Journal of Equine Veterinary Science 40 (2016) 62–73

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Review Article

Coccidioidomycosis and Histoplasmosis in Equines: An Overview to Support the Accurate Diagnosis

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ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form 9 February 2016
Accepted 9 February 2016
Available online 21 February 2016

Keywords:
Coccidioidomycosis
Histoplasmosis
Equines
Epidemiology
Pathogenesis and diagnosis

ABSTRACT

Fungal infections of the respiratory tract of horses are not as frequent as those of bacterial and viral origin, often leading to worsening of clinical conditions due to misdiagnosis and incorrect treatment. Coccidioidomycosis and histoplasmosis are systemic mycoses caused by the dimorphic fungi *Coccidioides* spp. and *Histoplasma capsulatum*, respectively, which affect humans and a variety of other animals, including equines. These systemic mycoses of chronic and progressive nature can exhibit clinical manifestations similar to other microbial infections. Thus, this article broadly discusses the epidemiology, etiology, virulence, pathogenesis, clinical presentation, treatment, and diagnostic strategies of coccidioidomycosis and histoplasmosis, to support accurate diagnosis.

1. Introduction

Coccidioidomycosis and histoplasmosis, respectively caused by *Coccidioides* spp. and *Histoplasma capsulatum*, share some similarities: both etiologic agents are saprophytic dimorphic fungi that can infect humans and several other mammal species, including horses, mainly through the inhalation of fungal propagules that are present in the environment, and both diseases have diverse clinical presentations [1–3]. Throughout the last decades, several researches focusing on novel diagnostic strategies for human coccidioidomycosis and histoplasmosis have been published, which have contributed for better understanding these diseases in human hosts. However, literature on equine coccidioidomycosis and histoplasmosis is still scarce, as it is mainly limited to a few case reports. Curiously, in some regions where these mycoses seem to be endemic, according to the notification of human cases, the diagnostic resources in veterinary practice are still insufficient. In fact, the broad spectrum of clinical presentations of coccidioidomycosis and histoplasmosis in horses can lead to misdiagnosis. Besides the difficulty of establishing the clinical diagnosis of systemic mycoses, these diseases are not of compulsory notification [4], which contributes for the little epidemiologic data on coccidioidomycosis and histoplasmosis in horses. Furthermore, it is important to highlight the technical limitations associated with the manipulation of the culture of these fungal pathogens, which are restricted to biosafety level 3 laboratories [5]. All these issues justify the development of this work that

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http://dx.doi.org/10.1016/j.jevs.2016.02.230

makes a parallel approach of these mycoses in humans and horses, mainly considering the great number of scientific papers on human medical literature addressing this theme, but at the same time highlighting peculiar aspects of these mycoses in horses. Initially, taxonomic, morphologic, and eco-epidemiologic aspects of *Coccidioides* spp. and *H. capsulatum* var. *capsulatum* were quickly presented, followed by the discussion of the virulence and pathogenesis of these fungal pathogens and a description of the clinical presentations and treatment of coccidioidomycosis and histoplasmosis. Finally, different strategies for the diagnosis of these mycoses are thoroughly discussed and presented in this article.

2. Taxonomy, Morphology, and Eco-epidemiologic Aspects of *Coccidioides* spp. and *Histoplasma* spp.

The genera *Coccidioides* and *Histoplasma* are classified in the Onygenales order, which is contained in the Eurotiomycetes class, in turn included in the Ascomycota phylum, belonging to the Fungi kingdom [6]. *Coccidioides immitis* and *Coccidioides posadasii* are the only species in the genus *Coccidioides*. The first species is native and restricted to San Joaquin Valley, California, United States, whereas the second presents larger geographic distribution, occurring in other states in the United States, and other countries, such as Mexico and Central and South America [7,8]. This genus belongs to the Onygenacea family [9].

In turn, the *Histoplasma* genus is part of the Ajellomycetaceae family and presents the species *Histoplasma capsulatum*, whose teleomorph is named *Ajellomyces capsulatus* [6]. This species has three varieties: *H. capsulatum* var. *capsulatum*, which is pathogenic to humans and a variety of mammals, including equines causing classic histoplasmosis [10–13]; *H. capsulatum* var. *duboisii*, restricted to Central Africa, which causes African histoplasmosis, mainly in humans; and *H. capsulatum* var. *farciminosum*, endemic to western, northern, and northeastern countries in Africa and Asia, including India, Pakistan, and Japan, causing epizootic lymphangitis in horses [2,14].

Coccidioides spp. have a mycelium formed by hyaline and septate hyphae that originate arthroconidia, which are asexual reproduction structures, measuring 2–4 μm by 3–6 μm, intercalated with sterile cells devoid of cytoplasmic material known as disjunctor cells (Fig. 1A). When reaching maturity, the infective arthroconidia are released, and the remnants of the disjunctor cells are seen at the end of individual arthroconidia. This characteristic is responsible for their easy aerial propagation. The yeast form is named spherule, which are large rounded structures with thick walls that measure 20–200 μm in diameter. Each spherule produces a large number of small endospores inside, measuring 2–4 μm in diameter (Fig. 1B) [15].

The filamentous form of *H. capsulatum* shows a mycelium composed of hyaline, septate, and ramified hyphae, with two types of conidia. The first, microconidia, are oval structures with smooth walls and a diameter of about 2–4 μm, which are easily dispersed in the air due to their small size and are considered the main infective propagules of this fungus [16]. The second type, macroconidia, also called tuberculate macroconidia, are round large structures, measuring 8–15 μm, with a rough wall covered with distinctive projections (Fig. 1C) [16–18]. The yeast form presents small oval uninucleate cells, measuring about 3–5 μm in diameter (Fig. 1D) [17,18].

Coccidioides spp. are geophilic fungi that develop in soils with high salinity and alkaline pH, usually found at a depth of 10–50 cm [7]. They are associated with arid and semiarid regions, which have an average temperature above 30°C, recurrent droughts, and poor and sparse xerophytic vegetation [19]. The survival of *Coccidioides* species is substantially reduced by competition with other microorganisms in the soil [15]. Several studies have confirmed the importance of armadillo burrows in the ecology of *Coccidioides* spp. especially in Northeastern Brazil [20,21]. There are also reported cases of coccidioidomycosis associated with contact with rodent burrows [22].

The saprophytic phase of *H. capsulatum* is also geophilic, but it has been associated with soils rich in nitrogen compounds, with acidic pH and high moisture, which are characteristics found in soils enriched with the feces of birds and bats [23,24]. Thus, places like caves, hollow trees, chicken pens, and abandoned houses and parks are likely sites of colonization by this fungus [25]. Bats are susceptible to infection caused by *Histoplasma capsulatum* var. *capsulatum* and actively participate in the epidemiology of histoplasmosis. By their migration, these animals can disseminate the pathogen from one location to another, through the elimination of viable forms of the fungus in

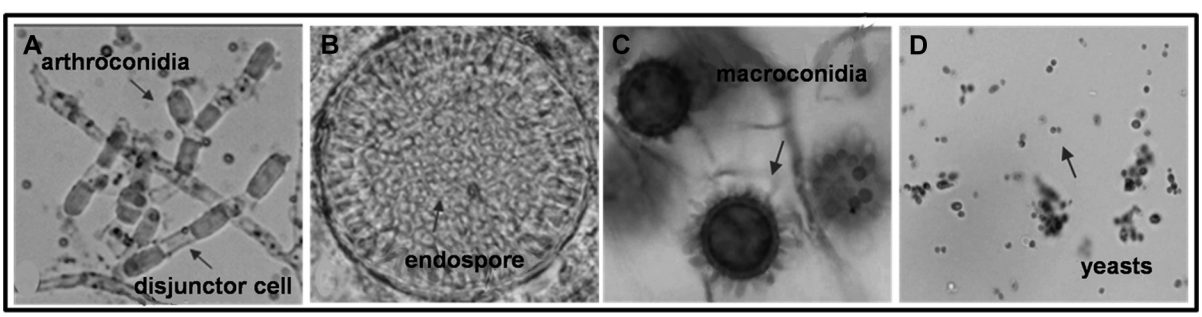

Fig. 1. Micromorphologic aspect of *Coccidioides spp.* (A and B) and *H. capsulatum* (C and D). (A) Filamentous phase, showing arthroconidia and disjunctor cells; (B) Yeast phase, demonstrating a mature spherule with numerous endospores; (C) Filamentous phase, showing tuberculate macroconidia; (D) Yeast phase, showing oval and rounded yeasts.

guano [26,27]. Birds are rarely affected, probably because of their high body temperature, around 41°C, but it is believed that they carry the organism on their wings, feathers, and beak [28,29]. The epizootic lymphangitis, disease caused by *Histoplasma capsulatum* var. *farciminosum*, rarely affect humans, cattle, and camels; however, its occurrence predominates in horses, donkeys, and mules [30].

Considering the reports of human cases and environmental isolation of both fungal agents, it is estimated that the prevalence of coccidioidomycosis and classic histoplasmosis is high in the Americas (Fig. 2). However, coccidioidomycosis has a limited geographic distribution, with the largest incidence of the disease being described in the United States, where several endemic areas have been identified, such as in Nevada, Colorado, Arizona, California, New Mexico, Texas, and Utah [5]. Outbreaks have also been reported in northeastern Mexico [31]. Additionally, several countries in Central and South America, such as Guatemala, Honduras, Argentina, Paraguay, Bolivia, Colombia, Venezuela, and Brazil, are also considered endemic areas [8,32]. Although human coccidioidomycosis has been known since 1889 [33], in equids, this disease was initially described in mules [34]. The oldest reports of this disease in horses were described in areas where coccidioidomycosis is endemic [35]. Since then, several researches have been published approaching epidemiologic surveys with the intradermic use of coccidioidin and isolated clinical cases of this mycosis in equids [36]. Cases of equine coccidioidomycosis have been reported by Zontine [37], Crane [38], Langham et al. [39], Reed et al. [40], De Martini and Riddle [41], Hodgin et al. [42], Stoltz et al. [43], Kramme and Ziemer [44], Ziemer et al. [36], and Walker et al. [45].

In contrast, *H. capsulatum* var. *capsulatum* has cosmopolitan distribution. Thus, classical histoplasmosis, although more prevalent in the Americas, has a wide geographic distribution, having been reported in more than 50 countries. There are several endemic areas in the central and southern regions of the United States in the valleys of the Mississippi and Ohio rivers. It is also found on several Caribbean islands and countries such as Mexico, Honduras, Guatemala, Nicaragua, and Panama. In South America, Venezuela, Colombia, Peru, Argentina, Uruguay, and Brazil also have high prevalence of histoplasmosis. Sporadic autochthonous cases of histoplasmosis have also been reported in Africa, Asia, and Europe [18,23]. Although this disease is considered to be the most common systemic mycoses in humans, the reports of histoplasmosis in horses are rare [46]. It is estimated that 50%–73% of horses from endemic areas are positive for the intradermic tests with histoplasmin. Moreover, cases of equine classic histoplasmosis have been reported by Hall [47], Goetz and Coffman [48], Johnston et al. [11], Richter et al. [12], and Nunes et al. [13].

Cases of infections caused by *H. capsulatum* var. *farciminosum* have been reported in Iraq, Egypt, Sudan, Central African Republic, Nigeria, Italy, Russia, UK and Ireland, Japan, China, and India [49]. Most of the recent reports are from Ethiopia [50–53], with high prevalence in towns in mid-altitude regions between 1500 and 2300 m above sea level. The Office International des Epizooties (OIE) previously listed epizootic lymphangitis as a list B disease; however, the infection is no longer listed as notifiable, although still prevalent in some countries [54].

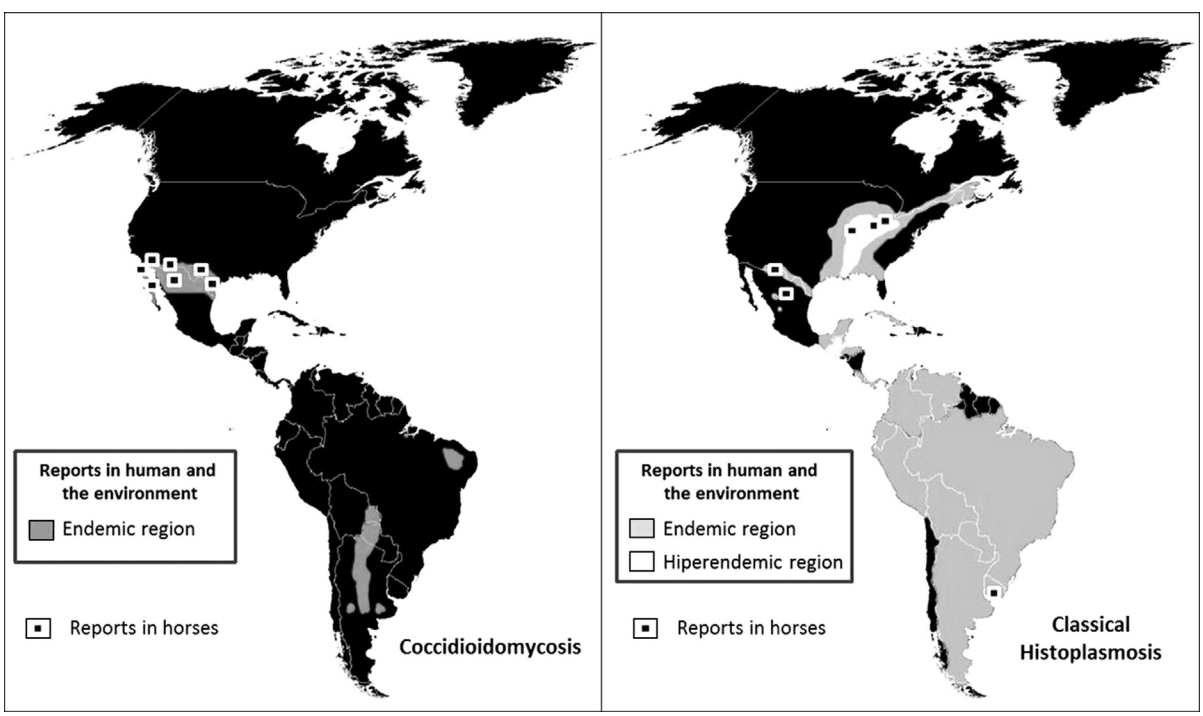

Fig. 2. Endemic areas for coccidioidomycosis and histoplasmosis in the Americas, considering the notifications of human cases, environmental recovery, and reports of cases of these mycoses in horses.

3. Virulence and Pathogenesis of *Coccidioides* spp. and *H. capsulatum*

Coccidoides spp. and *H. capsulatum* var. *capsulatum* are able to cause disease even in immunocompetent individuals. They have biochemical and morphologic characteristics that allow them to find a microenvironmental niche with sufficient nutritional substrates to survive and multiply in the host. Moreover, these two pathogens have virulence factors that allow them to avoid, subvert and/or rupture the immunologic defenses of the host. Therefore, they are classified as primary pathogens and considered to be the most virulent and infective fungal agents [55,56]. The dimorphism presented by *Coccidoides* spp and *H. capsulatum* is considered one of the most relevant virulence factors in the pathogenesis of these fungi. The establishment of these. pathogens in the host directly depends on the conversion of the filamentous phase to the parasitic yeast phase [57]. This phase transition results not only in a morphologic change of the cell but also in an alteration in the antigenic composition of the cell wall, as well as in the expression of virulence related genes. Thus, in response to high temperatures and other adverse conditions found in the host environment, these pathogens express several genes that are specific for the yeast phase, some of which seems to contribute for the virulence of these fungal pathogens [58].

One of the major virulence factors specific for *Coccidioides* spp. is the production of an immunodominant antigen, the glycoprotein SOWgp, which is deposited on the external wall of the spherules. The exposure of the host to the SOWgp modulates the immune response to the Th2 pathway. The persistence of a Th2 immune response is advantageous to the pathogen because it impairs the cellular immunity of the host [59]. Moreover, a metalloproteinase (Mep1) secreted during differentiation of endospores digests the cell surface of the spherules, thus resulting in a depletion of the immunodominant antigen SOWgp, preventing recognition of free endospores by the host's immune system during their development, when they are most vulnerable to phagocytic cells [60].

Histoplasma capsulatum, in the parasitic phase, modifies the biosynthesis of the glucans that makes up its cell wall, leading to the production of a layer of α-1,3 glucan. The presence of α-1,3 glucan is related to pathogenicity and virulence of the strain as it prevents the detection of the fungus by the innate immune system, hence, interfering with the activation of cellular immunity and allowing them to remain inside the phagolysosomes [55]. The YPS3p protein encoded by the *yps-3* gene is also found as a constituent of the wall of the *H. capsulatum* yeast. It is believed that this protein participates in the dissemination of the fungus to extrapulmonary sites [61]. Moreover, the ability of *H. capsulatum* to survive and proliferate within macrophages is mediated by factors such as siderophores associated with the *S1D1* gene [62], responsible for the acquisition of iron and calcium binding protein (CBP1), encoded by the *CBP1* gene, responsible for binding and transporting Ca^{2+} to the intracellular environment [63].

Human and animal coccidioidomycosis and classical histoplasmosis are acquired through inhalation of infectious conidia produced in the saprophytic filamentous phase of the fungi *Coccidioides* spp. and *H. capsulatum*, respectively. In soil, the conidia of these pathogens can be easily dispersed in the air, after which they can follow one of two paths: germinate and perpetuate the saprobic cycle or be inhaled by a susceptible host and start the parasitic cycle, converting to the yeast phase (Fig. 3) [64].

Histoplasma capsulatum var. *farciminosum* is a soil saprophyte fungus. Contact of injured skin with material contaminated by the mycelial form of the pathogen is the main via of infection [51]. A study showed the reproducibility of epizootic lymphangitis after experimental infection of two horses with yeast and mycelial form of the fungus [65]. Biting flies of the genera *Musca* or *Stomoxys* are believed to spread the conjunctival form of the disease [66]. Also, pulmonary infection can be acquired by inhalation of fugal conidia [54] however is uncommon. In endemic areas, the seasonal dusty winds expose horses to the inhalation of dust and spores, leading to pneumonia [30].

In coccidioidomycosis, the initial response of the host targets the arthroconidia and is characterized by influx of polymorphonuclear leukocytes that respond to chemotactic substances generated in response to the activation of the complement system. In the first 72 hours, the arthroconidia are converted to spherules. The spherules form many endospores inside their structures through cell wall thickening and fragmentation of the cytoplasm. Afterward, the inflammatory response shifts to infiltration of mononuclear cells, which persists throughout infection, leading to formation of granulomas [1]. The spherules rupture at maturity, releasing endospores, which in turn undergo isotropic growth and progressively mature. Thus, each endospore acts as a new infectious agent, growing and differentiating into a new spherule, which is again able to produce 200 to 300 endospores, restarting the parasitic cycle [67]. Mature spherules can easily evade phagocytosis because they are too large to be ingested by neutrophils, macrophages, and dendritic cells. Endospores released from immature spherules are susceptible to phagocytosis; however, *in vitro* studies have shown that some endospores may survive. Therefore, the participation of T cells is highly effective against *Coccidioides* spp., as they potentiate the action of phagocytes [67].

In classical histoplasmosis, inhaled spores reach the pulmonary alveoli, stimulating an inflammatory response in the host, composed of mononuclear cells and macrophages. After conversion to the yeast phase, the fungal cells bind to receptors (CD11/CD18) on the surface of resident alveolar macrophages and are, then, engulfed. Afterward, phagosomes containing the blastoconidia are fused with lysosomes. Thus, the yeast cells survive and multiply within phagolysosomes [68]. Activation of macrophages by the immune system results in a granulomatous reaction followed by scarring, fibrosis, and calcification [23]. In the absence of an immunologic barrier, the causative agent of histoplasmosis can be transported via the lymphatic system to the mediastinal lymph nodes and disseminate to other organs and systems through the blood, producing new inflammatory foci in the liver, spleen, and bone marrow. Immunocompromised patients present the disseminated form of the disease, as there is no

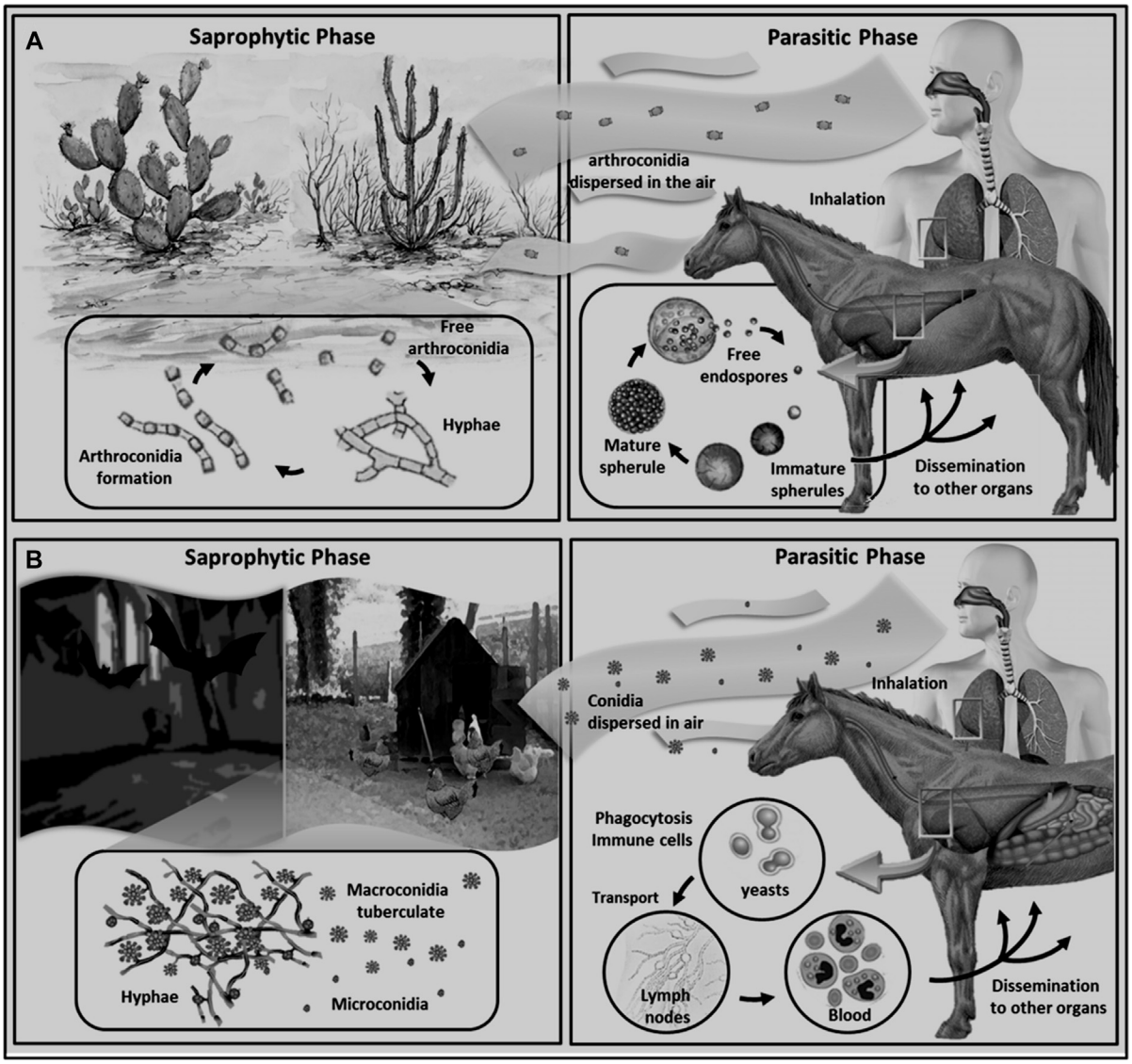

Fig. 3. Biological cycle of *Coccidioides spp.* (A) and *H. capsulatum* (B), showing the saprophytic phase in the environment (on the left) and parasitic phase after being inhaled by a susceptible host (on the right). Image created by the authors, based on an active search through the internet (www.medicalook.com/Lung_diseases/Histoplasmosis.html; acxmassa.blogspot.com/2012/02/vegetacoes-da-caatinga-ne-brasil.html; pt.dreamstime.com; fungalinfections.wordpress.com; www.cdc.gov; www.thinklikeahorse.org).

inflammatory reaction or efficient formation of compact granulomas to prevent the spread of the fungus. Moreover, the pathogen *H. capsulatum* can remain latent for many years before reactivating in cases of immunosuppression [18,23,24].

4. Clinical Presentation of Coccidioidomycosis and Histoplasmosis

Coccidioidomycosis and classical histoplasmosis are primarily pulmonary diseases but can present a wide spectrum of clinical manifestations. The severity and progression of these mycoses will be determined by the amount of inhaled particles, immune status of the host, and virulence of the infecting strain [17,69]. Thus, these infections can be merely asymptomatic or acute but self-limited in most hosts. However, in susceptible individuals, the infection can also progress to a chronic disease and lead to dissemination, infecting other organs and systems [17,69].

In this context, coccidioidomycosis has three main clinical forms: primary pulmonary, progressive pulmonary, or disseminated [70]. In humans, primary pulmonary infection is asymptomatic in 60% of cases. Infected individuals have no symptoms or exhibit common symptoms of upper respiratory tract infection [71], ranging from flu-like to severe nonspecific respiratory infection in more severe cases. Primary pulmonary coccidioidomycosis usually resolves spontaneously within 30–60 days, even without antifungal treatment [72]. In some cases, acute

pulmonary coccidioidomycosis regresses, without progressing to chronic pneumonia. This progressive pulmonary form is usually chronic and evolves from infections that do not resolve after 2 months. These forms can cause nodular or cavitary lesions, lung disease with fibrosis or miliary pulmonary dissemination with nonspecific clinical and radiological manifestations [72]. The most severe form of the disease is disseminated and can quickly reach several organs and systems, leading to death if not diagnosed and treated. However, only approximately 1%–5% of patients with primary pulmonary evolve to dissemination, with skin lesions as the most common extrapulmonary location. Disseminated lesions are also usually observed in bones, joints, central nervous system, and genitourinary system [69].

In equines, the cases described traditionally are severe and often fatal [73]. In general, when clinical symptoms are present, the disease is already severe. The animals may have fever, chronic weight loss, cough and/or tachypnea, and leukogram results show inflammatory characteristics, such as hyperfibrinogenemia, nonregenerative anemia, leukocytosis with mature neutrophilia and monocytosis, and hyperglobulinemia [74,75]. The clinical presentations include interstitial pneumonia with or without pleural or pericardial effusion, cutaneous lesions, and disseminated osteomyelitis [43,45,67]. Miscarriages accompanied by placentitis, fetal infection, and osteomyelitis with or without respiratory signs have also been reported [43,76]. Mastitis can also occur as a result of disseminated infections [45]. Apparently, horses have greater risk of developing the disseminated form of the disease, with unfavorable prognosis [46]. Risk factors for severity of infection include the breed, with Arabians being more susceptible than Quarter Horses and thoroughbreds, as well as mares during pregnancy [2]. In Arizona, about 4% of the horses in endemic areas show subclinical infections [4].

Classical histoplasmosis, in turn, can be clinically classified as acute pulmonary, chronic pulmonary, or disseminated [77]. In humans, the acute pulmonary disease is observed in most patients, of which about 90%–95% are asymptomatic, showing no clinical signs. Symptomatic cases generally show symptoms similar to a viral respiratory infection. A more serious acute pulmonary condition can also be observed, especially when there is massive aspiration of infective particles of the fungus. Symptoms disappear after 2 to 4 weeks, by spontaneous regression without treatment [17,23,24]. Chronic pulmonary histoplasmosis, in turn, affects people >50 years, smokers and patients with chronic obstructive pulmonary disease. The clinical signs are quite similar to those observed in chronic pulmonary tuberculosis, though, less severe [78]. Disseminated histoplasmosis is the most serious form of the disease, occurring extrapulmonary and extramediastinal foci of the infection, with a progressive course. The dissemination commonly results in infiltration of lymph nodes, liver, spleen, bone marrow, adrenal glands, skeletal system, and integument. The clinical signs vary according to the organs and systems involved [18,79]. About 90% of cases of dissemination are fatal when not properly treated [18,77]. Immunocompromised patients, especially HIV positive individuals, are the most affected, although a small number of people who are healthy or have transient immunosuppression develop disseminated histoplasmosis [80].

In equines, there are few classical histoplasmosis cases reported in the literature when compared to those described in humans, dogs, and cats. However, reports indicate that these animals are commonly exposed to the fungal propagules in the environment, most likely resulting in asymptomatic infections. This hypothesis is based on researches performed in endemic areas which showed that 50% of 467 healthy horses raised in Central Kentucky (USA) and 73% of 44 horses from Missouri (USA) were positive for histoplasmin intradermic test, as were 8.7% of 51 horses from Uruguay and 7.9% of 2221 horses from Mexico [46]. Histoplasmosis can be presented as localized infections in different organs and tissues, such as lungs, bone marrow, placenta, eyes, colon, cecum, and mesenteric lymph nodes [10,12,13,48]. The disseminated form is less frequent and can involve the lungs, pleura, spleen, kidneys, liver, small intestine, and colon [10,11]. In general, the described clinical signs include dyspnea, weight loss, diarrhea, granulomatous colitis, placentitis, miscarriage, and keratitis [2]. In the disseminated form, generalized lymphadenopathy as well as pleural and peritoneal effusion have also been reported [11]. It is believed that the systemic infection is the result of immunologic compromise [11]. Blood work usually reveals nonregenerative anemia and thrombocytopenia. Several cases of disseminated histoplasmosis were described by Razabek et al. [10] in fetuses and newborn foals. It is noteworthy that the absence of generalized clinical signs in mares with infected foals or miscarried fetuses has been reported, indicating that ascending infections of the reproductive tract can occur [10]. Similarly, cases of gastrointestinal infection in horses, with no pulmonary involvement, have been described, suggesting that in these cases, the acquisition of classical histoplasmosis may have occurred by the oral route [13].

Histoplasma capsulatum var. *farciminosum* is the causative agent of epizootic lymphangitis, a fungal disease occurring manly in equids. The disease can be grouped into four different forms, namely: cutaneous, respiratory, ocular, and asymptomatic. The cutaneous form of the disease, after which has a tendency to ulcerate, or to undergo alternating periods of discharge and closure for some weeks before healing which residual scar formation [30]. It is characterized by multi-focal pyo-granulomatous sub-cutaneous nodules caused by the dissemination via the lymphatic system [49]. Thickening of lymphatics with the formation of purulent nodes can occur, and regional lymph nodes may be enlarged and inflamed [51]. Lesions are most common in the forelimbs, the chest wall, and the neck. In severe cases, skin over the entire body may be affected. The lesions begin as indolent, chancre-like papules, becoming larger over the course of weeks, and eventually form irregular pyogranulomatous nodules [30]. Severe debilitation caused by chronic disease may lead to multisystemic involvement of the organism [66]. Mortality does not usually exceed 10%–15%, and the main loss results from the inability of animals to work for several weeks because of extremely painful lesions [30].

Histoplasma capsulatum var. *farciminosum* also can cause less frequent infections as ulcerating conjunctivitis or

multifocal pneumonia [54]. Respiratory form is characterized by pyo-granulomatous lesions within the nasal mucosa that can extend throughout the respiratory tract to the lung parenchyma [49]. This form usually occurs as a late development in the cutaneous form of the disease. On the nasal mucosa, the lesions begin as yellowish papules or nodules and these soon form crater-like granulating ulcers that bleed easily. The lesions are usually found near the external nares. These lesions may also occur in the lungs. Asymptomatic carriers can be identified clinically by the identification of fibrocalcific skin lesions at previous sites of infection. Such horses will give positive reactions to serological tests [30].

5. Treatment

To start the treatment of coccidioidomycosis and histoplasmosis, it is necessary to know the extent of the infection and identify factors that predispose the host to the disease. In both cases, patients with localized pulmonary infections and no risk factors for complications often require only periodic reevaluation to demonstrate spontaneous resolution of their infection, with rest and clinical observation as the most effective measures. On the other hand, patients with extensive spread of infection or who are at high risk of complications due to immunosuppression or other preexisting factors require appropriate treatment strategies [69,78]. In this context, the existing treatments for acute, chronic, or disseminated forms of coccidioidomycosis and histoplasmosis can be prolonged and difficult to tolerate, because the therapy takes months to years. In some patients, suppressive antifungal therapy is needed throughout life to prevent relapse [69,78]. Among the therapeutic options currently available, azoles such as itraconazole, fluconazole, and more recently voriconazole have replaced amphotericin B in the treatment of most infections. They are used especially in patients with mild or moderate symptoms. Azoles are also commonly used as maintenance therapy after initial treatment with amphotericin B in patients with the most severe forms of the diseases [17,69]. Thus, although the use of amphotericin B is considered the gold standard for the treatment of these systemic mycoses, this drug has been reserved only for the most serious cases, such as rapidly progressive infection, severe immunosuppression, respiratory failure, or pregnancy, due to its high toxicity [69,78,81].

In animals, according to the Infectious Disease Committee of the American Association of Zoo Veterinarians [82], the treatment of these mycoses traditionally includes the use of amphotericin B, itraconazole, and fluconazole as the drugs of choice, with the latter being indicated for ocular and central nervous system involvement. Posaconazole and voriconazole are newer and effective drugs, but are expensive and have little information in veterinary medicine literature. For coccidioidomycosis, itraconazole may be more effective for skeletal lesions, but bone infections may be incurable. In addition, the treatment for this mycosis is recommended for 1–6 months after resolution of clinical signs, however, relapses may occur after treatment. For histoplasmosis, treatment should be done for 4–6 months and at least 1 month after resolution of clinical signs and/or after *H. capsulatum* antigen concentrations reach levels <2 ng/mL [82].

In equines, therapeutic success of coccidioidomycosis was reported for pulmonary forms in horses treated with fluconazole, which showed resolution of clinical signs in just 2 weeks [72]. Itraconazole was also effective in treating a foal with vertebral osteomyelitis during 9 months, without showing signs of toxicity [75]. In histoplasmosis, there are reports of effective treatment with the use of a 5-week regimen of amphotericin B against the pulmonary form [83] and a 10-day regimen of topical fluconazole against corneal *Histoplasma* infection [12]. Moreover, therapy with itraconazole should also be considered against histoplasmosis, despite the lack of data in equine veterinary practice [84].

6. Diagnostic Methods

The diagnosis of coccidioidomycosis and histoplasmosis is determined through a combination of clinical, epidemiologic, and laboratory data, with the latter being essential for a definitive diagnosis. Currently, the laboratory tests used are based on mycologic, histopathologic, immunologic, and molecular techniques [23,85].

6.1. Mycologic Detection

The mycologic diagnosis of coccidioidomycosis and histoplasmosis includes direct examination of clinical specimens through optical microscopy to detect parasitic structures that are characteristic of *Coccidioides* spp. and *H. capsulatum* [23,55]. The most common clinical samples in coccidioidomycosis are fresh sputum obtained by bronchial lavage, bone marrow aspirates, biopsy material from bone lesions and joints, urine, lymph node aspirates or biopsy, and other similar clinical specimens. Clinical samples should be evaluated under the microscope in wet mounts with 10% KOH or calcofluor white or [86,87]. The sample is considered positive when intact or ruptured spherules are observed (Fig. 4A). In histoplasmosis, the most frequent samples are buffy coat smears and bone marrow aspirates, which can be stained with Giemsa. Positivity is indicated by the presence of small ovoid structures measuring about 3–5 μm, surrounded by a clear halo, inside the macrophages and monocytes (Fig. 4B) [23,88].

Concomitant to the direct examination, fungal culture continues to be the gold standard for definitive diagnosis of these mycoses. *Coccidioides* spp. and *H. capsulatum* are not particularly demanding, so a variety of primary isolation media can be used to obtain these cultures. Mycological media commonly used include BHI (brain heart infusion) agar, potato dextrose agar, and Sabouraud dextrose agar, with or without chloramphenicol and cycloheximide [88,89]. Generally, the colonies of *Coccidioides* spp. grow at 30°C, within 3–5 days. It is rarely necessary to maintain the cultures for >3 weeks, except when animals are under antifungal treatment at the time of sample collection. The colonies are initially white to cream, with glabrous texture, growing adhered to the medium. As they mature, they exhibit filamentous areas with cottony aspect (Fig. 5A). In general, the colonies are white to off-white, although other

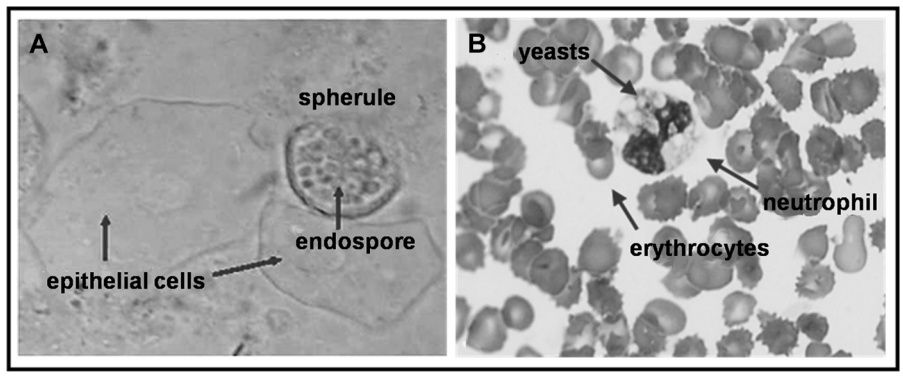

Fig. 4. (A) Direct examination of sputum in preparation with KOH 30%, showing spherules of *C. posadasii* in different stages of maturation; (B) Buffy coat smear stained with Giemsa, showing intracellular yeasts of *H. capsulatum*.

colors have been observed, such as variations of yellow, brown, gray, and lavender [56]. The *in vitro* reversion of the filamentous phase of *Cocdidioides* spp. to the parasitic phase is not routinely performed, not only because it requires specific growth conditions (high concentrations of CO_2 at 37°C in a rich medium), but mainly because of the biological risk of handling this fungal agent [90]. As for *H. capsulatum*, colonies grow well at room temperature, around 22–30°C, after 1–3 weeks of incubation, showing filamentous aerial mycelium with suede-like or cottony texture and white color that tends to darken over time (Fig. 5B) [88]. The *in vitro* conversion of filamentous phase to the yeast phase has also been used for a precise diagnosis of histoplasmosis. In this case, enriched nutrient media are required, such as BHI agar or Sabouraud agar supplemented with sheep blood, and the strains are incubated at 35–37°C. However, the dimorphic transition is a laborious process and requires 2–4 weeks, increasing the period to obtain diagnosis [23,88]. Macroscopically, the yeast colonies of *H. capsulatum* present white to brown color, creamy texture, and smooth or rough surface (Fig. 5C). Microscopically, small (3–5 μm) and oval yeast cells can be observed [18].

6.2. Histopathologic Detection

For proper analysis of histologic samples, special stains for fungi are generally used, such as Gomori-Grocott and periodic acid-Schiff (PAS). However, other stains such as hematoxylin-eosin and Giemsa have also been successfully used [15,17]. In coccidioidomycosis, histopathology is performed with tissue or fluid samples from cutaneous lesions, lungs, musculoskeletal system or brain, or with other suspicious material, including samples obtained from necropsy. In positive cases, spherules are visualized in different stages of maturation, with endospores inside (Fig. 6A) [15]. The inflammatory tissue consists of granulomas, with the presence of Langhans giant cells, plasmocytes, and lymphocytes [68]. In histoplasmosis, common biopsy samples include material from lungs, bone marrow, liver, and lymph nodes. The histopathologic examination is positive when macrophages containing spherical to oval yeast cells, surrounded by a very thin and hyaline cell wall, are visualized (Fig. 6B) [17,88,91]. The inflammatory tissue reveals the presence of granulomas with or without caseous necrosis in immunocompetent individuals, whereas in

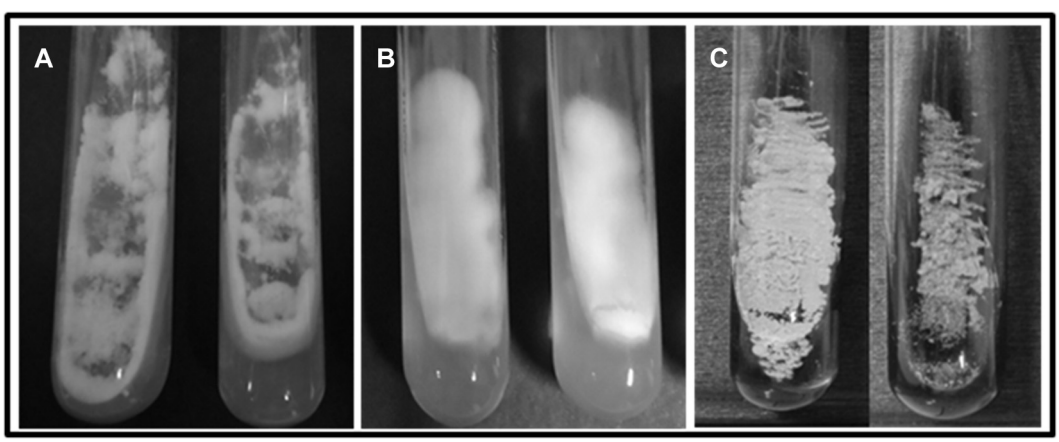

Fig. 5. (A) and (B) Macromorphological aspect of *Coccidioides spp.* (A) and *H. capsulatum* (B) after 15 days of incubation at 28°C on potato agar, demonstrating colonies with white color and cotton texture; (C) Macroscopic aspect of *H. capsulatum* in the yeast phase, after 15 days of incubation at 35°C on Sabouraud agar (on the right) and BHI agar (on the left) supplemented with 10% sheep blood, showing colonies with beige color and creamy texture, with smooth or rough surface.

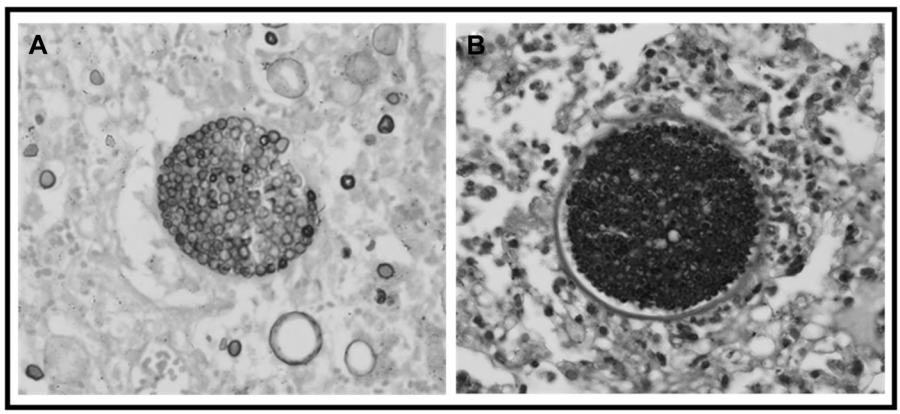

Fig. 6. Histopathologic aspect of *Coccidioides spp.* in biopsies from mice, showing mature spherules containing a large number of endospores stained with Gomori-Grocott (A) and PAS (B).

immunocompromised individuals, loose granuloma, lympho-histiocytic aggregates, or mononuclear diffuse infiltrates are often found [17].

6.3. Immunologic Detection

Serological tests are ancillary tools for the diagnosis of coccidioidomycosis and histoplasmosis, besides revealing important information on the epidemiology of the disease [78,92,93]. The immunologic techniques used have different levels of sensitivity and specificity [15].

In coccidioidomycosis, IgM antibodies become measurable early in the acute phase, between the first and third weeks of infection. IgG antibodies react at the end of the infectious process and remains for several months, so it can be used for the prognosis of infection [89]. The technique of complement fixation detects IgG antibodies in the progressive and disseminated forms of coccidioidomycosis and antibody titers are usually directly correlated with the severity of the disease. Immunodiffusion (ID) (Fig. 7A) can be applied to detect IgM and IgG antibodies, but they require longer incubation period (4 days) to reduce the occurrence of false negatives [89]. Enzyme immunoassays (EIA) are also available for the detection of IgM and IgG and apparently are more sensitive than the other methods. However, they may be less specific, especially if only the IgM test is positive. For instance, a retrospective analysis of EIA FC and ID of sera from human patients during the acute phase of the disease showed that the overall sensitivity of immunoassays is only 82%. Therefore, negative serology does not rule out coccidioidomycosis, especially in the early phase of infection [15]. A study conducted in the United States indicated that the number of animals with positive serology in endemic areas was low: only 4.1% (8 of 197 samples) of healthy horses had positive titers (IgG) for coccidioidomycosis [4].

In histoplasmosis, the serological diagnosis is based on the identification of anti-H and anti-M antibodies. Seroconversion occurs late in the course of infection, and 2 to 6 weeks are needed for the detection of antibodies. M and H antigens are the building standards used in double immunodiffusion (Fig. 7B) and complement fixation tests. The presence of the M and H precipitins is considered conclusive for diagnosis of histoplasmosis [88,94]. When properly executed, the serological tests assist the diagnosis of histoplasmosis, with sensitivity >90% [17]. Apart from immunodiffusion, the main techniques used for the serological diagnosis of histoplasmosis are complement fixation and radioimmunoassay. However, these methods have some limitations, mainly the occurrence of cross-reactions with other fungi that cause systemic mycoses such as blastomycosis, coccidioidomycosis, paracoccidioi-

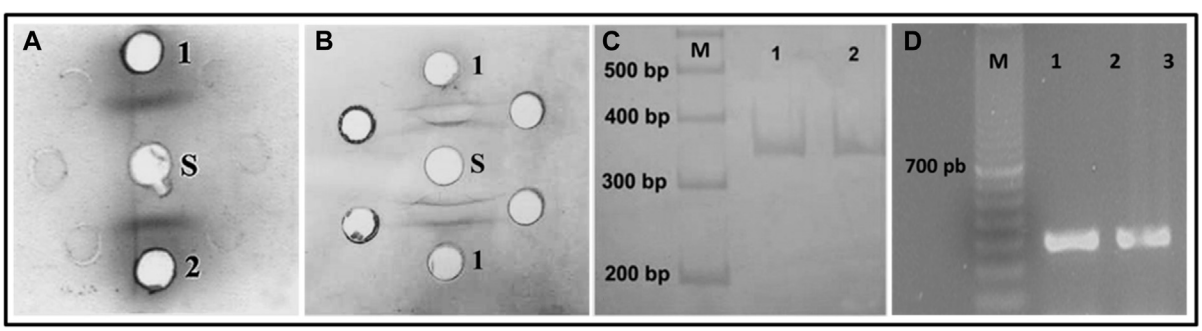

Fig. 7. (A) and (B) The immunodiffusion test positive for coccidioidomycosis in A and for histoplasmosis in B, showing the precipitation bands H and M, where: (S) patient sera, (1) commercial antigen and (2) experimental antigen; (C) and (D) PCR amplification product of gene Ag2/PRA of *Coccidioides spp.* in C and of gene 100 kDa-like protein of *H. capsulatum* in D, where (M) molecular marker (1) the test sample and (2) positive control.

domycosis, and aspergillosis [17,94]. Intradermal skin tests with histoplasmin are widely used in epidemiologic surveys but have no diagnostic value [88].

Methods for detection of antigens have also been used, especially when the detection of antibodies is unlikely, as in immunocompromised patients, whose antibody titers may not be reliable as they are unable to mount an immune response. Another important advantage is the ability to identify cases early in infection before seroconversion can be detected. For coccidioidomycosis, promising results in detection of specific antigens of *Coccidoides* spp. in urine by EIA were demonstrated by Durkin et al. [95], resulting in 71% of positivity [95]. For histoplasmosis, detection of antigens of *H. capsulatum* can be performed by radioimmunoassay or ELISA. These two methods are widely used in patients with acute histoplasmosis and/or severe disseminated histoplasmosis [18].

6.4. Molecular Detection

Among the molecular tests performed directly on clinical samples (the same specimens that are used for mycological diagnosis), polymerase chain reaction (PCR)–based tests are the most widely used techniques [88,96–98]. Different targets have been used in PCR reactions to identify *Coccidioides* spp. and *H. capsulatum*. Among the most used are the ITS region (internal transcribed spacer) of ribosomal DNA (rDNA) [99–102], followed by DNA sequencing for the identification of the fungal species. However, DNA sequencing-based techniques are not very useful in routine diagnostic laboratories, as they are costly and time demanding [103]. Other specific targets of each fungal genus have also been successfully explored. In *Coccidioides* spp., for instance, *in situ* hybridization targeting variable regions of the 18S and 28S ribosomal DNA has been used to identify *C. immitis* in formalin-fixed paraffin-embedded tissue sections. These procedures have shown a sensitivity of 94.3% and specificity and positive predictive values of 100% [96]. In addition, a PCR assay for the amplification of fungal DNA from serum samples has been described [98], and a nested PCR and a real time PCR were developed targeting the genus-specific antigen2/proline rich antigen of *Coccidioides* spp. (Fig. 7C) [97]. Both PCR assays correctly identified 120 clinical isolates, previously identified as *C. posadasii*. As for *H. capsulatum*, the gene encoding the 100-kDa protein HC100 (Fig. 7D), specific and essential for the survival of the pathogen in the host cells, as well as the gene sequence encoding the antigen M, a glycoprotein that activates the humoral and cellular immune response, have been used as targets, through techniques such as seminested and nested PCR [104,105]. This nested PCR targeting the Hcp100 gene has been used in a variety of clinical samples from humans, including respiratory specimens, fresh biopsies, and whole blood [106–109] reporting sensitivity values varying between 86% and 100% and specificity values between 92% and 100%. A real time PCR assay to detect *H. capsulatum* in formalin-fixed paraffin-embedded tissues that targeted the specific Hcp100 gene has also been described [110].

Although there are some available data on the use of molecular techniques for the diagnosis of coccidioidomycosis and histoplasmosis, these data are all from studies performed with human cases of these diseases. To our knowledge, there are no studies evaluating the performance of these techniques in the diagnosis of coccidioidomycosis and histoplasmosis in animals. Despite the significant and growing role of molecular techniques for the identification and detection of fungal agents in clinical mycology laboratories, there is a lack of standardization of methods; hence, results vary greatly across laboratories. In addition, it is important to highlight that there are no Food and Drug Administration–approved nucleic acid–based assays for fungal diagnosis [103].

7. Final Considerations

Classical histoplasmosis is considered to be the most common endemic mycosis in humans. However, bibliographic data indicate that this mycosis is less frequent in horses than coccidioidomycosis. Although this premise can be stated, it is known that the prevalence of both systemic mycoses in nonhuman animal species is highly underestimated, considering the total lack of epidemiological data from regions where microepidemics and epidemics of human coccidioidomycosis and histoplasmosis have been reported. This epidemiologic gap may be a consequence of the lack of diagnostic resources and the insufficient knowledge on the characteristics of these mycoses among animal health professionals, which may lead to diagnostic confusion with other infections, associated with the fact that these diseases are not of mandatory notification. Therefore, we believe that this review will draw the attention of the scientific community for the need of performing further studies focusing on the occurrence of coccidioidomycosis and histoplasmosis in horses.

Acknowledgments

This work was supported by National Scientific and Technological Research Council; Brazil (CNPq; process numbers 445670/2014-2; 552161/2011-0).

Conflict of interest statement: None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

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