# Chapter 6
# Histopathologic Diagnosis of Fungal Infections of Lab Animals


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## 6.1 Common Fungal Infections in Lab Animals

Fungal disease or mycosis can occur in laboratory animals due to several known or unknown reasons, such as exposure to fungal spores through contact, inhalation, or invasion through injury point. Lab animals which are experimentally stressed, diseased, or immunocompromised are at higher risk of gaining infections. Mycosis is caused in lab animals due to direct or indirect infections, which can originate from opportunistic and/or obligatory pathogenic fungi (Fisher et al. 2012). Opportunistic fungal pathogens mostly survive and replicate in the environment outside-host such as saprotrophs (Casadevall 2008) and invade normal hosts or immunosuppressed hosts on encountering them. Obligatory pathogens essentially need their hosts to complete their life cycle.

Fungal infections in lab animals can be categorized as superficial, subcutaneous, and systemic. Superficial fungal infections cause skin diseases and are called dermatophytosis. Common examples for fungal pathogens causing superficial infections are *Microsporum, Trichophyton*, or *Epidermophyton* (Connole et al. 2000). Subcutaneous or dermal mycoses infect the skin and extend into subcutaneous tissue and further to other tissues and organs. The infection occurs usually through a traumatic injury of the skin and direct inoculation of fungi into the subcutaneous tissue (Zijlstra et al. 2016); an example is candidiasis caused by *Candida* sp. which can be a superficial infection such as thrush; else it can be a disseminated infection

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affecting certain target organs, such as the eyes or kidneys. In systemic mycoses, the fungal pathogen infects the host at a single point of entry and spread to multiple organ systems. Opportunistic fungal pathogens which exist in the environment invade debilitated or immunosuppressed hosts causing opportunistic infections. The surrounding environment is the primary source of most infections, which can be acquired by inhalation, ingestion, or traumatic introduction of fungal elements. Types of systemic fungal infections that can be recognized in animals are intravenous; intraperitoneal; gastric; pulmonary such as intranasal, intratracheal, and inhalational; mucosal such as oropharyngeal and vaginal; and central nervous system mycoses such as intracranial, intracisternal, or intrathecal (Guarner and Brandt 2011). Primary systemic mycoses in lab animals are caused by fungal pathogens such as histoplasmosis, coccidioidomycosis, blastomycosis, and cryptococcosis. Lab animals under prolonged administration of antimicrobials or immunosuppressive agents are more likely to catch such infections by the opportunistic fungi that cause diseases such as aspergillosis and candidiasis, which may be focal or systemic (Dedeaux et al. 2018).

Many opportunistic fungi are able to inhabit a variety of substrates in the surroundings of lab animals, and some can be common contaminants of cultures (Dedeaux et al. 2018); therefore it is imperative to ascertain whether the fungus represents the true infection or comes from some contamination or colonization. Histopathology is regarded as one of the oldest, reliable, and cost-effective method of fungal diagnosis, which is largely based on the microscopic examination of the tissue, smears, lesions, and morphological features of the fungus. Sometimes, the morphological features of many fungi may be identical for histological and/or cytological features, and such overlapping features are likely to question the accuracy of histopthological identification (Schwarz 1982). In such cases, other diagnostic methods can be used which include serology, fungal culture, immunohistology, and molecular diagnosis such as in situ hybridization (Guarner and Brandt 2011).

## 6.2 Histopathology in Diagnosis of Fungal Diseases

In some fungal diseases such as cryptococcosis, blastomycosis, coccidioidomycosis, histoplasmosis, and rhinosporidiosis, this method is just adequate for diagnosis, and in certain diseases caused by *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*), *Lacazia loboi*, (formerly *Loboa loboi*), and *Rhinosporidium seeberi*, histopathology is recognized as the most robust method of identification (Schwarz 1982). In a number of other diseases such as candidiasis, aspergillosis, zygomycosis, phaeohyphomycosis, hyalohyphomycosis, and oomycosis (pythiosis and lagenidiosis), histopathological method provides only presumptive diagnosis and their definitive diagnosis requires other methods to be employed such as culture or PCR-based techniques (Dedeaux et al. 2018).

Since histopathological diagnosis of fungi is largely based upon microscopic observation of fungal morphology and fungal infestation in various tissue sections, use of special/specific stains enhance the accuracy of diagnosis and eliminates the chances of misidentification (Surendran et al. 2014). Different types of fungi are differentiated on the basis of morphological features such as size and appearance (yeast form, hyphae, or dimorphic form), presence of capsule, thickness of the capsule, presence of septae, and budding pattern (Chandler and Watts 1995).

Among all other modern modes of diagnosis, histopathology still remains indispensable as a presumptive and/or definitive diagnostic tool for fungal disease diagnosis. It is largely based upon morphologic interpretation of histologic tissue sections, cytologic preparations, biopsy material, and culture morphologies. It takes advantage of distinctive morphology of different fungal strains and requires observer's capability for accurate identification and subsequent pathological correlation (Reller et al. 2001). It is particularly advantageous in the instances where the microorganism fails to grow in culture or it is fastidious (Watts and Chandler 1995).

### *6.2.1 Histopathologic Diagnosis of Dermatophytosis*

*Dermatophytosis is caused by dermatophytes which are responsible for* most superficial fungal infections (Aly 1994) in lab animals generally known as "tinea." These fungal pathogens are recognized as keratinophilic fungi, i.e., they *usually* invade the keratinized tissues in animals such as *the skin, hair, or nails (*Dobrowolska et al. 2006). There are a number of animal species which are invaded by dermatophytes such as dogs, cats, cattle, sheep, goats, pigs, rodents, rabbits, and birds. These animals are likely to catch the infections through direct contact with the surroundings contaminated with the fungal spores.

Commonly found dermatophytic genera are *Trichophyton* and *Microsporum* of which zoophilic and zoonotic dermatophytic species are more pathogenic to laboratory animals than anthropophilic strains, for example, *Trichophyton verrucosum, T. mentagrophytes, Microsporum gypseum, and M. canis* (Fisher et al. 2012). Infection from these dermatophytes result into diseases such as tinea capitis, tinea pedis, and onychomycosis (Gupta et al. 2005). The fungi Trichophyton is geophilic, i.e., its natural habitat is soil; therefore the primary source of Trichophyton infection to animals is soil. The fungi Microsporum are naturally zoophilic, i.e., they are acquired from animals (Weitzman and Summerbell 1995). The dermatophytes infect both healthy and immunocompromised animals. These diseases are mostly contagious and are frequently transmitted from one animal to another, and if they are zoonotic, they can be transmitted from animals to humans (Bond 2010).

Accurate diagnosis of dermatophytosis in lab animals is necessary for prevention of its relapse or recurrence in animals and transmission of infection to humans. The symptoms of dermatophytic infections are superficial and therefore easy to diagnose

(Robert and Pihet 2008). These diseases in lab animals can be commonly and clearly manifested by gross lesions on skin, hair, and nails. However, to eliminate the possibilities of misidentifications owing to overlapping symptoms of few dermatological conditions, other diagnostic techniques such as antifungal susceptibility profiling or molecular diagnosis may be performed.

The conventional techniques for diagnosis of dermatophytosis include histopathology, trichography, and fungal culture (Moriello and De Boer 2014; Pollock 2003). The most convenient, simple, and rapid method of dermatophytosis identification is microscopic examination of the clinical specimen (Lousbergh et al. 1999). The lesions of alopecia, erythema, papules, scaling, or crusting at common locations such as head, ears, nails, and front limbs (Pollock 2003) are usually indicative of the dermatophytosis condition. The microscopic examination of hair or trichogram is carried out of diagnosis of fungal spores or hyphae. It usually involves a semi-invasive approach of plucking of hair and examining the hair tip, hair root, and shaft under the microscope (Bond 2010).

### *6.2.2 Histopathologic Diagnosis of Systemic Mycoses*

Systemic mycotic diseases such as histoplasmosis, coccidioidomycosis, and cryptococcosis are regarded as primary systemic mycoses and occur usually in mammals such as ruminants and equines; birds such as poultry; fish; and crustaceans, but are rarely seen in reptiles (Jacobson et al. 2000). It is frequently observed in farmed fish (De Hoog et al. 2011).

The histopathological examination of biopsy material demonstrates the nature of invasive disease, and further culturing of microorganisms reveals the identity of the pathogen. Preliminary identification of microorganisms in tissue sections may allow for the culture of fastidious microorganisms that require special media or growth conditions. Rapid histologic assessment of tissues can be done using frozen sections, which do not involve the fixation and routine processing. Such analysis is done in the cases of rapidly progressive diseases that require emergent surgical debridement, such as rhinocerebral zygomycosis or necrotizing fasciitis (Frater et al. 2001).

Disease characterization also involves the analysis of host inflammatory response, which is helpful in differentiating among acute, chronic, and/or granulomatous inflammation. Granulomas with or without necrosis usually appear in many fungal infections, which are differentiated as pyogranulomatous inflammation, suppurative granulomas, and palisading granulomas. Necrotizing granulomas often occur in systemic fungal pathogens, such as *Histoplasma* sp. and *Coccidioides* sp. (Woods and Walker 1996). Certain fungi such as *Aspergillus* and members of *Zygomycetes* are angiotrophic, i.e., they have a predilection for vascular invasion; therefore they often cause tissue infarction (Frater et al. 2001).

### 6.2.3 *Role of Histochemical Stains in Fungal Diagnosis*

Histochemical stains play a very vital role in detecting a large number of pathogens; however, sensitivity and efficiency of the stains depend upon several factors such as number of microorganisms present in the sample and methods adopted for sample procurement and sample preparation (Woods and Walker 1996). The hematoxylin-eosin (HE) stain is the most widely used stain for histopathologic evaluation of tissue sections. A wide range of yeast cells and fungal hyphae can be stained and visualized simply using HE stain. However, some specific stains such as methenamine silver stain (GMS) or the periodic acid-Schiff (PAS) stain are more useful to visualize subtle details of fungal morphology (Chandler and Watts 1995; Woods and Walker 1996). Papanicolaou stain has been used to identify the fungal elements from organisms *Aspergillus* and *Candida* species (Greaves and Strigle 1985). Mayer's mucicarmine and Alcian blue stain are specific mucin stains that stain the mucopolysaccharide capsule of *Cryptococcus neoformans* (Gazzoni et al. 2009) (Fig. 6.1).

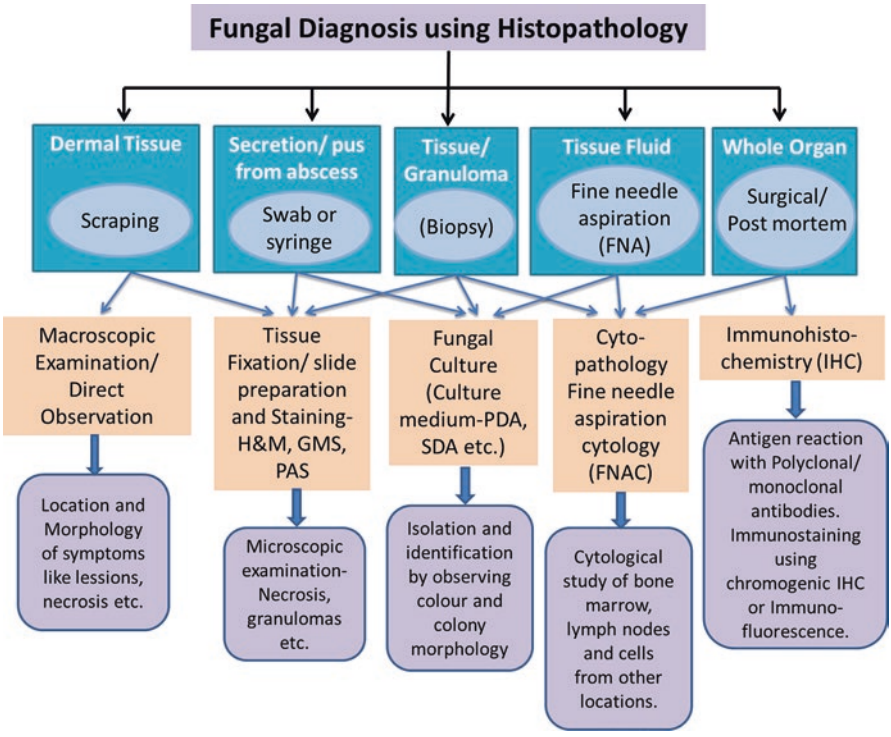


**Fig. 6.1** Diagnosis of fungal infections using histopathological tools and techniques

## 6.3 Common Fungal Infections in Rats/Rabbits/Guinea Pigs and Their Diagnosis

Murine animals and rodents are the laboratory animals commonly used as/for experimental models. The accuracy and reliability of experimental results largely depend upon the animals' physical and physiological condition; thus any fungal infection can have profound impact on it. While they are treated as experimental subjects, they are often rendered compromised due to exposure to various experimental conditions and become more susceptible to fungal infections. The previously infected lab animals also become carriers of infection for healthy subjects. It is therefore imperative to correctly diagnose and treat the fungal infections in lab animals (Weitzman and Summerbell 1995).

Laboratory rats have been found to be infected with *T. mentagrophytes* that causes enzootic dermatophytosis manifested by alopecia, scaling, and erythema. The identification scheme involves observations of skin lesions and microscopic examination of colonies followed by molecular analysis for definitive diagnosis of specific strains (Baker 1998; Pollock 2003). Laboratory mice and rats have been diagnosed with indigenous yeast species *Candida pintolopesii* in their gastrointestinal tract as normal flora (Baker 1998).

Guinea pigs have been used as animal models for dermatophytoses as they demonstrate the clinical features comparable to the disease in humans. Etiologic agents *Trichophyton mentagrophytes* and *Microsporum canis* produce acute inflammatory *infections in* guinea pigs clinically identified by semiquantitative redness and lesion scores. Histopathological diagnosis of the dermatophytes involves microscopy and fungal culture, while molecular diagnosis through pan-dermatophyte PCR is reported to be more sensitive than microscopy (Saunte et al. 2008).

A rapid, accurate, and highly sensitive method of clinical diagnosis of rabbit-derived pathogenic dermatophytes is reported using microsatellite-primed polymerase chain reaction (PCR) in combination with a clustering method. The method is based upon detecting the DNA polymorphism fingerprints of amplified DNA fragments of *Trichophyton mentagrophytes, Microsporum gypseum,* and *Microsporum canis* using specific primer. The method could be highly useful in epidemic research of the dermatophytes (Miao et al. 2014).

## 6.4 Common Fungal Infections in Cattle and Their Diagnosis

Cattle serve as experimental models in biomedical research as their physiology and genome sequence are found closer to the humans as compared to rodents. Many livestock models are used to identify new cellular and molecular mechanisms regulating infertility and reproductive disorders such as polycystic ovarian syndrome (PCOS) in humans. There are many limitations to investigation and treatment of several prenatal and postnatal disorders in human fetuses, which have been overcome

by using pregnant sheep as model for in utero treatments for congenital birth defects (Hamernik 2019). Some fungal infections in cattle have been discussed further, which are of high relevance.

Bovine alimentary mycosis caused by *Mucor* and *Aspergillus* is termed as mucormycosis and aspergillosis, respectively, both of which are systemic mycosis and involve rumen, omasum, abomasum, reticulum, and intestine to a larger extent while omasoabomasal orifice and tongue to a lesser extent. Identification can be done through visualization of lesions and macroscopic focal hemorrhagic necrosis on various parts of alimentary canal. Histological diagnosis reveals thrombosis, coagulative necrosis, and typical fungal hyphae (Chihaya et al. 1992).

A common superficial mycosis occurring in cattle is dermatophytoses or ringworm, which affects the superficial keratin-rich body parts such as skin, hair, nails, and horns. Three main genera of dermatophytes are *Epidermophyton, Microsporum,* and *Trichophyton*, of which the most common pathogens causing disease in cattle are *Microsporum canis, M. gypseum, Trichophyton mentagrophytes,* and *T. verrucosum*. General manifestations of ringworm include circular white and itchy lesions, alopecia or hair loss, crusting of skin, inflammation, and erythema (Ahmad and Gholib 2016). Besides observation of clinical signs and symptoms, the diagnosis is based on microscopic examination of isolated pathogen hyphae and spores. *T. verrucosum* is cultured on Sabouraud dextrose agar medium containing chloramphenicol and actidion and examined for physical appearance of cultures such as color, texture, and shape and microscopic identification of septate hyphae and microconidia. Definitive diagnosis using PCR-based methods such as random amplified polymorphic DNA (RAPD), multiplex PCR, and specific nucleotide sequence are also suggested (Walid and Eman-abdeen 2018).

Mycotic mastitis in cows is caused mainly due to mold and yeast infection that occurs in mammary glands. The mold genera generally responsible for mycotic mastitis are *Aspergillus, Alternaria, Penicillium, Phoma,* and *Geotrichum*. The predominant yeast causing this disease is *Candida* sp.; other genera are *Trichosporon, Cryptococcus,* and *Saccharomyces*. The disease is characterized by sporadically occurring mammary gland inflammations, edematous fluid exudates, pain, redness, and decreased milk production. Subclinical mastitis is diagnosed through somatic cell count in milk followed by isolation and identification of fungi in mastitis milk after culture. Histopathological identification of fungal hyphae and spores is done in infected tissue after slaughter (Ahmad and Gholib 2016).

Mycotic mastitis is also reported in farm goats in their postpartum period, mainly characterized by purulent mammary secretion and progressive induration of the affected glands. Postmortem histological diagnosis revealed widespread acute and chronic mycotic lesions in the infected mammary glands consisting of fungal hyphae. Immunohistochemical diagnosis *by* indirect immunofluorescent labeling led to the identification of *Aspergillus fumigatus* as main pathogen, while zygomycotic hyphae could also be identified in a granulomatous lesion (Jensen et al. 1996).

A number of fungal infections have been associated with mycotic bovine abortions and stillbirths. Most frequent cause was reported to be the *Aspergillus*

*fumigatus*, while other causative organisms were *A. terreus, A. nidulans, A. flavus,* and *A. rugulosus.* Some genera of *Zygomycetes* such as *Rhizomucor* and *Rhizopus*, yeast *Candida,* and *Ascomycota* fungi *Pseudallescheria boydii* were also associated with mycotic abortions with varying frequency. Coexistence of several fungi was found to cause mixed infection. The histological diagnoses revealed the occurrence of mixed infection due to the presence of both septate and nonseptate hyphae in placental tissues (Knudtson and Kirkbride 1992).

## 6.5 Common Fungal Infections in Swine and Their Diagnosis

Swine have been used commonly in cardiovascular research due to many anatomic and physiological similarities, such as size of heart, coronary blood flow, myocardial contractility, patterns of atherosclerosis, and hemodynamics. Besides, they are also used largely as preclinical models involving surgical and interventional protocols for studying other organ systems including integumentary, digestive, and urological (Smith and Swindle 2006). The human genome sequence is similar to the genome sequences of pigs to a higher degree as compared with rodents; therefore, pigs are also considered as better models to study many human genetic diseases (Hamernik 2019).

Respiratory disease in pigs is associated with the fungal infection by an opportunistic fungi *Pneumocystis carinii* which inhabits the respiratory tract of pigs and occasionally cause interstitial pneumonia (Binanti et al. 2014). The immunohistochemical detection of *Pneumocystis* organisms was done using Grocott's staining of lung tissue obtained from slaughtered pigs. The *Pneumocystis* organisms could be observed in histologically normal lungs as well as in lungs with histological lesions. The histopathological diagnosis revealed the occurrence of bronchointerstitial pneumonia in infected pigs (Sanches et al. 2007).

Pneumocystis infection in pigs was also diagnosed through in situ hybridization (ISH) technique which enabled unambiguous detection of pneumocystis in paraffin wax-embedded lung tissues obtained from pigs with interstitial pneumonia postmortem. ISH involved designing of an oligonucleotide probe which could potentially hybridize with all representatives of the genus *Pneumocystis*, making it a robust and highly sensitive technique. It also proved to be superior to the traditional Grocott's methenamine-silver nitrate (GMS) staining method which failed to identify sections with only few *Pneumocystis* cells (Binanti et al. 2014).

## 6.6 Common Fungal Infections in Birds and Their Diagnosis

The laboratory birds are used as subjects in a variety of experimental setup pertaining to fundamental and applied research in medical sciences, veterinary sciences, pharmaceutical sciences, ecotoxicology, zoology, and many others. Mostly

domesticated birds are used in laboratory such as domestic fowl, domestic poultry, turkey, and quail. Wild birds and pigeons are less commonly used as laboratory birds. Fungal pathogens can cause diseases in all birds; however, immunocompromised subjects are more susceptible toward infections. The predisposing factors for causing fungal infections are exposure to fungal spores, overcrowding, poor sanitation, poor ventilation, warm environment, humidity, poor nutrition, and age and poor sanitation. The most common fungal pathogens in birds are *Aspergillus* spp. causing respiratory tract infections and *Candida* spp. causing gastrointestinal (GI) tract infections (Tell 2005). Some less common pathogens are *Macrorhabdus*, *Cryptococcus, Rhodotorula, and Mucor* (Imran and Ali 2014).

Aspergillosis is an opportunistic but non-transmittable infection, caused by many species of which *Aspergillus fumigates* is the most common, others being *A. flavus*, *A. niger*, *A. glaucus,* and *A. nidulans*. (Perelman and Kuttin 1992). Acute aspergillosis occurs on inhalation of an overwhelming number of fungal spores over a short period of time, causing massive colonization of fungal mycelia in respiratory tract manifested as military granulomatous foci (McMillan and Petrak 1989). Chronic aspergillosis generally occurs in immunosuppressed or debilitating conditions. Diagnosis of aspergillosis is challenging due to nonspecific signs and involves reviewing history, physical examination, histopathology, biochemical tests, serology, hematology, radiography, endoscopy, and fungal culture. Mycotic keratitis, blepharitis, and dermatitis involving the eyelids and the head are observed. Histopathological diagnosis on the basis of lesions is not often definitive as the fungal filaments are quite similar and the manifestations of disease are not pathognomonic (Jones and Orosz 2000). Brain and heart along with organs of respiratory system such as larynx, trachea, and lungs are important for histopathological examination (McMillan and Petrak 1989).

Another fungal disease caused in birds is candidiasis, also known as thrush, which is common in domestic poultry and water fowls. The etiologic agent of this disease is the opportunistic yeast *C. albicans,* which is most abundant organism, while the less common pathogenic species are *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei,* and *C. lusitaniae* (Tiwari et al. 2011). *C. albicans* is usually present in the gastrointestinal tract and causes endogenous infection due to yeast overgrowth. Clinical signs of the disease in birds include anorexia, crop stasis, white plaques in the oral cavity, regurgitation, and weight loss. Complete crop and GI stasis occurs in case of severe infections (Campbell 1986). Identification of disease is done by observing lesions which usually appear in the upper digestive tract. Histopathological diagnosis involves microscopic examination of cultured colonies of *Candida*, which can be isolated from feces, crops, gizzards, lungs, and livers. *Candida* spp. is identified on a gram stain, Romanowsky-type, or new methylene blue stain of the digested smear, feces, crop contents, or regurgitated material. Scrapings or impression smears from the crop or pharynx are also used for microscopic examination which shows budding yeast and hyphae (Tiwari et al. 2011).

## 6.7 Common Fungal Infections in Reptiles and Their Diagnosis

Fungal infections of primary and secondary nature may also occur in reptiles primarily due to their terrestrial habitat. Most common fungal mycoses in reptiles are hyalohyphomycoses in skin caused by the dermatophytes *Chrysosporium* (Johnson et al. 2011). Other fungal species found in skin lesions in reptiles include *Paecilomyces, Penicillium, Fusarium, Geotrichum, Mucor*, and *Aspergillus* (Jacobson et al. 2000). Severe ulcerative dermatitises on the ventral scales in a reticulated python (*Python reticulatus*) and in a boa constrictor (*Constrictor constrictor*) have been observed (Frank 1976).

Systemic mycoses are less common in reptiles, but have occasionally been observed in many cases. Histopathologic diagnosis has revealed muriform fungal elements in different internal organs of a snake (Jacobson et al. 2000). Phaeohyphomycosis in eyes and lungs of Galapagos tortoise (*Geochelone nigra*) (Manharth et al. 2005) and subcutaneous inflammation in an eastern box turtle (*Terrapene carolina*) (Joyner et al. 2006), caused by *Exophiala* species, is diagnosed by gross necropsy. Microscopic examination is done to characterize pigmented fungal hyphae and conidia present at necrotic sites.

Fungal disease in bones and carapace in an aldabra tortoise (*Geochelone gigantea*) is identified on histological examination involving biopsy of infected tissue. Phaeohyphomycosis in tortoise caused by *Exophiala oligosperma* mainly occurs in the superficial keratinized layers and further extend to deep bones (Stringer et al. 2009). Histological examination of biopsy specimen and stained tissue sections also diagnosed dematiaceous hyphae of fungi *Ochroconis humicola* causing foot lesions in the tortoise (*Terrapine carolina* var. *carolina*) (Weitzman et al. 1985).

Certain emerging obligate pathogenic fungi causing dermatomycoses and systemic mycoses in reptiles are reported which belong to families Onygenaceae and Clavicipitaceae. Members of family Onygenaceae of Ascomycota – Nannizziopsis guarroi and *Ophidiomyces ophiodiicola* – cause deep fungal dermatitis in bearded dragons and free living snakes, respectively. Pathogenic fungi from family Clavicipitaceae are known to cause granulomatous glossitis, pharyngitis, dermatitis, and disseminated visceral mycosis in lizards, tortoises, turtles, and crocodiles. The pathogenicity of fungi is determined through histopathological evaluation involving the isolation and differentiation of fungal agents (Schmidt 2015).

Most common fungal infections in captive reptiles occur in integumentary system, respiratory system, and the gastrointestinal tract. Systemic mycoses are only diagnosed during postmortem investigations. Histopathological diagnosis involves microscopic identification and biopsies of infected tissues (Schumacher 2003).

## 6.8 Common Fungal Infections in Amphibians and Their Diagnosis

Amphibians catch fungal infections in a variety of ways such as directly from contaminated environment, traumatic injury, or transmission of pathogen from fish population. Some common fungal diseases identified in amphibians are chytridiomycosis, phaeohyphomycosis, zygomycoses, saprolegniasis, and ichthyophoniasis (Densmore and Green 2007). Chytridiomycosis is a fatal fungal disease of amphibians caused by a ubiquitous chytrid fungus *Batrachochytrium dendrobatidis* (Daszak et al. 1999). A number of amphibian species such as frogs, toads, and salamanders are susceptible to chytridiomycosis. It is keratinophilic or chitinophilic fungus, and histologically the infections of this fungus are limited mostly to keratinized epithelial cells of the skin and oral disc. The range of signs and symptoms of the disease include hyperkeratosis associated with dysecdysis, excessive skin shedding, erythema, and skin ulcerations, as seen in green tree frogs (*Litoria caerulea*) (Berger et al. 2005), while abduction of the hind legs and tail autotomy as observed in salamanders (Pasmans et al. 2004).

Many wild and captive frogs (*Hyla caerulea*, *Pternohylaf odiens*, *Phyllobates trinitatis*, *Rhacophorus* spp., and *Hyla septentrionalis*) are diagnosed with the disseminated systemic mycosis caused by fungi that belong to the genus *Phialophora*, *Fonsecaea,* and *Rhinocladiella.* The disease is manifested in form of granulomatous infection of internal organs in frogs. Histopathological diagnosis involves ulcers or nodules in the skin and lesions of internal organs such as spleen, liver, and kidney (Densmore and Green 2007). The lesions are observed to consist of granulomatous encephalomyelitis (Daszak et al. 1999).

Zygomycosis or mucormycosis is caused in many species of toads such as Wyoming toad (*Bufo baxteri*), giant toad (*Bufo marinus*), Colorado River toad (*Bufo alvarius*), and White's tree frog (*Philodryas caerulea*). The etiologic agents are *Mucor* spp. and *Rhizopus* spp. that belong to the fungal subclass *Zygomycetes*. External dermatitis involving multifocal hyperemic nodules with visible fungal growth in ventral integument are histologically identified. Systemic disease is manifested with nodules and granulomatous inflammation in internal organs (Densmore and Green 2007).

*Saprolegnia* or water mold causes saprolegniasis in freshwater salmonids. Usually it is a secondary pathogen but can infect the host directly on encounter. It is often called cotton mold due to its cottony appearance. The infection restricts to the epidermal layers causing necrosis on the skin, but may become fatal (Densmore and Green 2007).

## 6.9 Common Fungal Infections in Invertebrates and Their Diagnosis

Many invertebrates are used as subjects for laboratory experimentations such as annelids, arthropods, and molluscs. These are also susceptible to various fungi that are mostly acquired from their surroundings and can cause diseases. Crustaceans such as crabs and lobsters are infected by *Exophiala cancerae* (De Hoog et al. 2011) and *Fonsecaea brasiliensis* (Vicente et al. 2012) causing lethargy and loss of balance in animals (known as lethargic crab disease or LCD). Histopathological diagnosis is done on the basis of symptoms such as claw tetany, weak motor control of chelae and pereiopods, necrosis, and tissue degeneration followed by the death in severe cases. Mollusks such as mussels are found to be infected with epizootic black yeast that belongs to the order *Chaetothyriales*. Most of these pathogens are detected in wild mussels while farmed mussels are mostly devoid of it.

Histological examination revealed occurrence of a variety of fungal spores between the digestive tubules of mussels. These spores belonged to *Psilocybe* sp., *Ulocladium* sp., and *Alternaria* sp., which were identified on the basis of their morphology (Kovacic et al. 2018). The histological identification is done by observing the features such as discoloration of mantle from creamy to brown or black. The brown/black coloration is due to accumulation of dense population of yeast cells which eventually infiltrate to the connective tissue and gonads.

Annelids such as earthworm can be infected by fungi *Exophiala jeanselmei, identified as black spherical bodies in* embryonic stages of *Octolasian tyrtaeum* and in cocoon albumen of *Eisenia foetida. The black granules consist of fungal hyphae and cell aggregates. This fungus is saprotrophic and is commonly* found in a variety of habitats such as soil, plants, water, and decaying wood. It has further adapted to become an opportunistic human pathogen causing phaeohyphomycosis, causing cutaneous and subcutaneous inflammation (Vakili 1993).

There is a large number of entomopathogenic fungal species which mostly belong to the phyla *Microsporidia*, *Chytridiomycota*, *Entomophthoromycota*, *Basidiomycota*, and *Ascomycota* (Vega et al. 2012). Certain fungal pathogens may cause fatal diseases in insects; these are *Beauveria* spp., *Cordyceps* spp., *Conidiobolus* spp., *Metarhizium* spp., and *Hirsutella* spp. Entomopathogenic fungi exhibit a wide range of mortality rates among infected populations of insects. The signs of mycoses in insects are identified on the basis of morphological and cultivation characteristics using taxonomic guidelines and characterization of fungal fruiting bodies. For example *Myriangium duriaei* causes mycoses on elongate hemlock scale (EHS), a small insect pest of order Hemiptera, which is characterized by the formation of black sclerotized masses on the scale surface. The early mycelial biomass on EHS typically shows white morphology and progressed to brown and finally black (Gouli et al. 2013).

**Mycotoxins:**
It is actually chemicals of fungal origin and toxin for animals; it is also known as mycotoxicoses. It is secondary metabolite produced by enzymatic action by metabolism of acetates, mevalonates, and malonite pathways. Mycotoxin is a big problem worldwide, as it leads to contamination of food. It is categorized by fungal species, structure, and mode of action. There are different mycotoxins such as aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, and tremorgenic toxins. These mycotoxins are agro-economically and public health-wise very important, causing severe diseases called acute or chronic mycotoxicoses. Mycotoxins produced by invading fungi can suppress immunity.

| S. No | Fungal toxin (mycotoxin) | Toxicity |
|---|---|---|
| 1. | Trichothecenes, verrucarins, sporidesmins | Dermatotoxic |
| 2. | Aflatoxins, ochratoxin A, zearalenone, trichothecenes | Hematotoxic |
| 3. | Trichothecenes, verrucarins, sporidesmins | Dermatotoxic |
| 4. | Aflatoxins, ochratoxins, rubratoxins, sterigmatocystin | Hepatotoxic |
| 5. | Zearalenone | Estrogenic |
| 6. | Ochratoxin A | Nephrotoxic |
| 7. | Trichothecenes | Gastrotoxic |

Treatment of fungal diseases in animals: There are a number of methods for the treatment of fungal infection in lab which are known and are able to control it. In this section few important methods have been discussed in short. These are:

1. *Antifungal treatment:* A number of excellent therapies are used for the treatment of fungal infections in animals and in humans. Many pathogenic fungi are able to produce chemicals which can work against many human and animal diseases and are called as antifungal agents, such as bifonazole, clotrimazole, econazole, enilconazole (imazalil), ketoconazole, miconazole and parconazole, and triazole and fluconazole. Studies at the molecular level of fungal infection are very complex; fungal cell adopt specific mechanism in the presence of toxic drugs. Fungi generally mutate its drug target protein, and it causes reduction of drug target affinity, overexpression of target protein by mutating promoter region, degradation of drug, etc. These are few important resistance mechanisms of fungi. The similarities in biological aspects of fungi and animal host led to a drug development, which is based on features of fungal cells, for example, cell wall of fungi contains ergosterol, and it is a drug target because it is distinct from the cholesterol present in mammalian cells, azoles, echinocandins, and polyenes such as commercial antifungal drugs that successfully act on the cell wall.

There are specific mechanisms of resistance to antifungal drugs resistance, some of which are mentioned below:

1. Non-synonymous point mutation includes alteration of genes at genetic level, which changes the enzymes at amino acid sequence level and changes the function of enzymes (Lamb et al. 1997, Lamb et al. 2000, Katiyar and Edlind 2001, Sanglard et al. 1999, Miyazaki et al. 1998, Parkinson et al. 1995, Lopez-Ribot et al. 1999, Loffler et al. 1997, Vanden Bossche et al. 1990, White 1997, Franz et al. 1998).
2. The transcription level of genes encoding the enzymes gets increased which enhances the level of its expression (Marr et al. 1998).
3. The decreased concentrations of the drug molecules within fungal cells due to drug (Vermeulen et al. 2013; Seyedmousavi et al. 2014; Howard et al. 2009).

In recent years, researches identified and analyzed antifungal targets that improved due to improvements in genetic tool for the manipulation of fungal pathogens. This helps in the standardization of animal models for fungal infection.

**Burch and Russell concept of three Rs**

*Reduction:* Extensive literature search and based on experiment design; organizes a plot before large animal experimentation; collection of data for every animal and appropriate statistical test must apply for perfect analysis.

*Refinement:* Elimination of pain and search of perfect alternative animal, perfectly trained before execution of procedure, proper use of doses of anesthetics for painful procedure, uses of perfect thermoregulation system for experimentations.

*Replacement:* Analysis by using computers that is in silico method followed by in vitro such as cell culture and cell tissue culture methods.

There are different antifungal compounds that work against different fungal infections in different animals, and few of them are listed in Table 6.1.

4. Alteration of the biochemical and biosynthetic pathways results in lower level of target of antifungal drugs (Mellado et al. 2007)

There are many reports about different animal species such as yeast cells showing higher level of azole resistance. Azole and echinocandins are mainly used for treatment of animal fungal diseases. Along with this there are many limitations similarly, a lot of variation found in animal species too which have variable pharmacokinetics, drug interactions, and antifungal resistance.

2. *Drug combination:* Antifungal treatment has limitation, and based on this fact combination of drug is the new method for the treatment of fungal infections.

**Table 6.1** List of antifungal agents and different disease controlled by them in different animals

| S. No | Antifungal | Animal | Diseases |
|---|---|---|---|
| 1. | Amphotericin B | Birds, dogs, cats, horses | Aspergillosis, candidiasis, histoplasmosis, coccidioidomycosis, sporotrichosis, mucormycosis, cryptococcosis, blastomycosis, histoplasmosis |
| 2. | Thiabendazole | Birds, horses, rodents, rabbits, ruminants | Dermatophytosis |
| 3. | Ketoconazole | Birds, dogs, cats | Aspergillosis, candidiasis, blastomycosis, histoplasmosis, cryptococcosis, coccidioidomycosis, malassezia dermatitis, and dermatophytosis |
| 4. | Fluconazole | Birds, dogs, cats | Candidiasis, cryptococcosis, blastomycosis, aspergillosis, blastomycosis, coccidioidomycosis |
| 5. | Itraconazole | Birds, dogs, cats, horses, rodents, rabbits, and fur animals | Aspergillosis, candidiasis, blastomycosis, histoplasmosis, cryptococcosis, sporotrichosis, coccidioidomycosis, dermatophytosis, malassezia dermatitis, mycotic keratitis |
| 6. | Griseofulvin | Dogs, cats, horses, rodents, rabbits, and fur animals | Dermatophytosis, sporotrichosis, ruminants |
| 7. | Clotrimazole | Birds (raptors), dogs, cats, rodents, rabbits, and fur animals | Aspergillosis, dermatophytosis, and malassezia dermatitis |
| 8. | Enilconazole | Birds, dogs, cats, horses, ruminants, rodents, rabbits, and fur animals (dermatophytes and other pathogenic fungi) | Aspergillosis, dermatophytosis, dermatitis, malassezia |
| 9. | Voriconazole | Birds, dogs, cats, horses | Aspergillosis, scedosporiosis, keratitis |
| 10. | Flucytosine | Cats | Cryptococcosis |
| 11. | Griseofulvin | Dogs, cats, horses, ruminants, rodents, rabbits, and fur animals | Dermatophytosis and sporotrichosis |
| 12. | Posaconazole | Dogs, cats | Aspergillosis, mucormycosis |

Combination of more than one drug is used for the treatment as it increases the efficacy and possibility of action on multiple targets at a time. The level of toxicity is reduced as less of drug is used (Chen et al. 2014). Combination of drug is used as it leads to improved activity, and using more than one drug can increase efficacy due to the possibility of action on more than one target; in addition, toxicity is reduced because less of the drug is used.

3. *Vaccines:* Vaccination is used against high-risk groups of fungal infections. Understanding the mechanism of fungal infection and pathogenic mechanism has supported the development of novel vaccines against fungal diseases.

Researchers have developed many vaccines which are durable, safe, and robust and able to control endemic infections (Shahid 2016).

4. *Alternative animal model for drug discovery:* This model was given by Burch and Russell in 1959, and it was described as "alternative animal model." It has three action rules and is based on three Rs, refinement, reduction, and replacement.

In this, an alternative model or mini host is used for in vivo testing. Amoeba, nematodes, fish, insects, and chicken embryos are used for this, and the reasons behind its selection are that the neural system is poorly developed in these animals and almost they are painless, large number of animals can be used for experiments, the maintenance is cheaper as compared to traditional animals, and also there is a correlation between mammalian animals and alternative animals.

## 6.10 Conclusion

Fungal infections in laboratory animals may cause major loss of researches based on animal models. Fungal infections can be avoided in labs by taking some important precautions along with perfect experiment design. There are many more techniques which can help for the cure, and different fungal stains can be cured by using these methods. Uses of animal model is the base of high-level research for the welfare of human beings, and it is expensive in many ways, so proper care is necessary or successful results. Drug discovery research and validation of in silico experimental results need healthy animal model.

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