentagrophytes, T. tonsurans*, and *E. floccosum,* although others agents have been sporadically reported, such as *M. audouinii, T. verrucosum, M. aenigmaticum*, *M. praecox,* and a new species of the *M. gypseum* complex [97, 86, 87, 98]. The species within the *genus Microsporum* cause a microsporic *tinea corporis*, in which a large number of small plaques are observed. They can become confluent and form large plaques, whereas species of the *genus Trichophyton* cause a trichophytic *tinea corporis* in which a smaller number of large plaques are formed and become confluent, resulting in an even larger plaque with an irregular size that affects a large extent of the body surface [99, 3]. It is manifested as flaky, erythematous, annular, and localized plaques, with papules, pustules, or vesicles and an active margin. It can be observed either as a single or multiple lesions. Immunosuppression is one of the predisposing factors to this disease (diabetes, use of topical corticosteroids, AIDS, among others) [77, 3, 79]. Other varieties are present less frequently, such as gluteus dermatophytosis, or dermatophytosis of the diaper area, which affects infants younger than 3 years of age in the diaper area and neighboring zones such as the perineum, legs, and abdomen, among others. The main causal agent is *E. floccosum*, although sporadic cases caused by *T. rubrum* and *T. verrucosum* have also been observed *verrucosum* [100, 101]. It is manifested as multiple circular, flaky, and erythematous lesions, which can be circinate and ser-

piginous; macules, papules, and vesicles can also be observed [100, 102]. A less frequent form of this variety is *corporis gladiatorum*, which is observed among wrestlers, in which the most affected areas are skin-to-skin contact areas in wrestling sports, such as the face, neck, and extremities. In the last few years, the incidence of the disease has increased alarmingly, thus gaining the attention of researchers. Pruritus is common, provoking malaise and impacting work performance during training and competitions [103, 3], moreover, an erythematous, annular plaque with an active peripheral margin is commonly observed (vesicles) [78].

Trichophytic granuloma. Also known as Majocchi granuloma, the infection is caused by *T. rubrum, T. tonsurans*, and *T. mentagrophytes var. mentagrophytes*, and it can be manifested in the dermis as well as in subcutaneous tissue. Some predisposition has been observed in immunocompetent or immunosuppressed patients, although the latter are more severely affected. Frequently affected body areas are the scalp, beard, and the upper and lower extremities, with a few instances in either the face or neck and rare occurrences in the vulva or ankles [104–106]. Females are commonly afflicted, which has been attributed mostly to leg shaving, during which the follicle is ruptured and subsequently infected by fungi carrying keratin and necrotic material from the dermis. Immunosuppressed persons are also commonly affected. This immunosuppression can be in the form of diabetes, AIDS, the use of topical corticosteroids, autoimmune disease, or physical trauma [105, 107, 106]. This disease is characterized by the presence of dark, inflammatory nodular lesions, which can be observed in the active margin of erythematous plaques. Papules are discreet or clustered, and pustules can also be observed. It is important to highlight the rarity of cheloid or verrucous lesions [108, 105, 107, 106].

Mycetoma (Pseudomycetoma). The term pseudomycetoma is applied to mycetomas caused by fungi such as *M. canis*, *M. gypseum*, *M. audouinii*, *M. ferrugineum*, *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *T. schoenleinii*, and *T. mentagrophytes* var. *interdigitale* [109], which present the characteristic triad of eumycetic mycetoma: (1) tumefaction of the affected tissue; (2) the appearance of microcolonies (grains); and (3) the formation of fistules for the external draining of grains. Unlike mycetoma, which is initiated by external inoculation, this disease is initiated as tinea.

Dermatophytic disease. Described by Hadida [110], this is a rare, chronic infection (Hadida disease); the few known cases have mostly been reported in Algeria in association with the high percentage of consanguineous matrimonies, which would explain the recessive autosomal heredity predisposition to this disease [111]. Immunological studies have also reported a poor cellular response against the causal agents, which may be *T. rubrum*, *T. violaceum*, *T. mentagrophytes*, and *T. schoenleinii* [111, 112]. Affecting mostly males, with an onset at infancy, this condition is usually manifested as a superficial infection that later evolves and invades the dermis, lymph nodes, and potentially even the lungs, bones, liver, intestines, and central nervous system. Lesions are manifested as polymorphic, granulomatous, and flaky plaques. Granulomas can also be found in lymph nodes [113, 111, 112].

Tinea unguium. Also known as onychomycosis, this condition is manifested in fingernails or toenails. It has an estimated incidence of 2–13% worldwide, and it is strongly associated with the presence of *tinea pedis*. The major causal agents are fungi of the *genera Trichophyton*, *Microsporum*, and *Epidermophyton* [114], which produce keratinase and degrade the keratin of nails [115], although damage to the nail plaque by other filamentous, non-dermatophyte fungi has also been observed (non-dermatophyte mold), such as dematiaceous and hyaline fungi. These fungi do not express keratinase, however, and instead use residues from hyperkeratosis that are left behind by dermatophytes. Yeast are also known to frequently affect fingernails (70–80%). According to the International Society for Human and Animal Mycology, the infection is defined according to the etiological agent, i.e., *tinea unguium* when caused by dermatophytes, onyxis when caused by yeasts, nail candidiasis when the yeast in question is from the *genus Candida*, and nail mycosis when the causal agent is a filamentous, non-dermatophyte fungi [116].

Certain clinical criteria must be considered in evaluations of fungal infection in nails, such as (1) spots or stripes with a white–yellow or orange–brown tint; (2) onycholysis; (3) subungual hyperkeratosis; (4) thickening of the nail plaque; and (5) mycological criteria such as a positive dermatophyte microscopy test and culture in vitro.

The most recent classification of *tinea unguium*, or onychomycosis, was described by Hay and Baran [117] and Baran and Hay [118], who propose the classification according to clinical manifestations during the course of infection: (1) distal and lateral subungual onychomycosis, in which fungi invade the subungual region distal to the matrix and lateral to the nail, and some alterations in tint (opaque, white, yellow, orange, grey, brown, or black) can be observed. Other alterations are observed, such as onycholysis, paronychia, or complications of dermatophytoma, which does not allow the proper function of antifungal treatments. The causal agents are *T. rubrum*, *T. mentagrophytes* var. *interdigitale*, *T. mentagrophytes* var. *nodulare*, and opportunistic fungi such as *Scopulariopsis brevicaulis, Paecilomyces,* and *Neoscytalidium* spp.; (2) superficial onychomycosis, manifested in two modalities, white or black. The white modality is also called trichophytic leukonychia, which is characterized as white, rugged patches at onset that later extend over the surface of the nail. The main etiological agents are *T. rubrum*, *T. mentagrophytes* var. *interdigitale* and the keratinase-expressing opportunistic fungi *Aspergillus*, *Fusarium*, and *Acremonium*. The black variety, melanonychia, can be caused by dermatophytes such as *T. rubrum*, opportunistic fungi such as *N. dimidiatum* and *Scytalidium* (*Neoscytalidium*) *hyalinum*, and yeasts such as *Candida albicans* and *C. parasilopsis*; (3) proximal subungual white onychomycosis, in which fungi invade the subungual area of the nail, directly on the cuticle, and progressively advance toward the lunula and the remaining the nail. The last two varieties have been associated with immunosuppression; (4) endonyx onychomycosis, which presents distal and superficial damage, without inflammation or hyperkeratosis. The associated causal agents are *T. soudanense* and *T. violaceum;* (5) mixed pattern onychomycosis, which can be manifested in several combinations of any of the previously mentioned onychomycoses; (6) total dystrophic onychomycosis, which manifests as the final stage of

any variety initiated by subungual infection. The nail bed is thickened and obliterated, and the nail surface becomes disintegrated. The etiological agents are usually *T. rubrum* and filamentous, non-dermatophytic fungi; (7) secondary forms of associated diseases, in which fungi tend to invade nails that have been previously afflicted by another type of primary pathology, such as psoriasis and dystrophia caused by trauma; (8) paronychia is a secondary onyxis caused by *Candida*. Females are commonly affected due to continuous contact with predisposing factors such as humidity and carbohydrates, although individuals with metabolic disorders, such as diabetes mellitus and other hormonal disorders, are also predisposed to this infection [117, 118].

13.6 Pathogenesis

The process of dermatophyte infection must first overcome a series of obstacles, including the innate immune response of the host, the physical structure and chemistry of the skin, constant UV exposition, temperature, lack of humidity, normal microbiota, sphingosomes produced by keratinocytes, and fatty acids produced by sebaceous glands. The infectious process requires the initial inoculation and adherence of fungal elements to the corneal epithelium [119]. Arthroconidia adhere through mannose and galactose ligands to the corneal epithelium of the host; however, the host organism has several defense mechanisms against the adherence of infectious agents, consisting of renewal of the corneal layer leading to epithelial shedding and possible fungal elimination. Therefore, epidermal invasion is only possible if tissue adherence is achieved before elimination by epithelial cell shedding. Moreover, during the initial interaction between arthroconidia and the corneal layer, within 3–4 h after initial contact, elongated fibrillar structures are produced that connect the arthroconidia to the keratinocytes, thus attaching to the corneal epithelium. After germination of the arthroconidia, hyphal growth is radial and multidirectional, stabilizing the permanence of the fungi within the host; this process takes 24 h [120]. During the invasion of deeper epidermal layers, recently formed arthroconidia covering the surface become finer and shorter, taking on a flat morphology that increases the surface area in contact with the invaded tissue and resulting in greater adhesion and the capacity for nutrient acquisition. In addition to adhesion to the corneal layer, these fibrillar structures enable the connection between adjacent arthroconidia, suggesting the formation of a stable complex similar to a biofilm or even an intercellular communication mechanism [121, 122]. Once the fungal elements have adhered to the host and germinated, the invasion of hyphae into the stratum corneum occurs rapidly, followed by the formation of additional hyphae that grow and spread in a circular pattern, particularly within the inferior layers of the stratum corneum. Once established, dermatophytes seek growth-enabling nutrients and respond to the environmental pH by activating the expression of proteins and enzymes that are favored by an acidic environment, i.e., adhesins, lipases, phosphatases, DNases, and keratinolytic proteases (serine-subtilisin and

fungalisin), allowing the degradation and processing of keratin and other proteins, as well as lipids and DNA, in order to thrive [119, 123]. Moreover, once within the keratinized tissue, dermatophytes must catalyze the degradation of the surrounding keratin into oligopeptides or amino acids that can be assimilated by the fungi. Regardless, these enzymes cannot function until disulfide bonds reduction (sulfitolysis) within the compact net of keratin has been achieved. It is suggested that this process is managed by a sulfite efflux pump encoded by the gene *TruSsu1,* which dissociates the disulfide bonds of keratin and releases cysteine and S-sulfocysteine. Disulfide bonds are responsible for the hardness and difficult degradation of keratin; therefore, the sulfite produced by dermatophytes from environmentally available cysteine reduces and directly dissociates these bonds, facilitating digestion by exo- and endo-peptidases, thus providing an adequate nutrition source for these pathogens [124]. An additional source of nutrition is obtained from surrounding host macromolecules, which can be degraded into basic carbon, nitrogen, phosphorus, and sulfur components. Nonetheless, the high molecular weight of proteins, starch, cellulose, and lipids complicates their transport through the cytoplasmic membrane, making their degradation a necessary process. For this purpose, dermatophytes secrete proteases, lipases, elastases, collagenases, phosphatases, and esterases that hydrolyze the structural components of the epidermal tissue, in turn providing the invasive characteristics of these pathogens. These enzymatic mechanisms are well-characterized virulence factors of dermatophytes [120, 125, 126]. In addition, the process of invasion, production, and secretion of proteases contributes to the degradation of inner epithelial layers in immunocompromised hosts [120, 127]. Furthermore, it is thought that deeper penetration is inhibited by the limited availability of iron due to the ferritin activity present within the dermal subjacent layer of the host [128].

As previously mentioned, the secretion of proteases is an important virulence factor of dermatophytes, supporting the colonization of these fungi within keratinized structures of the host [124]. The resulting proteolytic damage in the invaded tissues varies markedly according to species [129, 130]. Therefore, the pattern of excreted proteases likely determines the survival of the fungi and evolution of the infection in the host tissue. Thus, the production level of proteases, as well as the level of adaptation of a dermatophyte to its host, contributes to the intensity of the inflammatory response. Therefore, it can be stated that increased adaptation results in a less intense inflammatory response. In contrast to deep infection, superficial infections are less likely to promote inflammation, providing a superior survival strategy for dermatophytes [131]. The clinical presentation of dermatophytosis is diverse and depends on both the host and fungal species. The more severe lesions produced by dermatophytes (acute infection) present faster and more efficient resolution through a specific innate immune response; however, more adapted species (e.g., *T. rubrum* and *T. interdigitale*) cause chronic infections with few or absent symptoms [120]. In the case of *tinea unguium*, nail localization is ideal for long-term survival because the dermatophyte is inaccessible to a host immune response. Likewise, the immune response of the host against dermatophyte infection depends on several factors, including defense against fungal metabolites, strain virulence,

and the anatomical site of infection. These factors, as well as those related to the host (entry site, nonspecific defense, immune response, and synchronic microbial colonization), play an important role in the inflammatory process [130, 132]. Furthermore, several factors participate in both the host and fungi, resulting in a predisposition to the development of dermatophytosis and affecting the degree of the associated inflammation [129, 130].

Dermatophytes also have the capacity to manipulate the immune response of the host, i.e., the localization within the stratum corneum, preventing the development of a strong cellular immune response. The manifestation of an immune response depends on the degree of inflammation, which varies depending on the dermatophyte species, host, and physiopathological state [120]. Zoophilic species cause more inflammatory infections that may heal spontaneously, providing resistance against reinfection. Anthropophilic species, however, usually cause chronic infections that are less likely to provide resistance against future incidents [133]. Some species promote increased production of IL-8 and TNFα, explaining the occurrence of an acute inflammatory response. Mannan-oligosaccharides, a component of the cell wall of dermatophytes, seems to be involved in suppressing the inflammatory response [134]. Furthermore, it has the capacity to suppress the expression of Toll-like receptors on the surface of keratinocytes, compromising the Th1 cellular response. Thus, infection by these pathogens becomes chronic [130].

13.7 Molecular Taxonomy

As mentioned previously, to distinguish between different dermatophyte species, certain morphological criteria are used, including macromorphology, size and pigmentation of the colonies, shape of macro- and microconidia, growth rate, physiological properties (e.g., urease production), in vitro tests for hair perforation [135], growth in rice, alkaline production in bromocresol purple medium [136], sorbitol assimilation [137], vitamin or amino acid requirement, and certain biochemical tests [138]. Regardless, these markers are limited in number (there is a finite number of measurable characteristics) and must be assessed at predetermined stages of fungal growth. Moreover, these microorganisms tend to have a variable appearance and some go through degenerative and irreversible changes associated with pleomorphism. This phenomenon, which may occur shortly after isolation and following culture reseedings, results in colonies that turn white in color and lose their sporulating capacity. Furthermore, the culture media and other growth conditions influenced by the environment can affect the macro- and microscopic morphology of dermatophytes. Therefore, the utilized criteria lack definition and objectivity in some instances. The advent of molecular biology and the development of molecular markers has become a useful tool for the elimination of inconvenient means of identification based on the exclusive analysis of phenotype, enabling the rigorous and reproducible identification of species and varieties. The different types of molecular markers used to differentiate species have a remarkable capacity to detect

polymorphisms in unique or multiple *loci*. In this regard, the available techniques include restriction fragment length polymorphism (RFLP) from mtDNA, restriction enzyme analysis (REA), and markers based on DNA amplification such as polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD), arbitrary primer-PCR (AP-PCR), simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP), single nucleotide polymorphisms (SNPs), inter single sequence repeat (ISSR-PCR), microsatellites, and multilocus sequence typing (MLST) [12].

The use of these markers has made possible numerous studies of dermatophyte taxonomy. The first of such studies, by Symoens et al. [139], employed isoelectric focusing to analyze four isolates of *A. benhamiae, A. vanbreuseghemii, T. mentagrophytes*, and T. *interdigitale* to differentiate the species of the *T. mentagrophytes* complex and those in close proximity to *T. interdigitale.* Their results revealed two groups, the first of which fit the profile of *A. vanbreuseghemii*, and grouped the isolates of *T. interdigitale* and most isolates of *T. mentagrophytes*. The second fit the profiles of *A. benhamiae* and *T. mentagrophytes*. Therefore, they suggested that *T. interdigitale* could be an anamorph species of the teleomorph *A. vanbreuseghemii* and that *T. mentagrophytes* could correspond to either *A. vanbreuseghemii* or *A. benhamiae* teleomorph species.

Mochizuki et al. [140], using mtDNA restriction analysis, reported the relationship between *T. interdigitale* (*T. mentagrophytes* var. *interdigitale*) and other members of the *T. mentagrophytes* complex. The restriction profiles were compared with those of *A. simii*, *A. benhamiae*, and *A. vanbreuseghemii*, and they revealed that *T. interdigitale* is identical to *A. vanbreuseghemii*. Therefore, the authors consider these two species to be closely related.

Aiming to define the divergence between *Nannizzia incurvata*, *N. gypsea*, *N. fulva*, and *N. otae*, Kawasaki et al. [141] compared these isolates through mtDNA restriction profiling, and they observed that only *N. fulva* showed two restriction profiles. The phylogenetic analysis suggested that *N. gypsea* is more closely related to *N. fulva* than *N. incurvata* and that there is a larger phylogenetic distance between *N. otae* and the remaining species.

Kawasaki et al. [142] researched the phylogenetic relationship between the *genera Arthroderma* and *Nannizzia* using mtDNA RFLP. The phylogenetic tree was constructed with 10 species: *A. benhamiae, A. insingulare, A. quadrifidum, A. simii, A. vanbreuseghemii, N. fulva, N. grubyia, N. gypsea, N. incurvata*, and *N. otae*. Their results did not reveal any differences between the *genera Arthroderma* and *Nannizzia*, confirming that the *genera Arthroderma* and *Nannizzia* are congeneric, as previously reported by Weitzman et al. [143].

Another study, using mtDNA analysis to determine the phylogeny of the *genus Trichophyton*, showed the division of *T. rubrum* isolates into two groups, Type I and II, suggesting that the *genus* is composed of a species complex. *A. benhamiae* was closely related to *T. mentagrophytes* var. *erinacei* and *T. rubrum* Type II, whereas *T. tonsurans* and *A. vanbreuseghemii* showed a similar restriction profile, which suggested that they are identical. *T. quinckeanum* and *T. schoenlenii* also showed an identical restriction profile, differing slightly from *A. vanbreuseghemii* [144]. These results are consistent with those reported by de Bievre et al. [145].

Harmsen et al. [146], studied the phylogenetic relationship of isolates representative of the *genera Trichophyton*, *Microsporum*, and *Epidermophyton* by analyzing the 18S rDNA sequences, and they demonstrated a monophyletic origin of dermatophytes, which were further classified as a subgroup within the *order* Onygenales.

Gräser et al. [147], with the goal of identifying 26 species of the *genera Trichophyton*, *Microsporum*, and *Epidermophyton,* utilized an RAPD approach with the primers $(AC)_{10}$, $(GTG)_5$, M13, and AP3. From the obtained polymorphic patterns, 17 species showed characteristic patterns, suggesting that dermatophyte species can be identified by the characteristic amplicon patterns of each species.

Another report, utilizing rDNA RFLP to differentiate isolates of *T. rubrum* and other dermatophytes, found that RFLP analysis together with nontranscribed spacer (NTS) and ITS intergenic regions could differentiate isolates of *T. rubrum*. They further identified fourteen types (A-N) among clinical isolates, the most predominant of which were A and B. The authors observed that the NTS region was highly polymorphic in *T. rubrum* compared with the ITS region; regardless, some closely related taxa such as *T. rubrum-T. soudanense* and *T. quinkeanum-T. schoenlenii* could not be distinguished from each other [148].

Makimura et al. [149], constructed the phylogeny of a group of dermatophytes, including the *genera Trichophyton, Microsporum*, and *Epidermophyton*, identifying the species through sequence comparison of the rDNA region ITS1 with the database. The study demonstrated that species of pathogenic dermatophytes for either humans or animals, *T. tonsurans*, *T. schoenleinii*, and *T. verrucosum*, are members of group *A. vanbreuseghemii-A. simii* or group *A. benhamiae*.

Furthermore, Gräser et al. [150] delimited the molecular taxonomy of *T. mentagrophytes* and *T. tonsurans* through morphological and physiological characterizations in addition to molecular techniques (i.e., rDNA ITS regions, AFLP, and PCR fingerprinting), using the primers T3B, M13, and $(AC)_{10}$. The 24 species and/or varieties analyzed in that study were reclassified into five species: *T. tonsurans*, *T. interdigitale*, *T. mentagrophytes*, *T. simii*, and *T. erinacei.*

Using the rDNA ITS region (ITS1, ITS2, and 5.8S), Gräser et al. [151] evaluated the phylogenetic relationships within the *family* Arthrodermataceae. The authors concluded that *Trichophyton* and *Microsporum* are conserved as separate *genera*, whereas within the *Trichophyton*, there is a separation of human pathogenic and primary geophilic species. Moreover, the two species of *Epidermophyton* were not related, suggesting that *E. stockdaleae* should be reclassified. Likewise, the authors proposed three species for *T. mentagrophytes* and a reduction of the number of varieties of *T. mentagrophytes*; however, they also suggested that the *genus Keratinomyces* should be restored. They further demonstrated that the taxa *T. rubrum*, *T. megninii*, *M. gallinae, A. grubyi*, *M. audouinii* var. *rivalieri*, *M. audouinii* var. *langeronii, M. audouinii*, *M. canis*, and *M. equinum* are so closely related and likely conspecific.

Summerbell et al. [152] analyzed the shared characteristics of dermatophytes with *T. rubrum* using representative isolates of recently described species such as *T. krajdenii*, a *T. mentagrophytes* isolate that is related to the yellowish variants of *T. rubrum*. These isolates were analyzed by sequencing the ITS1 and ITS2 regions. The results showed that the isolates of *T. raubitschekii*, *T. fischeri*, and *T. kanei* share

identical sequences in the ITS region with *T. rubrum, T. soudanense*, and *T. megninii*, with only a small number of differences between *T. rubrum* compared with *T. soudanense* and *T. megninii. T. krajdenii* carries a sequence that is similar to that in isolates of *T. mentagrophytes* and others related to the *A. vanbreuseghemii* teleomorph.

Weitzman et al. [143] classified dermatophytes in the *genera Epidermophyton*, *Keratinomyces*, *Microsporum*, and *Trichophyton*. The anamorphic, nonpathogenic fungi were assigned to the *genus Chrysosporium*. Teleomorphic fungi, however, were classified within the *family* Arthrodermataceae. Teleomorphic fungi of the *genus Microsporum* were classified in the *Nannizzia*, and those from the *genus Chrysosporium*, *Keratinomyces*, and *Trichophyton* were classified in the *genus Arthroderma*.

Gräser et al. [153] evaluated the taxonomy of *T. rubrum* and *M. canis* using phenotypic characteristics (macro- and micromorphology, urease production, hair perforation) and genotyping (PCR fingerprinting, rDNA ITS region, and AFLP) techniques. The species that were closely related to *T. rubrum,* including *T. circonvolutum*, *T. fischeri*, *T. fluviomuniense*, *T. glabrum*, *T. gourvilii*, *T. kanei*, *T. kuryangei*, *T. megninii*, *T. pedis*, *T. raubitschekii*, *T. rodhaini*, *T. rubrum* var. *nigricans*, *T. soudanense*, *T. violaceum* var. *indicum*, and *T. yaoundei,* were reclassified as *M. canis* (teleomorph: *A. otae*), *M. ferrugineum*, and *M. audouinii*.

Hirai et al. [154] studied dermatophytes and fungi related to the *genera Chrysosporium, Epidermophyton, Microsporum*, and *Trichophyton* by analyzing the sequence of the chitin synthase 1 (*CHS1*) gene. The phylogenetic tree revealed that the *genus Microsporum* was genetically different from *Chrysosporium* and *Trichophyton.* Furthermore, the *Trichophyton* species were divided in two groups, one consisting of pathogenic geophilic fungi including *A. curreyi*, and the other consisting solely of geophilic fungi.

Years later, Nenoff et al. [155], based on previous studies, modified the taxonomic classification of the *T. mentagrophytes* complex to include the relationship with teleomorph species.

Species classified as *T. mentagrophytes* var. *interdigitale* (anthropophilic), *T. mentagrophytes* var. *nodulare* (anthropophilic) (also *T. krajdenii*), *T. mentagrophytes* var. *goetzii* (anthropophilic), and *T. mentagrophytes* var. *granulosum* (zoophilic) were reclassified as *T. interdigitale* (anthropophilic and zoophilic) (anamorph) and its teleomorph *A. vanbreuseghemii* [156]. Species classified as *T. mentagrophytes* var. *quinckeanum* (zoophilic) (also *T. sarkisovii* and *T. langeronii*) were reclassified as the anamorphic species *T. mentagrophytes* and the teleomorph *A. simii*. Species classified as *T. mentagrophytes* var. *granulosum* (zoophilic) were reclassified as *Trichophyton* sp. (zoophilic) and *T. mentagrophytes* var. *erinacei* (zoophilic) were reclassified as *T. erinacei* (zoophilic) and the teleomorph *A. benhamiae.*

To define the biological species in the *T. mentagrophytes* complex, several authors performed a sexual stimulation between fungi, seeking to reveal the sexual phase of the dermatophytes. To achieve this goal, Anzawa et al. [157] performed crosses between *A. simii* with asexual species of *Trichophyton*, including *T. rubrum, T. concentricum*, and *T. interdigitale.* The cross between *T. rubrum* with *A. simii*

produced several ascomata, suggesting that *T. rubrum* is neither an asexual nor a clonal species Symoens et al. [158] also tried to improve the definition of teleomorph species in the *T. mentagrophytes* complex by performing a crossing analysis with isolates from humans, animals, and reference strains of *A. benhamiae* and *A. vanbreuseghemii*, comparing the mating competence with the sequencing of the ITS and 28S rDNA regions; this was the first study to report the taxonomy of dermatophytes associated with a mating analysis. Based on the ITS sequence and mating capacity with *A. vanbreuseghemii* strains, the authors identified all of the *tinea pedis* and *tinea unguium* isolates as *T. interdigitale*. Therefore, *T. interdigitale* should be considered a humanized species derived from the cross with *A. vanbreuseghemii*.

Kawasaki [159] verified the taxonomy of dermatophytes based on a phylogenetic analysis and mating capacity through genealogical concordance phylogenetic species recognition (GCPSR). They utilized several DNA regions: 5.8S rDNA, ITS regions 1 and 2, a partial sequence of the actin gene including two introns (ACT), a partial sequence of the DNA topoisomerase gene including one intron (TOP), and a partial sequence of the GAPDH gene including one intron (GPD). The authors found that *A. simii, A. vanbreuseghemii, T. mentagrophytes* var. *interdigitale*, and *T. tonsurans*, as well as *A. benhamiae*, *T. concentricum*, *T verrucosum*, and *T mentagrophytes* var. *erinacei*, can be considered phylogenetic species.

Tartabinia et al. [160] utilized a set of phenotypic methods to identify fungal isolates within the *genus Trichophyton*, as well as PCR fingerprinting using the primer $(GACA)_4$. The phenotypic tests allowed the differentiation of *T. rubrum* from the isolates of the *T. mentagrophytes* complex but not from the species within the complex; regardless, the molecular technique allowed the characterization of both isolates from *T. rubrum* as well as from the *T. mentagrophytes* complex, which were identified as *T. interdigitale* and *Trichophyton* sp., anamorphs of *A. benhamiae*.

Ahmadi et al. [161] performed phylogenetic and taxonomic analyses based on inter- and intraspecific variations using a partial region of the calmodulin gene. Some intraspecific differences were found in the isolates from *T. interdigitale*, *A. simii*, *T. rubrum*, and *A. vanbreuseghemii*, whereas *T. tonsurans, T. violaceum, E. floccosum, M. canis, M. audouinii, M. cookei, M. racemosum, M. gypseum, T. mentagrophytes, T schoenleinii*, and *A. benhamiae* were conserved. The isolates from *E. floccosum/M. racemosum/M. cookei*, *A. obtosum/A. gertleri, T. tonsurans/T. equinum*, and *T. interdigitale* presented identical sequences for the calmodulin gene. The topology of the phylogenetic tree for this gene concurred with the results obtained for the ITS, β-tubulin (BT2), and Tef-1α molecular markers.

13.8 Molecular Epidemiology

The molecular epidemiology study of infectious diseases evaluates the dynamics of such diseases within a population. These dynamics are determined by the transmission of a parasite to its host and by how this transmission affects the dispersion of the microorganism within the host population. Moreover, molecular epidemiology

also identifies the existing clonal relationship between several isolates of the same species, particularly during outbreaks caused by multiresistant strains. Applying molecular techniques permits the calculation of the number of circulating clones and the identification of the infection source or reservoir as well as the vehicle for disease transmission, which are important for the control of infectious diseases [162].

The following table (Table 13.2) shows, in chronological order, relevant reports examining the molecular epidemiology of dermatophytosis based on different molecular techniques to provide a better understanding of this disease.

13.9 Diagnosis of Dermatophytosis

The diagnosis of dermatophytosis is determined based on clinical and epidemiological data; however, diagnosis must be confirmed using a laboratory test because there are non-mycotic dermatophytosis that cannot be clinically differentiated from a mycotic dermatophytosis, resulting in poor handling of the affected patient. Prior knowledge regarding the etiology of dermatophytosis is essential for the election of an effective antifungal treatment, as well as in facilitating the determination of the origin of the causal agent (zoophilic or anthropophilic) and preventive measures to treat the infection [180]. To achieve these goals, a broad number of conventional and molecular laboratory techniques are currently available.

Conventional techniques. Before suspecting a dermatophyte skin infection, either in glabrous or hairy skin, a preliminary test can be conducted in the affected skin area through a Wood lamp examination (UV light) in a dark room. Dermatophytes typically show a greenish fluorescence, even when the fungi are no longer viable, although some dermatophytes, such as *T. tonsurans* and *T. violaceum*, lack fluorescent properties [10]. However, a KOH test for the microscopic analyses of the clinical specimen (nail, skin, or hair) and cultures in vitro are the gold standard for the diagnosis of dermatophytes because these methods allow the detection of fungal elements and, in some instances, their morphology based identification. The KOH test (10–20%) is a fast and economic tool for the diagnosis of fungal infection; however, it does not allow the differentiation between a dermatophyte and a non-dermatophyte fungal infection, especially when the analyst lacks proper experience [181, 182].

The KOH test can present false positive results, which are observed in 15% of cases, partly due to the presence of artifacts constituted by lipid vesicles, air bubbles, and textile fibers, which may be mistaken for dermatophytes [181–183]. Instead, chlorazol black and DMSO have greater sensitivity because they stain fungal structures in a black-blueish color, facilitating their identification. The use of specific dyes targeting the cell wall of fungi has long been implemented, including Periodic acid-Schiff (PAS), Parker blue, and Congo red, as well as fluorescent dyes such as Calcofluor white, rhodamine, and diaminostilbene acid [180, 182, 184]. Despite facilitating the analysis, the detection of fungal elements by fluorescence microscopy is limited in its routine application due to the high cost of reagents and equipment [185].

Table 13.2 Application of molecular epidemiology in dermatophytosis

Objective	Molecular method	Conclusion	References
Identify *T. tonsurans* and determine if there are genetic differences in the phenotypic function or the geographical region of isolation	RAPD with three random primers	*T. tonsurans* is a genetically homogenous species, independent of geographical localization	Kim et al. [163]
Detect genetic variability in *T. rubrum* isolates from Europe, North America, Africa, and Asia	RAMD, SSCP, and RFLP	No genotypic variability was revealed, indicating clonal reproduction	Gräser et al. [151]
Analyze the genetic variability between isolates of *T. mentagrophytes* and *T. rubrum*	REA and RAPD	Little variability was observed between *T. mentagrophytes*, and none was detected in *T. rubrum*	Howell et al. [164]
Analyze the genetic variability between isolates of *T. rubrum* and *T. mentagrophytes*	NTS region amplification, 18S probe, and RFLP	Few differences were found among *T. rubrum*, *T. mentagrophytes*, and *T. tonsurans* by observing intraspecific isolates of *T. rubrum* and *T. mentagrophytes*	Gupta et al. [165]
Determine if nail infection caused by *T. rubrum* is due to one or more fungal isolates	NTS region amplification	Two or more types of *T. rubrum* were identified	Yazdanparast et al. [166]
Investigate the genetic variants of *T. tonsurans*	NTS and ITS region amplification	12 strains were identified, evidencing the genetic heterogeneity of *T. tonsurans*	Gaedigk et al. [167]
Study of a scalp tinea outbreak in a school population due to *M. canis* and environmental isolates	RAPD, NTS, and ITS region amplification	Clinical and environmental isolates showed identical patterns	Yu et al. [168]
Investigate the phenotypic and genotypic correlation of *M. canis* isolates from cat, dog, and human	ITS1 and ITS2 region amplification, REA, and RAPD	Microscopic and culture characteristics revealed the variability of *M. canis*. Genotypic characteristics showed that isolates of *M. canis* from cat, dog, and human were genetically identical and can therefore be considered clonal	Brilhante et al. [169]
Typify the isolates of *M. canis* obtained from humans and animals (dogs and cats)	ISSR-PCR	The isolates of *M. canis* obtained from animals were closely related to those isolated from humans	Cano et al. [170]

(continued)

Table 13.2 (continued)

Objective	Molecular method	Conclusion	References
Analyze the genetic variability of *T. tonsurans* isolates from Japan	VIR region in IGS of rDNA	All isolates had the same genotype, suggesting that a specific genotype predominated in the studied area	Sugita et al. [171]
Study the genetic variability of *T. rubrum* isolates obtained from patients with onychomycosis	RAPD, NTS region amplification	The isolates showed great diversity in the genome of *T. rubrum* and inter-strain variations	Baeza et al. [172]
Confirm that the distribution of genotypes in *T. rubrum* is associated with the geographic origin of the human host	MLMT	A subdivision of the species into two populations was revealed, showing clonal reproduction and differences in human host predilection (scalp vs. feet), as well as a geographical differentiation	Gräser et al. [173]
Study the genetic variability of isolates of *M. persicolor* from India	Microsatellites	The analysis revealed a high intraspecific variability in isolates from India, indicating that this species was not recently introduced into the country	Sharma et al. [174]
Characterize the isolates of *M. canis*, *M. fulvum*, *M. gypseum*, *T. mentagrophytes*, and *T. terrestre* from animals (dogs, cats, rabbits) and humans	PCR and sequentiation of CHS-1 and ITS	Two genotypes of *M. fulvum* were identified and grouped more closely with *M. gypseum* than with *M. canis;* in addition, two genotypes of *T. mentagrophytes* were detected; likewise, *M. fulvum* and *M. gypseum* were grouped with *T. mentagrophytes*, and *M. canis* and *T. terrestre* were in separate groups	Cafarchia et al. [175]
Differentiate isolates pertaining to *T. mentagrophytes sensu lato*	ITS region amplification and PCR-RFLP	The sequences of *T. interdigitale/A. vanbreuseghemii* isolates revealed a unique polymorphic pattern. This differentiation was essential for the selection of an antimycotic therapy	Heidemann et al. [176]
Identify dermatophytes isolated from workers in rabbit farms with a clinical history of skin lesions	Amplification of CHS-1, ITS1, 5.8S, and ITS2 regions	The close phylogenetic relationship between strains *T. interdigitale* (zoophilic) and isolates from dogs, pigs, and mice could indicate that these animals represented an infection reservoir for dermatophytes in rabbit farms	Cafarchia et al. [1]

Evaluate the inter- and intraspecific variation in dermatophytes	BT2 PCR	*T. tonsurans, T. equinum, T. concentricum, T. verrucosum, T. rubrum, T. violaceum, T. eriotrephon, E. floccosum, M. canis, M. ferrugineum,* and *M. audouinii* were not variable. The more closely related species were *A. benhamiae* and *T. concentricum* and *T. tonsurans* and *T. equinum*; more distant species were *M. persicolor* and *M. amazonicum*	Rezaei-Matehkolaei et al. [177]
Evaluate the inter- and intraspecific variation in dermatophytes	Tef-1α PCR	*T. rubrum, T. tonsurans, T. schoenleinii, T. concentricum, T. violaceum, E. floccosum, M. ferrugineum, M. canis, M. audouinii, T. equinum, T. eriotrephon,* and *T. erinacei* were not variable	Mirhendi et al. [178]
Genotype isolates of *T. mentagrophytes sensu lato* obtained from humans and diverse animal species with clinical symptoms of dermatophytosis	ITS-PCR-RFLP sequentiation of ITS	Identified *T. interdigitale*, *A. benhamiae*, and *A. vanbreuseghemii*	Ziółkowska et al. [179]

MLMT multilocus microsatellite typing, *RAPD* randomly amplified polymorphic DNA, *NTS* nontranscribed spacer, *BT2* β-tubulin, *RFLP* restriction fragment length polymorphisms, *REA* restriction enzyme analysis, *ISSR-PCR* inter single sequence repeat, *ITS* internal transcribed spacer, *rDNA* ribosomal DNA, *RAMD* random amplified monomorphilic DNA, *SSCP* single-strand conformation polymorphism, *IGS* intergenic spacer, *Tef-1α* translation elongation factor 1-α, *CHS-1* gen chitin synthase-1

In vitro culture has some advantages over microscopy analyses because it can provide higher sensitivity thresholds due to the greater quantity of available material for analysis, and in some cases, the fungi can be identified based on their morphology alone [186]. Sabouraud dextrose agar (SDA) is the most commonly used medium for dermatophyte isolation, and it is responsible for enabling the description of several morphological characteristics of these fungi. The development of these colonies in vitro is slow, lasting between 7 days and 6 weeks [186], although Rezusta et al. [187] suggested that an incubation period of 17 days was sufficient for proper interpretation of results.

The addition of antibiotics (gentamicin, chloramphenicol, and cycloheximide) to SDA culture medium confers selectivity for the development of dermatophytes by preventing the growth of nonpathogenic fungi or bacteria. Once these colonies have developed, dermatophytes can be identified based on their macro- and micromorphology [182], although sometimes it is necessary to perform subcultures in specific media (lactrimel, potato dextrose, malt extract, Candi-Select(™) 4, SDA, 1% Tween-80), hair perforation tests in vitro, or to evaluate the capacity to degrade or assimilate compounds such as keratin, urea, and special amino acids or vitamins, especially when trying to identify *T. rubrum* or members of the *T. mentagrophytes* complex [180–182, 188, 189].

Another commercially available medium, the selective culture medium BBL DTM (Becton, Dickinson and Company, USA), can be used for the detection and presumptive identification of dermatophytes from clinical samples, providing the advantage of direct inoculation in the clinician's office. Despite the multiple options for in vitro culture, the obtained results are often false negative (40% of analyzed samples) compared with microscopic analyses, which may be due to the inviability of fungal elements obtained from medical samples [190]. Therefore, several authors concur that the success of a culture in vitro lies in proper sampling and transport to avoid potential contamination with other microorganisms that limit detection and identification, especially when nails are concerned [181, 191].

Therefore, serial repeats with intervals of 2–5 days between samplings of in vitro microscopy and culture tests are highly recommended to improve the results [192].

Furthermore, when the results are negative despite strong clinical suspicion, a histological test provides the best alternative, possessing a higher sensitivity than in vitro culture or a KOH test [193, 194]. These histological analyses commonly employ staining methods such as silver methenamine, PAS, hematoxylin and eosin, and Gomori to highlight hyphal structures against the stratum corneum [182, 195]. Such analyses assist clinicians in deciding whether to continue or terminate treatment [196]. Regardless, histological analyses are unable to determine the viability of the analyzed fungi, nor are they able to identify the pathogenic species [180, 186].

It is important to mention that regardless of the modifications implemented to improve conventional methods, a series of factors still impede the identification of dermatophytes using these tests, including intraspecific morphological variation, atypical or similar interspecific morphology, and pleomorphism. Moreover, taxonomic changes in the classification of dermatophytes have motivated the development of new methods for the identification of these species, including molecular techniques [197].

13.10 Molecular Techniques

Among the molecular techniques most commonly used to identify dermatophyte species are different formats of PCR: endpoint, multiplex, nested, RT-PCR, qPCR, PCR-ELISA, and PCR-electrospray ionization mass spectrometry (PCR-ESI/MS).

These PCR-based methods have enabled a more precise identification of dermatophytes at both inter- and intraspecific levels, depending on the amplification target. The main targets for amplification are the genes: topoisomerase II, chitin synthase, actin, regions D1/D2 and ITS, and ribosomal subunits 28S and 18S [198–200]. PCR is sensitive enough to detect 1 fg DNA or 1 colony forming unit (CFU), particularly with modalities of nested PCR, qPCR [198, 201, 202], or PCR-ELISA, [203]. Likewise, RAPD [204], amplification of ribosomal subunits followed by restriction enzyme digestion [205, 206], and the amplification of microsatellite regions (GACA)4, (AC)10, and (GTG)5, have also been utilized for the identification of dermatophytes by comparing the migration patterns of amplicons with reference strains [147, 207, 208]. Nonetheless, these techniques are recommended for epidemiological studies rather than diagnosis.

PCR tests enable a swift identification of the etiological agent in question; therefore, these assays contribute to the selection of a proper therapeutic course [209, 210], which is important because of the growing resistance to antimycotics. Most PCR tests have been developed using clinical samples (either freshly collected or mounted in paraffin blocks), and their efficiency in comparison to conventional methods reveals that the results obtained with PCR are, in some instances, consistent with those obtained by conventional methods [211] and, in some other cases, show a higher sensitivity and specificity [198–200, 211–213]. Regardless, PCR is not exempt from false positive/negative results, even when employing commercial kits such as Dermatophyte PCR (Statens Serum Institut; Copenhagen, Denmark) [214, 215]. qPCR, in contrast, greatly reduces the incidence of false results, although it depends on the adequate collection and handling of the samples [216–218], thus recommending that negative samples are cultured in vitro and re-tested if a positive microscopy result is obtained [216]. Another important advantage of qPCR for the diagnosis of dermatophytosis is the capacity to evaluate the viability of these fungi by quantifying actin mRNA or by amplifying ITS and D1/D2 regions. Moreover, several samples from skin scales or nails have been analyzed before and after treatment with terbinafine; as therapy progressed, the results revealed a reduction in the number of actin mRNA copies per mg of analyzed sample. These results were consistent with the in vitro culture and KOH test [212, 219]. Therefore, qPCR is useful for determining whether to continue treatment [220]. Furthermore, Worek et al. [221] developed a genome in situ hybridization method (GISH) using specific probes for the successful identification of *T. interdigitale*, *T. rubrum*, and *M. canis*. The authors propose the use of this method when proper identification of a dermatophyte remains elusive due to ambiguous PCR-RFLP patterns.

Another technique that has been developed for the diagnosis of dermatophytes is soft matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)

spectrometry. This technique enables the identification of dermatophytes by comparing peptide patterns from proteolytic degradation with a database containing the peptide spectral library of different dermatophytes. L'Ollivier et al. [222] reported a database including *T. mentagrophytes*, *T. interdigitale*, *T. erinacei*, *T. simii*, *A. otae*, *M. canis*, *M. audouinii*, and *M. langeronii*, as well as *T. terrestre* and *T. ajelloi*, two nonpathogenic dermatophytes. This is a time-saving procedure that allows the simultaneous and precise identification of up to 64 dermatophyte strains in 24 h from clinical samples or in 3–6 days when analyzing cultures in vitro [182]. In addition to the identification of species, MALDI-TOF MS technology offers additional possibilities, such as the detection and prediction of resistance phenotypes in fungi and clinically relevant subtypes [223]. These tests are based on pure in vitro cultures of dermatophytes as well as on skin and nail samples, achieving the correct identification of dermatophytes of the *genera Trichophyton*, *Microsporum*, *Epidermophyton*, and *Arthroderma* [224, 225].

Nenoff et al. [225] compared the findings for 285 clinical isolates obtained by mass spectrometry against conventional methods and ITS sequencing and discovered consistent results in 68.9% and 98.8%, respectively. Therefore, MALDI-TOF MS has been recommended as a routine tool for the precise and reliable identification of dermatophytes.

Regardless, MALDI-TOF MS has limitations. For example, the peptide spectra can be variable, although in some cases a sufficient number of signals are conserved and are still useful for the unequivocal identification of dermatophyte species. In other cases, however, the spectra can be uninformative, resulting in an impossible or erroneous identification [182, 222].

As previously observed, both PCR and MALDI-TOF MS also have some limitations, and thus, they do not provide a complete substitute for conventional methods; however, these techniques can still be used as complementary methods to obtain a precise diagnosis [226].

New techniques are currently utilized, such as confocal reflection microscopy, which provides in vivo imaging of the epidermis and dermis at cellular resolution, revealing the presence of dermatophyte fungi and other parasites. The advantage of this test relies on its noninvasiveness and high sensitivity, which has been rated at 100%. Moreover, it is comparable to other conventional diagnostic methods with good results [227]. Another technique, immunochromatography, is currently being tested for the detection of dermatophytes in skin and nail samples with good results [228, 229]. In addition, Raman spectroscopy, a photonic test that allows the identification of organic and inorganic materials based on the scattering of monochromatic light, has also been employed for the detection and identification of *T. rubrum*, *T. tonsurans,* and *T. mentagrophytes* in nail samples; it is also capable of identifying non-dermatophyte species such as *S. brevicaulis* and *C. albicans* [230]. The results obtained using confocal reflection microscopy, immunochromatography, and Raman spectroscopy support their potential use as diagnostic tools. Regardless, further research is needed to validate these results.

13.11 Detection Methods

Wood lamp skin examination (UV light). Utilized for the direct diagnosis of *tinea* capitis caused by a species of *Microsporum* of zoophilic origin, this test is performed in a dark room, in which UV light is projected onto the infected hair and scalp, resulting in blue-green fluorescence emitted by these organisms.

KOH test (10–40%)/Chlorazol Black E (Delasco, Council Bluffs, LA). Hair or hair scales are transferred onto a slide, and KOH is added with or without DMSO. The slides are then sealed with a coverslip and analyzed under a microscope to visualize the hyphae. Regarding the hair itself, two types of microscopic invasions can be observed: the hair shaft and medulla are uniformly invaded with spores (ectothrix), or the spores are contained within the hair structure (endothrix).

Sample collection. This process is dependent upon the anatomical area of the infection. In the case of *tinea* in glabrous skin, a No. 15 scalpel blade is used to scrape the active edge of the lesion. When the scalp is sampled, hairs must be torn out, and the scalp must also be scraped using a surgical blade or a curette. When sampling a potentially infected nail, either the nail plaque or the nail bed, depending on the localization of the infection, is carefully scraped to avoid damage or bleeding in the patient. The obtained samples are placed in a slide or petri dish and sent to the laboratory for further analysis.

Calcofluor white. This is a direct test substituting KOH with a fluorescent stain (Calcofluor white M2R 1 g/L and Evans blue 0.5 g/L). The sample is placed on a slide and observed under a fluorescence microscope (380–440 nm wavelength, 100x), revealing a bright green or blue signal.

Fungal culture. The following culture media are used for the isolation of dermatophytes: Sabouraud Chloramphenicol and Actidione agar and SDA. Although fungal colonies grow slowly, taking up to 10–15 days, these media are useful for confirming a diagnosis of *tinea*. The samples used in this process are obtained from skin, scales, nails, and hair, which are transported to the mycology laboratory in sterile containers for later inoculation in SDA with or without antibiotics. For a culture to be considered negative, it must be incubated for 21 days.

13.12 Molecular Identification

ITS PCR. The primers used were as follows: ITS1-F (5′-TCCGTAGG TGAACCTGCGG-3) and ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′), as reported by White et al. [231]. PCR was performed in a final volume of 25 μL as follows: 10 ng gDNA, 2.0 mM $MgCl_2$, 200 μM dNTPs, 1.0 U *Taq* polymerase, and 0.5 μM of each primer. Amplification was conducted under the following conditions: 96 °C/6 min; 35 cycles at 94 °C/1 min, 56 °C/1 min, 72 °C/45 s; and a final

extension at 72 °C/10 min. The amplicons were analyzed by electrophoresis in a 1.5% agarose gel stained with gel red (30X, Biotium) (100 V/60 min, TBE 0.5X), and a 100-bp molecular weight ladder was used to determine band sizes. The results were visualized under UV light (254/365 nm) and documented electronically (e.g., GelDoc™XR). Amplification and sequencing of other genes are recommended to aid in the molecular identification of species that form complexes.

BT2 gene PCR. The primers used for PCR amplification were as follows: T1-F (5′-AACATGCGTGAGATTGTAAGT-3′) and Bt2b-R (5′-ACCCTCAGTGTAG TGACCC-TTGGC-3′), as reported by Rezaei-Matehkolaei et al. [177]. The PCR was performed in a final volume of 25 μL as follows: 10 ng gDNA, 2.0 mM $MgCl_2$, 200 μM dNTPs, 1.0 U *Taq* polymerase and 0.5 μM of each primer. Amplification was conducted under the following conditions: 96 °C/6 min; 35 cycles at 94 °C/1 min, 56 °C/1 min, 72 °C/45 s, and a final extension at 72 °C/10 min. The amplicons are analyzed by electrophoresis in a 1.5% agarose gel stained with gel red (30X, Biotium) (100 V/60 min, TBE 0.5X). A 100-bp molecular weight ladder was used. The results will be visualized under UV light (254/365 nm) and documented electronically (e.g., GelDoc™XR). Amplification and sequencing of other genes are recommended to aid in the molecular identification of species that form complexes.

Amplicon sequencing. Amplicons were sequenced, and the electropherogram is edited using analysis software such as BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) or Geneious (http://www.geneious.com) [232, 233]. The edition criteria are based on consensus sequences.

Sequence analysis. The obtained sequences from the analyzed gene(s) were compared against the GenBank (URL4) database using the Basic Local Alignment Search Tool (BLASTn) algorithm [234]. The aligned sequences are analyzed for similar percentage, expectation and identity values.

Phylogenetic analysis. After editing and analysis, the sequences are aligned using ClustalΩ, MAFFT, KALIGN, MUSCLE, and MultiAlin (URL5-9), seeking the best alignment through pair sum and minimum entropy criteria, followed by election of the best model of evolution using the software Jmodeltest [235]and considering the parameters of likelihood, Bayesian information criterion, Akaike information criterion, and decision theory.

The phylogenetic analysis can be performed using several methods, including maximum verisimilitude, Bayesian inference, and maximum parsimony with software programs such as MEGA 7.0, Mr. Bayes 3.2.2, and TNT [236–238].

13.13 Treatment

Scalp ringworm is effectively treated with griseofulvin in daily doses of 10–20 mg/kg for 8–12 weeks [239]. Selenium disulfide (2.5%) or azole shampoos can be added to eliminate viable spores from the scalp surface. Concerning kerion, some

clinicians recommend prednisone at daily doses of 2 mg/kg during the first 2 weeks along with antimycotics to decrease scabs and alopecia. Terbinafine can be used with equal efficacy, at a dose in infants of 3–6 mg/kg/day and in adults of 250 mg/day. Another alternative treatment of *Mycrosporum* tinea is itraconazole at 100 mg/day for 4 weeks or 3.3–6.6 mg/kg/day in infants. It is possible to use fluconazole at weekly doses of 6–8 mg/kg/day for 8 weeks [240].

In other anatomical locations, such as the body, groin, hands, and feet, only systemic antimycotics are recommended for the treatment of disseminated infection that is resistant to local treatment, recurrent infection or infection with severe inflammation. In adults, the oral doses administered are griseofulvin 500 mg/day and ketoconazole 200 mg; the latter is recommended only for short term treatments given the risk of hepatitis; itraconazole 100 mg/day, terbinafine 250 mg/day for 2–4 weeks, and fluconazole 150 mg in a single weekly dose.

In frequent, noncomplex manifestations, the use of topical drugs is usually sufficient. Iodine creams (0.5 and 1%), Whitfield ointment (Vaseline with 3% salicylic acid and 6% benzoic acid), tolnaftate, tolciclate, tolindate, pyrrolnitrin and undecylenic acid, topical imidazoles, naftifine or terbinafine, cyclopyroxolamine, and butenafine, are used once or twice daily.

Onychomycosis are generally resistant to topical treatment, and drug penetrance is frequently increased by occlusion and with transport coadjuvants. It is usually convenient to eliminate the infected keratin by partial surgical removal or via chemical means such as 40% urea [241]. The suggested systemic drugs are itraconazole 200 mg/day for 3 months or 400 mg/day for 1 week a month in intermittent therapy (4 months); terbinafine 250 mg/day for 3–4 months or 500 mg/day for 1 week a month in intermittent therapy (3–4 months); or fluconazole 150 mg/week for 8–24 months or at a 300 mg dose for a shorter time period. Other local treatments are 28% tioconazole, 5% amorolfine, 8% ciclopirox (alone or combined with hidroxypropilchitosan), or 1% bifonazole combined with 40% urea [242]. Additional treatments currently in trials include CO_2 laser, Q-switch, and photodynamic therapy. A combined systemic and topical treatment is more efficient because it allows the use of itraconazole, terbinafine, or fluconazole for shorter periods of time, thus avoiding secondary effects.

13.14 Conclusions

Dermatophytoses are the most common fungal infections in the world, with both public and veterinary health relevance; however, many features of their taxonomy, epidemiology, pathogeny, and diagnosis remain unknown.

In the taxonomy of dermatophytes, no consensus has been achieved regarding the number of species that make up each complex, supporting the suggestions of Guarro et al. [243] who suggest that some of the different concepts related to species being utilized are difficult to grasp. Therefore, mycologists should clearly define the criteria they are using to identify species.

Epidemiology is a subject in which new data are often made available, sufficiently altering established views regarding the epidemiological status of these fungi. Seeking to correct this situation, advances in molecular biology have provided invaluable knowledge on the identification, taxonomy, and epidemiology of dermatophyte species and have greatly contributed to unraveling the role played by dermatophytes in their associated diseases. The taxonomy of these fungi has been reviewed by phylogenetic analyses of the ITS region in particular. Although, to date, additional markers with good resolution have been used, no specific markers are yet suitable for the classification of dermatophytes. Furthermore, it must be highlighted that a single marker is insufficient to determine the relationship between isolates from different geographical areas or to place the dermatophytes within the appropriate taxon [244]. Therefore, the use of several markers is recommended to obtain optimal and reliable results for the taxonomic and epidemiological classification of dermatophytes.

During pathogenesis, dermatophytes can produce diverse clinical manifestations that develop in a manner that is dependent on a series of virulence factors. These factors act in a sequential manner to confer the capacity to adhere to the keratinized epithelium and degrade keratin. It must be mentioned that such mechanisms are potential targets for disease control concerning fungal infection, and studies are currently underway with the goal of developing an effective vaccine. Profound knowledge of these pathogenic mechanisms will provide greater insight into the development of future treatments against these microorganisms.

It is of utmost importance, when concerning dermatophytosis, to be aware of their localization and major clinical characteristics, as both shall undoubtedly assist clinicians in proper identification amidst the broad variety of skin maladies with similar symptoms. The clinician should also rely on laboratory tests to act accordingly.

Concerning diagnosis, previous knowledge regarding the etiology of dermatophytoses is imperative before the election of an adequate antifungal therapy. Moreover, it will aid in assessing both origin and prevention measures. Therefore, clinical suspicion must be promptly followed by proper confirmation with laboratory tests to determine the specific etiology of the infection in question, as fungi are notably diverse in their response to the available antifungal treatments. Electing a unique method for diagnosis confirmation in the laboratory is a complex task due to the different advantages and disadvantages of either conventional or molecular techniques. Correct identification of the pathogen will unquestionably lead to proper handling of the patient.

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