Fungal Biology

Arti Gupta
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Recent Developments in Fungal Diseases of Laboratory Animals

Fungal Biology

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Fungal biology has an integral role to play in the development of the biotechnology and biomedical sectors. It has become a subject of increasing importance as new fungi and their associated biomolecules are identified. The interaction between fungi and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these eukaryotic microorganisms are very diverse; fungi are present in every ecosystem on Earth. The fungal kingdom is equally diverse, consisting of seven different known phyla. Yet detailed knowledge is limited to relatively few species. The relationship between fungi and humans has been characterized by the juxtaposed viewpoints of fungi as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different fungi in diverse ecosystems as well as their interactions with living and non-living is essential to underpin effective and innovative technological developments. This series will provide a detailed compendium of methods and information used to investigate different aspects of mycology, including fungal biology and biochemistry, genetics, phylogenetics, genomics, proteomics, molecular enzymology, and biotechnological applications in a manner that reflects the many recent developments of relevance to researchers and scientists investigating the Kingdom Fungi. Rapid screening techniques based on screening specific regions in the DNA of fungi have been used in species comparison and identification, and are now being extended across fungal phyla. The majorities of fungi are multicellular eukaryotic systems and therefore may be excellent model systems by which to answer fundamental biological questions. A greater understanding of the cell biology of these versatile eukaryotes will underpin efforts to engineer certain fungal species to provide novel cell factories for production of proteins for pharmaceutical applications. Renewed interest in all aspects of the biology and biotechnology of fungi may also enable the development of “one pot” microbial cell factories to meet consumer energy needs in the 21st century. To realize this potential and to truly understand the diversity and biology of these eukaryotes, continued development of scientific tools and techniques is essential. As a professional reference, this series will be very helpful to all people who work with fungi and should be useful both to academic institutions and research teams, as well as to teachers, and graduate and postgraduate students with its information on the continuous developments in fungal biology with the publication of each volume.

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Recent Developments in Fungal Diseases of Laboratory Animals

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Preface

Fungal growths may significantly impact human and animal well-being. Numerous natural pathogens of laboratory animals may alter host physiology, rendering the host unsuitable for many experimental uses. While the number and prevalence of several pathogens have declined considerably, many still appear in laboratory animals and represent unwanted variables in research. Investigators using laboratory animals in biomedical experimentation should be aware of the profound effects that these agents can have on research. What does the future hold regarding the natural pathogens of laboratory animals? The choice of which animal model to use must be considered carefully, addressing issues on the type of human disease to mimic, the parameters to follow, and collection of the appropriate data to answer the questions being asked. Overall, animal models of fungal infection will continue to deepen our understanding of how these infections occur, progress, and can be controlled and eliminated. This book provides a valuable source of information for biological and biomedical scientists, and clinical and academic researchers working the area of fungal infections or diseases of laboratory animal species including mice and rats. To overcome this problem of fungal infections, we are trying to filling this knowledge gap by introducing some antifungal medicines that work by either killing the fungal cells, for example, by affecting a substance in the cell walls, causing the contents of the fungal cells to leak out and the cells to die, or preventing the fungal cells from growing and reproducing. We will attempt our level best to incorporate what we consider the most vital infectious agents found in lab animals as of now. Also, endeavors have been made to incorporate however much data as could reasonably be expected from natural outbreaks of disease. However, a lot of data will likewise be incorporated from experimental/lab-based in vitro studies and trials that may be based on conditions like the course and dosage to those of natural infections.

This book covers fungal infection as a keystone reference, reflecting state-of-the-art research and current emerging issues in fungal biology and biotechnology in the animal system. It reviews the methods and experimental work used to investigate different aspects of fungal infection and provides examples of the diverse applications of fungal infection. This book has been edited by an experienced team, with contributions from international specialists. It should be an invaluable resource

for industry-based researchers, academic institutions, and professionals working in the area of fungal biology and associated infection and prevention for their applications. It should be immensely useful to those in the field of biological sciences, especially microbiologists, microbial biotechnologists, biochemists, researchers, and scientists of fungal biotechnology. We have been honored that the leading scientists who have extensive, in-depth experience and expertise in fungal systems and microbial biotechnology took the time and effort to develop these outstanding chapters. Each chapter was written by an internationally recognized researcher or scientist with up-to-date and detailed knowledge of white biotechnology and the innumerable industrial applications of fungi.

This volume comprises ten chapters. In Chap. 1, emphasis is given on emerging infectious diseases in a variety of animals and their control measures are discussed. Additionally, the factors affecting the spread of fungal pathogens are also described by Shukla. Chapter 2 presented by Suman et al. covers the importance of honey bee, their role in the ecosystem and the impact of microsporidian disease, one of the most significant and less studied diseases, on the honey bee population worldwide. In Chap. 3, Avnish et al. suggested that limitations of the currently available rapid tests for point-of-care diagnosis of fungal diseases can be surpassed by fusing molecular biology techniques and immunodiagnostic procedures, along with other conventional devices and techniques. Chapter 4 by Tell et al. highlights the broad health and economic impacts of aspergillosis on veterinary medicine across so many animal species, making additional studies crucial. In Chap. 5, Hilal describes fungal diseases of animals, detailing symptoms and their cure by natural products. Chapter 6 by Kamal et al. represents an overview of the different categories of fungal infections that can be encountered in animals and also assesses the effect of climate change on mycotic diseases. Chapter 7 by Saurabh and Suchit deals with the epidemiology, pathophysiology, and symptoms of chronic obstructive pulmonary disease, together with invasive pulmonary aspergillosis. In Chap. 8, Shiv et al. place emphasis on the incidence of drug resistance and the importance of adjusting antifungal therapy and finding a newer drug to treat sporotrichosis. Plant-derived drugs may be an alternative approach for curing sporotrichosis and combating antifungal drug resistance. Ravindra and Ruhel focus, in Chap. 9, on invasive fungal infections in humans, diagnostic methods, and their treatment, including future implications of these infections, where more research in this field is required to facilitate the development of better diagnostic tests, therapies, and preventive vaccines. In Chap. 10, Yadav and Malvi give an overview of diseases in animals caused by fungi forming biofilms, which is a huge challenge in veterinary medicine.

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Poster Presenter awarded by the International Consortium of Contemporary Biologists (ICCB) and Madhavi Shyam Educational Trust, Ranchi, Fellowship Award awarded by the International Consortium of Contemporary Biologists (FICCB) and Madhavi Shyam Educational Trust (FMSET), and Dr. V.P. Agarwal Gold Medal awarded by D.A.V. (P.G.) College, Muzaffarnagar. Dr. Arti has lifetime memberships with the Indian Science Congress Association, Biotech Research Society of India, Zoological Society of India, and International Consortium of Contemporary Biologists.

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Chapter 1
Emerging Infectious Diseases Caused by Fungi in Animals and Their Prevention

Awadhesh Kumar Shukla

1.1 Introduction

It is well known that fungi are found to be ubiquitous in various ecological niches on planet Earth (Sharon and Shlezinger 2013). In nature, fungal species live as symbiotic, parasitic and saprotrophic mode (Kauserud et al. 2005; Sevindik et al. 2017). It is reported that there are approximately 140,000 fungi found on the earth and they play a pivotal role in the global ecosystem. Organic matter decomposes in the ecosystem due to the conversion of nutrients and recycling, soil formation and other activities of fungal species for the development of plants (Akgul et al. 2017; Bal et al. 2017). Fungi reproduce through their spores and, further, these spores spread in the environment and negatively influence living as well as non-living organisms (Sevindik et al. 2017).

The relevant environmental conditions vary from one species of fungi to another. Fungal species that are pathogenic in nature derive their nutrients from the host, while other species require specific environmental conditions for proliferation. It is reported that fungi and their host restrictions are variable in nature due to the species-specific interaction that can cause infectious diseases in a variety of animals. Pathogens are generally defined due to the nature of their relationship with their hosts. Some fungal species are totally dependent on their host in order to complete their life cycle, which are known as obligate parasites. On the other hand, some opportunistic fungal species live as saprophytes on dead and decaying organic matter and also invade living organisms, often called facultative pathogens. Previous research has noted that the fungus genus *Fusarium* has the potential to cause diseases in animals and humans, as well as in plants (Sexton and Howlett 2006). Emerging infectious diseases caused by fungi are a major global concern and

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recognised as a potential serious threat to animals and human beings. It has been established that zoonoses are potential infectious agents that are naturally transmitted between vertebrate animals and human beings (Seyedmousavi et al. 2015). It is reported that zoonotic infections have been a global concern for many centuries and contribute to the emergence of the majority of infectious diseases worldwide (Jones et al. 2008). Morse (1995) described the term "emerging infection", which is used to indicate an infection that has appeared for the first time amongst populations of living beings. However, there are various factors that trigger the emergence and re-emergence of infectious diseases in the arena of public health, including animals. These factors may include an increase in the population, technological and industrial development, environmental changes, resistance of microbes against drugs, a sudden outbreak of fatal diseases at local and global levels etc. (Lederberg et al. 1992). This chapter highlights the infectious diseases caused by environmental fungi in a variety of animals and also attempts to summarise the prevention and cure of infectious diseases in animals.

1.2 Fungal Diseases in Animals

Fungal diseases are widespread in a variety of animals. It is reported that the dispersion of pathogenic fungal spores in the environment is mainly attributed to anthropogenic activities. Additionally, the habitat flexibility, environmental and climate changes, and various reproductive modes of fungi are the major reasons for the emergence of disease (Fisher et al. 2013). It has been demonstrated that various fungal species possess inherent potential in a flexible manner for genetic recombination and horizontal gene transfer from species to species, and this is one of the plausible reasons for the genesis of new pathogens in the environment (Richards et al. 2011). Crawford et al. (2010) reported that amphibian populations and their diversity are being reduced in the highlands of central Panama due to the epidemic diseases spread by a chytrid fungus called *Batrachochytrium dendrobatidis*. The details of some other fungal diseases that spread in a variety of animals are summarised in Table 1.1.

1.3 Factors Affecting the Transmission of Diseases and Their Control Measures

It seems that the rampant industrialisation and overcrowding of the population pose serious threats in the foreseeable future. It is observed that there is continual change in climatic conditions and, consequently, increase in the global temperature and atmospheric gases alter humidity and rainfall (Pachauri and Reisinger 2007). The fungal phenology is directly affected by the changing environmental conditions. Due to the physiological and spatio-temporal changes, plants as well as pathogens

Table 1.1 Fungal species and their potential infections in animals

S. no.	Fungal species	Target animal	Disease	Reference
1.	*Basidiobolus ranarum*, *Conidiobolus coronatus*	Horse	Entomophthoramycosis	Smith (1989)
	Paecilomyces fumosoroseus	Aldabra tortoises	Pulmonary disease	
	P. lilacinus	Aldabra tortoise	Systemic	
	Fusarium solani	Aquatic animals, lobsters	Cutaneous	
	Pythium insidiosum	Horse	Cutaneous infection	
2.	*Cryptococcus neoformans* var. *neoformans*, *C. gattii*	Dog, cat	Cryptococcosis	O'Brien et al. (2004)
3.	*Exophiala pisciphila*, *E. salmonis*	Fishes	Cutaneous and central nervous system infection	Smith (2006)
	Aspergillus terreus, *A. deflectus*	Dog, German Shepherds	Aspergillosis	
4.	*Coccidioides immitis*	Armadillo hunters, hunters' dogs	Valley fever or desert fever	Tewari (2010); Sevindik (2018)
5.	*Geomyces destructans*	Bat	White-nose syndrome	Fisher et al. (2012)
6.	*Coccidioides immitis*, *C. posadasii*	Horses	Coccidioidomycosis	Cafarchia et al. (2013)
7.	*Microsporum* spp.	Domesticated and pet mammals	Dermatophytosis	Seyedmousavi et al. (2015)
8.	*Candida albicans*	Birds	Pulmonary candidiasis (sun conure, raptors)	Seyedmousavi et al. (2018)
	C. krusei	Ruminants, cattle	Bronchopneumonia	

are highly affected. Further, the release of fungal pathogens from the plant host causes dispersal into the environment and, consequently, creates health hazards for living beings. Earlier researchers have reported on the fact that there is less information available about the effects of climate change on the dynamics and distribution of animal-infecting fungi (Fisher et al. 2013). Other studies reported that warming of the climate has contributed to the appearance of *B. dendrobatidis*, which is a chytrid fungus causing disease in amphibians and reducing their population in Central America and Europe (Fisher et al. 2013). Additionally, agricultural processes also play an important role in climate change and alter the conditions of disease spread in the environment (Wilkinson et al. 2011). Globalisation is another major process that disseminates diseases, due to the export and import of plants and animals at the local, national and international levels and tourists and travellers potentially contribute to the transportation of disease organisms (Wilkinson et al. 2011).

Fungal diseases in animals are becoming a serious issue at the global level. The prevention of epidemic diseases is the prime concern for workers in this particular area. The curing of diseases may be possible by means of using antibiotics against the fungal species. However, sometimes, the pathogen becomes resistant to particular antibiotics; hence, broad-spectrum antibiotics could be useful for the mitigation of fungal diseases in animals. Chytridiomycosis is a serious disease among amphibians. Various studies have reported that immunisation, disinfection and the use of biocontrol are effective means for controlling this disease (McMahon et al. 2014; Bosch et al. 2015). Another fatal disease, coccidioidomycosis, is an important re-emerging disease of public health concern in the Southwestern United States. The infestation of this disease can be prevented by vaccination (Warnock 2006).

1.4 Conclusions and Future Prospects

It has been well proven for the past two decades that fungi are recognised as potential infectious agents for spreading diseases in animals. In the recent past, various organisms have emerged as new pathogens. It is essential to establish a microbial diagnosis because these organisms have potentially variable susceptibilities against antifungal drugs. The alteration of hosts' susceptibility to infection, diagnostic methods and changes in climatic conditions influence the mode of infection. It is very important to investigate the fungal spores and their concentration in order to counteract the fungal infection that causes emerging diseases in wild and domestic animals.

Emphasis should be given on the monitoring of fungal inocula in wild populations. Also, safety precautions focusing on the international trade of biological materials would be helpful in minimising the spread of fungal pathogens. Integrated approaches based on the theory and practical aspects of epidemiology, prediction of climate change and genomic analyses are warranted for the reduction of fungal pathogenicity.

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Chapter 2
Fungal Diseases of Honey Bees: Current Status and Future Perspective

Dipti Kashyap, Harshita Pandey, Kamal Jaiswal, and Suman Mishra

2.1 Introduction

Fungi are a eukaryotic, heterotrophic microorganisms having exclusive characters (decomposers, parasites, pathogens, symbionts) to adapt to different environments and the ability to grow and develop in both invertebrates as well as vertebrates. For instance, temperature, nutrient, and light conditions are critical environmental factors for the fruiting bodies of mushroom-forming fungi. Also, fungi are considered to be opportunists to flourish in a wide range of environmental conditions. The main factor that might have caused many saprotrophic fungi to became opportunist and, ultimately, pathogenic is the changing environment, and fungal pathogenesis is common for animals, as their host-specific adaptation is not that strong (Dworecka-Kaszak and Parazyto 2008). There was a perception that pathogenic fungi have not been posing serious threats to animal health. However, this perception has been changing rapidly due to the decline in wildlife caused by the emergence of unknown fungi (Daszak et al. 2000; Smith et al. 2006). Microsporidia, the most reduced and highly specialized fungi, have been adapted to an intracellular lifestyle and are also now considered as the major cause of the depopulation of honey bee colonies.

Honey bees are considered as a keystone species and contribute majorly to ecosystem functions because of the large number of pollination services that they provide for various ecosystems. Honey bees pollinate approximately 70–80% of crops worldwide, while the rest of the pollination in plants is done by other insects. As reported by McGregor (1976) and Delaplane and Mayer (2000), more than half of the human food supply is expected to be dependent on honey bee pollination. Honey bees are presently under threat because they are being attacked by different ordinary and anthropogenic aspects. Prominent natural factors which attribute their role in

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harming honey bees are mites, bacteria, viruses, and fungal spores (microsporidia). These factors act along with other pressures and contribute significantly to colony loss (Cox-Foster et al. 2007; Ratnieks and Carreck 2010).

The depopulation of honey bee colonies has a significant ecological and social impact; in fact, an ecosystem will experience a dramatic shift if the bee is removed. The direct cause of honey bee colony loss has not yet been determined, but microsporidian infection in bees' colonies has been reported by many researchers worldwide. Generally, *Nosema* spp.-infected bees have higher energy demands for escalating an immunological response. During nosemosis infection, a layer of the bee's intestine is covered by spores that ultimately deprive the physiological function of the alimentary canal. The nosemosis disease negatively affected survival and productivity, brood rearing, and pollen collection, eventually affecting bee behavior, as reported by Botias et al. (2013). Their study has shown that the interaction among these potential factors creates an immunosuppressive condition in the honey bees, which leads to mass infection in the honey bee colonies and further conditions that were detrimental to bee health.

This review chapter discusses one of the major factors in bee depopulation: the microsporidia, a group of obligate, spore-forming fungal pathogens found in all taxa from invertebrates to vertebrates. The impact of its infection on the honey bee colonies is reviewed and brief information is given about the importance of honey bees, their role in the ecosystem, and the impact of microsporidian disease on honey bee rearing and economic considerations. Also, the incidences of other fungal pathogens that have played a significant role in bee depopulation are discussed.

2.2 Major Milestones in Microsporidia Research

Nageli (1857) gave his first talk about *Nosema bombycis*, in which he first proposed that the organism responsible for the disease affecting silkworm was *Nosema bombycis*. Similarly, in Zurich, Nageli proposed the same causal organism for this disease. The first literature to mention a phenomenon of microsporidium was in the Beilage zur Botanischen Zeitung. In the nineteenth century, the European silk industry was facing a devastating scenario due to disease caused by microsporidium in France and Italy. The French sericulture industry was a growing and renowned industry in 1853 and had gained a tenth rank in silk production worldwide, but due to the arrival of this disease, the overall production of silk had become very low and almost 70% of silk manufacture became affected (Quatrefages 1860). The outbreak of the disease was not confined to only France but had extended to Italy, Spain, and Germany (Lebert 1856).

Unfortunately, in Germany (1860), silk manufacturing completely vanished because of this infection. A researcher named Lebert (1856) was the first person who recognized the oval creatures in unhealthy silkworms and proposed the name of the causal organism as *Panhistophyto novatum*, but it was not globally accepted. Later, after 5 years of investigation, Pasteur shared his valuable research and published it, namely, *Études sur la maladie des vers à soie* (Studies on the disease

of the silkworms) (Pasteur 1870). Pasteur not only described precautionary and preventive methods to overcome this infectious disease, but he also suggested that this disease was affecting both ova and moths. Pasteur advised silkworm rearers to examine worms under a microscope and discard unhealthy ones as a disease prevention technique.

Balbiani (1882), after a series of studies on the silkworm pathogen, was the first to suggest the separate taxon Microsporidia for *Nosema bombycis*, which was the only named microsporidian parasite up until then, followed by designation of the phylum Microspora by Sprague and Vavra (1977). Many important species were described later, with the first description of the microsporidium *Nosema apis* being the causative agent of an epidemic disease among honey bees by Zander (1909).

2.3 Morphology of Microsporidia

Microsporidia are intracellular, obligate, eukaryotic creatures. They are found in the form of spores, ranging in size from 3 to 6 μm, but 20 μm has also been reported (Sprague 1982). Spores of microsporidia are highly resistant and exhibit characteristic features, i.e., a coiled polar tube, its anchoring tool, and its genetic material, in which one or more filaments are present. This genus possesses unique aplanoblastic development, disporoblastic sporogony, and diplokaryotic nuclei in all developmental stages. The architecture of spores displays posterior vacuole and body, and this whole structure is bounded with a highly developed and specialized wall comprising the exosporium (electron dense) and the endosporium (electron lucent).

The life series of the multisporous microsporidian parasite can be divided into three phases; proliferative, sporogonic, and infective (Cali and Takvorian 1999). The dividing phase is intracellular and facilitated by host cytoplasm. The detailed morphological structure of microsporidia has been studied by Lom and Vavra (1961), and further electron microscopy of spores revealed new characteristic features of microsporidia which could become landmarks for distinction of the different species of *Nosema*. Under the microscope, the surface structure of spores looks unique and makes each species specific, which further helps the researcher to place the species accordingly in the hierarchy (Fowler and Reeves 1975). Chen et al. (2009a) conducted an ultrastructural study of *Nosema* spores and reported round spores that are bounded with endospore and exospore with ridges and furrows, while internal structures like polar filament, posterior vacuole, ribosomes, and a slightly separated diplokaryon nuclei were also observed and noted.

2.3.1 Life Cycle

The microsporidian spores enter through the bees' mouth, reach the midgut, and undergo development directly in contact with host cell cytoplasm. Mature microsporidian spores germinate by releasing their polar tubule and liberation of the

sporoplasm into the host cell. Inside the host cell, the spores undergo merogony and sporogony. Merogony is the proliferative stage of microsporidian spores in which meronts divide by binary fission to produce numerous spores and sporogony involves the spore maturation and differentiation stage, in which sporonts, sporoblasts, and, in due course, a mature spore is formed that is released outside the host cell to infect nearby cells (Poddubnaya et al. 2006).

Microsporidia of the genus *Nosema* are diplokaryotic and in direct contact with the host cell cytoplasm, and its life cycle is completed in less than 3 days. Spore activation takes place under appropriate conditions by shifts in pH and cation/anion concentration in the midgut. To date, signaling pathways that initiate germination have not been elucidated, but reports are present which suggest that calcium/calmodulin binding at the spore surface may play a role and start the signaling for spore germination.

2.4 Geographical Distribution

The European honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), a eusocial insect, is an ecologically and economically important species found throughout the world. The two most often studied species of *Nosema* commonly infecting honey bees are *Nosema apis* and *Nosema ceranae*. These parasites strictly follow the obligate mode of survival, as they multiply inside the digestive tract of bees and adversely affect their health. Both species of *Nosema* have, nowadays, received the extensive attention of researchers, but the latter species is relatively "new" and not as well understood. Researchers are not denying that variants of *Nosema* are now evolving and existing in a shifting environment. The active part of *Nosema* in current honey bee winter colony losses is blurred but has been implicated as a latent cause of losses of *Apis mellifera* in Europe (Higes et al. 2009), Asia (Huang et al.2007), the United States (Chen et al. 2008), and Canada (Williams et al. 2008).

Recent studies on the factors which are responsible for honey bee colony loss indicate that fungal infection is one of the most important causes related to honey bee disappearance, although the mortality of honey bees is a multifactorial process (Barcandritsos et al. 2010). Van Engelsdrop et al. (2009) carried out a descriptive study on colony collapse disorder and found that, over the last 2 years, a sudden decline was observed in the United States. Due to the lack of a recognized cause, this syndrome was named colony collapse disorder (CCD), as the key trait was a rapid destruction of adult worker bees.

Higes et al. (2009) described the horizontal transmission of microsporidian infection from worker honey bees to queens in a laboratory assay. According to these investigators, this can be a possible route of *Nosema ceranae* contagion in field colonies and have pathological consequences on honey bee queens. The main finding of the investigation was lesions which were found in the epithelial ventricular layer of the infected queens, with death following within 3 weeks, while the nurse workers were experimentally and collectively infected with almost 5000 viable spores per bee. Data of the research suggested that the greater number of infected house bees were positively related to the huge risk of transmission to queens.

An experiment was performed by Paxton (2010) to examine the impact of *Nosema ceranae* on the depopulation or colony collapse of the honey bee and individual bees also. That attempt was a step to pin down a causal relationship between *Nosema ceranae* and CCD. Whether *Nosema ceranae* is displacing *Nosema apis* was uncertain. Thereafter, Varvra and Lukes (2013) discussed intracellularly an obligate parasite which had the ability to reproduce and mature in the host cell cytoplasm. Additionally, they explained the various escaping features of microsporidia through which it manipulated not only infected cells but the host themselves as well.

Huang et al. (2012) carried out a comparison study on the immune response of selected and non-selected honey bee (*Apis mellifera*) lineage, taking the benefit of the haploid males to investigate its potential influence on the tolerance to *Nosema ceranae* spores. The lineage particular for *Nosema* tolerance showed higher *Nosema ceranae* spore load, with reduced mortality, and, surprisingly, an upregulated immune response. The findings of their study revealed that the specific gene of the Toll pathway was being regulated in the specific strain. Therefore, they concluded that this immune pathway was the probable route involved in *Nosema ceranae* infection response.

In India, a 50-million-hectare area of crops is dependent on bees for pollination and, in order to meet this target, 3 colonies/hectare are needed, but, at present, only 1.2 million colonies exist. A range of studies has shown that sexual reproduction in plants facilitated by honey bees plays a major role in agricultural production, including crops, fruits, fiber crops, nuts, etc. (Roubik 2002; Michener 2000). Honey bees are not only responsible for seed setting but they also maintain the quality of seed and fruits of various flowers. The incidence of disease-free bees with strong immune systems is a vital requirement for the managed pollination of food crops.

In India, *Nosema* disease is known for its devastating effects on *Apis mellifera* colonies in various parts of the country. Singh (1975) reported the first infection of *Nosema* in *Apis cerana* colonies from the Nainital district of Uttarakhand. The infrequent incidence of *Nosema* was logged from eastern states of India, i.e., Assam and Nagaland (Kshirsagar 1978). Kshirsagar (1982) also detected the contamination of *Nosema* disease from Jammu and Kashmir. Moreover, Rana and Katna (2011) reported that, in the northern states of India, more than 50% of *Apis mellifera* gatherings were found to suffer from this disease. Along with that, they also reported 30% mortality of forager bees during early spring and often during autumn for the last 5 years due to nosemosis.

Vavilova et al. (2017) performed an experiment to study the incidence of *Nosema* with *Crithidia* and *Lotmaria* pathogens in honey bee and bumble bee colonies of Jammu and Kashmir and South India. This was the first report of *Nosema bombi* infection in honey bee colonies. Further, they reported the presence of *Nosema ceranae* in the samples collected from India. Later, Kashyap et al. (2018) also described morphology with developmental stages of a microsporidian parasite of the honey bee in India. Their investigation was focused on the shape and size of microsporidia. They found shining green spore with typical Brownian movements under a light microscope. Morphometrics analysis was done by scanning electron microscope (SEM) study and the investigators found features similar to the genus *Nosema* and supported the fact that honey bee is infected with microsporidian spores collected from Lucknow state, India.

2.5 Factors Affecting Microsporidian Infection

Temperature, high level of pesticides, plant extracts, and the use of chemicals affects microsporidian infectivity. According to Retschnig et al. (2017), cold temperature increased the intensity of *Nosema ceranae* infection. Also, the use of high levels of pesticides in brood combs increased the level of *Nosema ceranae* infection. Moreover, several plant extracts have been used to reduce the parasitic effect of microsporidia. It was found that *Laurus nobilis* extract inhibited *Nosema ceranae* development (Porrini et al. 2011). Li and Fayer (2006) reported that chlorine exposure at variable concentrations inhibited microsporidia infection.

2.6 Lacunae in Knowledge

Microsporidia act as a stress-causing factor that has the ability to affect hives and create a devastating effect on the honey bee colony (Huang et al. 2012; Paxton 2010). The microsporidian *Nosema* spp. causes the very serious *Nosema* disease, also known as "nosemosis". As it is an unpredictable eukaryotic organism; an infection develops in the midgut of the host bees and, like other parasites, it consumes nutrition from the host tissue for survival (Campbell et al. 2010). Although this parasite does not create any lethal symptoms because it is an insidious, persistent disease with no outward symptoms, the beekeeper is not aware of the fact that the bees are infected (Higes et al. 2007). This disease is prevalent in the adult honey bees with no signs of disease and poses a worldwide concern to the honey bee apiaries through the reduction in their strength, multiplication, and honey production (Bailey 1981; Matheson 1993; Fries 2010).

According to Berrilli et al. (2012a, b), the composition of intestinal microbiota is an essential factor in controlling infection dynamics. However, there is only a partial understanding of the mechanism involved in interactions between the intestinal microbiota and the parasite. Ultimately, these parasites can be beneficial, neutral, or harmful to their hosts and, accordingly, on the basis of the host physiological state, these parasites can shift from an opportunistic to an infectious pathogen.

2.7 Knowledge of the Pathogen So Far

Microsporidia are micron-sized, obligate, multiporous fugal parasites that previously fell under the protozoan group. These eukaryotes are generally intracellular in nature and harbor all the taxa of the animal kingdom. These are opportunistic minute creatures belonging to the multitudinous regime with 1200–1400 species, infecting both vertebrates and invertebrates. Earlier, they were reported in acquired immune deficiency syndrome (AIDS), immunosuppressive, and immune-competent patients, but, later on, they were recognized as a major group of parasitizing agents, which parasitize insects.

Though these pathogens are an obligate parasite in the adverse condition, they exist in the form of spores as the escaping technique (Fries 1993). *Nosema apis* was the well-reported microsporidia infecting honey bees by Zander (1909). In addition to that, another species of microsporidia, *Nosema ceranae*, has been reported in many countries during the examination of seemingly healthy apiaries (Higes et al. 2006; Huang et al. 2007). Later on, research was focused on the rate of infectivity to different age groups of the honey bee. The findings indicated that the adult worker bee was prone to infectivity, even though they used to go outside the hive for pollen collection. Queens and drones were also found to be infected by *Nosema*, as the oral–fecal transmission route is also reported (Furgala 1962a; Bailey 1972; Higes et al. 2009).

Emergent members of the hive were found to be free of infection (Smart and Sheppard 2012). The ingestion of a viable spore is the first route of entry to the host; thereafter, upon reaching the midgut, they invade the epithelial cell and grow intracellularly by multiplying. Parasites infect their host by the invasion technique and pour their sporoplasm into the host tissue, where they grow rapidly and apply force on the cell wall. As a result, the wall ruptures and sporoplasm containing infective spores oozes out (Fries et al. 1992; Higes et al. 2007). Developmental stages in the form of spores can be observed under the microspore at 40× magnification. Spores bear a coiled polar tube, which acts as invasion organelle. Polar tube uncoiling takes place when triggered by an appropriate stimulus and, through this tube, sporoplasm transports from the infective spore to the host cell (Vavra and Larsson 1999). The number of coils in the microsporidian spores is species specific.

In *Nosema apis*, coiling of the polar filament is greater, as it is found to be more than 30, while in the case of *Nosema ceranae*, coiling of the polar filament reduces and it becomes only 20–23 (Fries 1989; Fries et al. 1996). Size variation in both species was observed and it was found that *Nosema apis* spores were large compared to *Nosema ceranae* spores. According to Ptaszynska et al. (2014), the surface of *Nosema ceranae* spores was crumpled; this feature was more prominent in the spores of this species (Figs. 2.1 and 2.2).

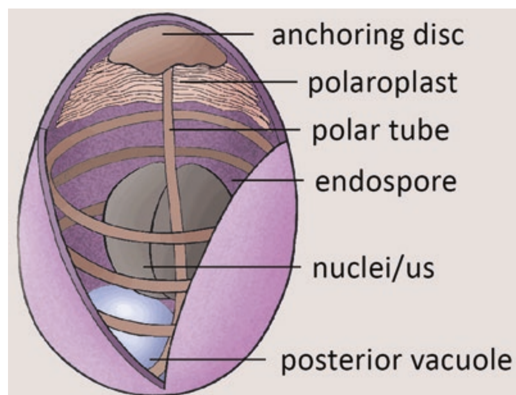

Fig. 2.1 Morphology and diagram of microsporidian spores. Parasitology Laboratory, Universidad San Pablo CEU. (Source: *The DMU eParasitology Project*)

Fig. 2.2 Life cycle of *Nosema ceranae* in the honey bee. (Source: http://scientificbeekeeping.com/the-nosema-twins-part-1)

2.8 Pathogenesis

Microsporidian spores are obligate, intracellular, eukaryotic pathogens, which need a host for their growth and development. For studying the pathogenicity, morphology, and life cycle of *Nosema*, the culture of *Nosema* spp. was done on lepidopteran cell lines by Gisder et al. (2011). This study was basically an attempt to discover the developmental stages as well as the mode of infectivity of nosemosis disease. It has been observed that this contagious disease is spread in bee colonies and individual bees by various means. In a study conducted by Higes et al. (2009), horizontal transmission has been confirmed as a possible route of *Nosema* infection in colonies.

The cleaning of infective excreta is one of the common causes of infection within a hive (Bailey and Ball 1991). Apart from that, bees usually get infected by visiting infected flowers (Evison et al. 2012). Prophylaxis was also reported by some researchers as a mode of infection (Smith 2012; Huang and Solter 2013). In case of severe infection in bees, millions of *Nosema* spores were observed in the infected midgut (Bailey and Ball 1991). Infected bees defecate and spread *Nosema* spores in the environment and, even in adverse conditions, the viability of spores do not get affected, but it has been reported that the sudden freezing of *Nosema ceranae* spores made them less viable (Fries 2010; Sanchez Collado et al. 2014).

Microsporidian spores unanimously affect all the phyla of the animal kingdom and, in case of a group of arthropods, it infects wasps, bees, bumblebees, house flies, etc. The occurrence of microsporidian infection is widely reported in adult honey bees. It has been mentioned that queen bees are less likely to get infected with *Nosema* disease because they are not involved in cleaning and feeding work; their

work is specific and this infection is spread via the orofecal route, so the queen bee is naturally prevented from getting infected (Wang and Moeller 1970; Dussaubat et al. 2013; Goblirsch et al. 2013). In some cases, it has been found that young queen bees gave a positive result for *Nosema* infection because of their weak immunity at earlier stages (Chaimanee et al. 2014).

As young queen bees are prone to infection, it is necessary for queen bee rearers to keep their comb healthy and clean to prevent devastating economic loss. The packed environment for mating (Gregorc et al. 1992), infected queen (Czekonska 2000), and increased queen mandibular pheromones (Alaux et al. 2011) are some of the reasons for infected combs at the queen level individually. The main symptoms reported among queen bees infected with *Nosema* spp. are shorter lifespan (Higes et al. 2009), less functionally active ovaries, and early supersedure (Hassanein 1951; Furgala 1962a; Loskotova et al. 1980). In forager bees, the symptoms become more prominent and affect the whole comb by interfering with the normal physiology of bees.

Immunosuppression and decreased vitellogenin level are the major alterations reported due to *Nosema ceranae* infection. Vitellogenin plays an important role in the regulation of the maturation of nurse bees to worker bees and it is an antagonist to juvenile hormone (Antunez et al. 2009; Goblirsch et al. 2013). The mortality of bees infected with *Nosema ceranae* is higher in Mediterranean states (Martin-Hernandez et al. 2011; Higes et al. 2008a), while in other states, the mortality rate is not as high (Forsgren and Fries 2010). The case of co-infection of both species is also reported (Milbrath et al. 2015). As *Nosema* infection adversely affects the physiology of bees, this disease enhances the capability of sugar syrup consumption in infected bees as compared to healthy ones (Martin Hernandez et al. 2011; Mayack and Naug2009). Bees consume trehalose for their flight mechanism and it is found that infected bees displayed a lower rate of trehalose compared to normal bees.

The lack of trehalose in bees leads to impaired wing formation and causes difficulties during flying. Along with that, it is reported that degenerative, discolored, or less functional hypopharyngeal glands are also observed in infected worker bees (Wang and Moeller 1969, 1971; Liu 1990). Loss of polytheism is the main reason for the less developed glands, as bees infected with *Nosema ceranae* start their foraging behavior at an early stage (Holt et al. 2013). A study on the protein content of food glands in infected bees revealed that diseased worker bees showed reduced protein levels in *Apis florae* (Suwannapong et al. 2010).

At the colony level, it has been observed that the ability of pollen collection is becoming reduced in infected bees (Anderson and Giacon 1992). It is also notable that, if 50% of bees in a colony are *Nosema*-infected, the colony may die off soon (Pickard and ElShemy 1989; Higes et al. 2008b).

An experiment carried out by Fries et al. (1984) revealed that the total honey yield was reduced to half when the colony was infected with *Nosema apis* infection. Simultaneously, they also found a 15 million *Nosema* spores count per bee with >50% of bees infected with *Nosema apis*. In case of *Nosema ceranae* infection, an increased level of ethyl oleate was found in worker bees, which plays an important role in the division of labor, but due to the misbalancing of this chemical, the whole comb could become adversely affected (Dussaubat et al. 2010, 2013).

Table 2.1 Microsporidia species and their different host groups

Microsporidia	Host
Cystosporogenes legeri	*Lobesia botrana* (Lepidoptera)
Cystosporogenes operophterae	*Operophtera brumata* (Lepidoptera)
Endoreticulatus schubergi	*Choristoneura fumiferana* (Lepidoptera)
Endoreticulatus bombycis	*Bombyx mori* (Lepidoptera)
Endoreticulatus sp.	*Lymantria dispar* (Lepidoptera)
Liebermannia patagonica	*Tristira magellanica* (Orthoptera)
Nosema bombycis	*Bombyx mori* (Lepidoptera)
Nosema ceranae, *Nosema apis*	*Apis mellifera* (*Hymenoptera*))
Orthosomella operophterae	*Operophtera bruma* (Lepidoptera)
Paranosema grylli	*Gryllus bimaculatus* (Orthoptera)
Paranosema locustae	*Locusta migratoria* (Orthoptera)
Pleistophora bombycis	*Bombyx mori* (Lepidoptera)
Systenostrema alba	*Aeshna* sp. (Odonata)
Thelohania solenopsae	*Solenopsis invicta* (*Hymenoptera*)

Adapted from Sokolova et al. (2006)

A very common symptom observed at the colony level is depopulation of the colony because, due to the *Nosema* infection, unhealthy worker bees lose their ability to find their return route to the hive. These unusual happenings occur due to compromised orientation skills (Kralj and Fuchs 2009) and weakened bodies (Mayack and Naug 2009, 2010). A study conducted by Huang et al. (2007) suggested *Nosema ceranae* infection to be dominant because of its wide genome diversity and single nucleotide polymorphisms (SNPs) per gene. Therefore, it is highlighted that apoptosis of bee cells decreased during microsporidian infection (Table 2.1).

2.8.1 Physiological and Behavioral Perturbation in Honey Bees

There is a rapid increase in the prevalence of nosemosis disease (Higes et al. 2008a). This disease causes a change in the feeding behavior of honey bees and is also correlated to a decrease in pheromone production, the ethyl oleate that, in due course, affects the behavioral maturation of bees (Leoncini et al. 2004). Apart from affecting gut microbiota, the disease suppresses the cellular immune response, impairing the defense system of the host (Alberoni et al. 2015). The nosemosis infection in honey bees also affects the ecological niche of beneficial host microbes by inhibiting the expression of genes that are involved in gut tissue regeneration, e.g., Wnt signaling (Dussaubat et al. 2012). Forager bees infected with the microsporidia *Nosema* are a useful indicator to determine colony-level infection (Fries et al. 1984).

2.9 Treatment

Any abnormal condition of an individual for which they can't perform their normal physiological and physical activity is known as a diseased condition. Nosemosis is one of the major outbreak diseases affecting bees, bee colonies, and the apiculture industry. To control this disease, various drugs have come into practice but the side effects of these drugs are another major problem. These side effects can be in the form of decreased yield of honey, compromised quality of honey, lethargic activity of bees, etc. An antibiotic named fumagillin was a popular drug used in the treatment of *Nosema* disease (Furgala 1962b; Williams et al. 2008), but was found to be genotoxic (Stanimirovic et al. 2007), and exposure to a dose lower than that prescribed increased the multiplication of *Nosema ceranae* in the host body (Huang and Solter 2013). Other therapeutics used as control measures for reducing the *Nosema* spore count is surfactin S2 (Porrini et al. 2010), thymol (Costa et al. 2010), and Nozevit (Tlak Gajger et al. 2009). Apart from using chemicals, researchers are directing attention to precautionary measures and suggesting that comb replacement, maintaining hygiene inside out in the comb, and replacement of the queen and eradication of dead bees are valuable practices that can be carried out as cheap and effective prevention methods (Fries 1988; Loskotova et al. 1980). A sophisticated method for reducing the spore count in the midgut of caged bees is the gene silencing method (RNAi) used by Paldi et al. (2010). Development or promotion of *Nosema*-tolerant honey bee strains may reduce the *Nosema* infection in the comb as this strain is capable of maintaining an optimal death rate of infected epithelial cells of the midgut (Kurze et al. 2015).

2.9.1 Diagnosis

Nosema spp. spores can be diagnosed by the microscopy technique by observing the crushed preparation of abdomen homogenate of bees. However, there is a lack of accuracy due to the small size of the spore. Various staining techniques, such as Giemsa staining, Calcofluor white, and the Chromotrope 2R method, have been chosen to increase *Nosema* spore identification (Weber et al. 1992). For diagnosing microsporidia in insects, the polymerase chain reaction (PCR) method has been demonstrated to be a specific method and, therefore, restriction mapping and hybridization probes have been widely used for epidemiological studies (Weiss and Vossbrinck 1999). So, for diagnosing *Nosema* spores, molecular biology techniques seem to be essential. By using a multiplex PCR assay, Hamidyzzaman et al. (2010) determined *Nosema* infection in honey bees. In their study, they amplified the 16S rRNA gene by the multiplex PCR technique. Additionally, they performed triplex PCR for spore quantification connecting co-amplification of the *Nosema apis* and *Nosema ceranae* 16S rRNA gene. Hence, it is concluded that semi-quantitative triplex PCR is more perceptive than standard PCR methods.

Also, multiplex species-specific quantitative real-time PCR can be used to determine *Nosema* infection in hives and seasonal differences as well (Traver and Fell 2012). To study surface morphology and the level of infection in the midgut of honey bees, Ptaszynska et al. (2014) used the SEM technique, which revealed the cause of bees' malnutrition. Also, they used bright field, phase contrast, and differential interference microscopy for the identification and differentiation of microsporidian spores. A transmission electron microscope can also be used for identification and intracellular parasite development. In many cases, no clinical signs of infection were reported, so, in order to determine the prevalence of nosemosis in honey bees, both light microscopy as well as multiplex PCR based on 16S rRNA should be used (Papini et al. 2017).

For determining the spore count, apart from a traditional hemocytometer, a Coulter counter has been proved to be more efficient (Crnivec 2016). Yucel and Gogaroglu (2005) determined the level of infection by microsporidia of the genus *Nosema* by spore counting and classified it as low infection if it was less than 5 million spores/bee, medium infection if it was between 5 and 10 million spores/bee, and high infection if it was more than 10 million spores/bee. This analysis suggests that dead bees are extremely infected with microsporidian parasites. Some serological methods such as enzyme-linked immunosorbent assay (ELISA) and Western blot have also been proved to be useful in diagnosing microsporidiosis (Singh et al. 1982). Also, some of the sequences of the rRNA operon have been used as a molecular marker for diagnosis (Chen et al. 2009b).

2.10 Occurrence of Other Fungal Pathogens

The pathogenic fungi in insects are capable of invading their specific host and overcoming immunity. Ascomycetes, such as *Ascosphaera* and *Aspergillus*, cause chalkbrood and stonebrood diseases (Humber 2008). Chalkbrood disease in honey bees has a narrow host range, while stonebrood disease is demonstrated to have a broad host range.

2.10.1 Stonebrood Disease

Apart from the high prevalence of nosemosis in honey bees, stonebrood disease with low virulence is also one of the causes of bee depopulation. This disease was first described by Massen (1906) and *Aspergillus* spp., which is a saprophytic, cosmopolitan, filamentous fungus, was the causative agent. Aflatoxins are produced by this fungus and have carcinogenic properties; therefore, when this disease affects bees, it might also be a grave cause of depopulation (Burnside 1930).

2.10.2 Chalkbrood Disease

This disease has been affecting bees worldwide, causing mycosis, which is produced by *Ascosphaera apis* (Spiltoir 1955). It is mostly fatal to bees' larvae but does not damage the bee hive; however, it causes losses in the number of bees as well as productivity (Bailey 1963).

By its hyphae, the *Ascosphaera* spp. penetrates the larvae intestinal wall and their mycelium grows within the body cavity. After a few days, on the outside of dead larvae, fruiting bodies with new ascospores are developed (Maurizio 1934). The larva is covered by a thick layer of mycelium with brown or black spots due to Ascomycota. During spring, the chalkbrood disease is more prevalent and the severity of infection is influenced by both abiotic (cold and wet hives) as well as biotic factors, such as fungal strains (Aizen et al. 2009). Bees associated with this disease are insensitive to olfactory stimuli with sick brood (Wilson-Rich et al.2009). The accumulation of spores in the hive might be the cause of long-term infection. The spores ingested by bees germinate in the intestinal lumen by the action of CO_2 (Nelson and Gochnauer 1982). Therefore, according to a 6-year database, the incidence of chalkbrood disease increases in the spring, with hives having a greater number of *Varroa* mites and *Nosema ceranae* (Hedtke et al. 2011). In a previous study, it was reported that the overuse of antibiotics (oxytetracycline) used for preventing bacterial disease in bees can change the intestinal microflora of bees that favors the growth of fungi (Menapace and Wilson 1979). Therefore, it is better to use natural compounds for controlling fungal diseases. Products derived from plants such as citral, citronella, and geraniol were tested in vitro, showing an inhibitory effect on the fungus (Bogdanov et al. 2004). To control disease in bees, proper management and sanitation practices, along with preventing the use of pesticides, should be followed.

2.11 Parasitized Honey Bee Immune Gene Suppression

To combat infection, insects have a diverse mechanism. Though insects have efficient cellular and humoral immune defense, some pathogens defeat the morphological defenses in insects. Gregory et al. (2005) studied the humoral immune response of bees that were parasitized by *Varroa destructor*. They studied the expression levels of the antibacterial peptides abecin and defensin, and found non-linearity with respect to the number of mites parasitizing the bees. Therefore, they concluded that mites overcame the host immune response by suppressing the immunity of bees. It is important to find out the immune response of honey bees and foragers to co-evolved fungal pathogens, mostly *Nosema apis* and *Nosema ceranae*, that causes epizootic disease within bee colonies and infect the gut epithelium of insects (Schmid-Hempel and Schmid-Hempel 1998). Honey bees have evolved

individual-level defense as well as group defense. Grooming and behavioral defenses reduce the impact of pathogens. A total of six antimicrobial peptides (AMPs) have been found. Defensin-1 and defensin-2 have been found in many insects but apisimin and hymenoptaecin have been reported only in honey bees (Yoshiyama and Kimura 2010). A study was conducted by Richard et al. (2007) on modulating social interaction by immune stimulation in honey bees. In that study, the investigators developed an adaptive strategy to prevent non-infected conspecifics. Apart from individual immunity, social immunity also plays an important role, such as colony-level pathogen protection; thus, cooperative behavior helps reduce the parasitic load (Cremer and Sixt 2009).

Venom peptides that have an antimicrobial property are present on the cuticle of adult bees and have been acting as a substance that possess an antiseptic property. The presence of this antimicrobial property helps in developing community immunity in social insects (Baracchi et al. 2011).

Most of the pathogens infecting bees are sensitive to temperature, so bees develop fever to kill *Nosema*-like diseases and chalkbrood diseases (Martin-Hernandez et al. 2009). Behavioral fever is a frequent response to an infection in many animals. Honey bees uphold high temperatures in the brood nest to speed up brood expansion and provide an effective means of protection from predators.

Antimicrobial effects have been found in secondary metabolites in the nectar of plants and the health of honey bees ultimately depends on the floral source of the honey. Therefore, the honey itself may act as a self-medicating agent to protect bees from infection (Simone et al. 2009)

2.12 Conclusion

The depopulation of honey bees is a major concern for researchers nowadays. It is a multifactorial process in which anthropogenic activity, industrial interference, and viral, bacterial, and fungal attacks are common factors. Out of all these factors, attack of fungal pathogen, i.e., microsporidia, is now catching the attention of researchers because of its universal presence in all taxa. Bees are highly susceptible to this pathogen as they visit outside the comb for foraging purposes. Urbanization prompts bees to fly to different areas where they get exposed to pathogens, while intensive monoculture encourages bees to change their habitat for fulfilling the nutritional requirement. The sudden exposure of bees to new environments increases their chance of getting infected by pathogens via various means. Loss of the whole colony due to microsporidian infection has been reported by many researchers and named as the phenomenon colony collapse disorder (CCD) (Bromenshenk 2010). A united approach to overcome this disease is needed; simultaneously, new eco-friendly therapeutic practices also need to be evolved to counter the negative side effects of chemical drugs.

2.12.1 Future Directions

Nosema bombycis was discovered a long time ago and plenty of research on microsporidiosis is still ongoing, though microsporidia infected a vast range of host species, including parasites, as they also exhibit hyperparasitism. For instance, *Desmozoon lepeophtherii* is a microsporidian hyperparasite in salmon louse causing opaque inclusions throughout the body (Freeman and Sommerville 2011). Thereafter, this has been reported by many researchers in a range of invertebrate hosts. The microsporidia not only infect healthy individuals but they also infect parasites which themselves harbor on other hosts. So, it appears that they have developed immunity against the parasite. It is imperative that research now must be focused in a manner such that the host–parasite relationship is studied in depth, with a special emphasis on *Nosema* and other microsporidian species. Studies on the immune response to microsporidian infection affecting parasites will give a better understanding for future studies on therapeutics to cure immunosuppressive diseases like AIDS. This may give new insights for researchers to observe every response given by parasite immunity which can further be correlated to deal with *Nosema* or other infections by developing targeted, cell-mediated, eco-friendly drugs. In the case of honey bees, this may lead to very useful tools that can be used without any side effects.

2.12.2 New Insights

In recent years, there has been an increase in invasive fungal infections in a wide range of invertebrates, as well as vertebrates. This increase may be related to an increase in immune-compromised patients, increasing incidence of cancer, prolonged invasive treatments, etc. Consequently, medical mycology has been tending towards the development of new vaccines against fungal pathogens. The development of anti-fungal drugs should now become a priority for the industry and academics because the world's population is increasing and the number of immune-compromised individuals will continue to rise, resulting in the enhancement of fungal diseases.

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Chapter 3
Immunological Diagnosis of Fungal Disease in Animals

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3.1 Introduction

Fungal species can be obtained from habitats like the ocean, land, air, animals, plants, etc. Although there are a large number of fungi in the world, only a small number can cause diseases. Infectious fungi can be classified as true pathogens or opportunistic pathogens. True pathogenic fungi cause localized or systemic infections, while opportunistic fungi cause disease under conditions of weakened immune system. Such conditions may result from viral infections, immune disorders, chemotherapy, immunosuppressive drugs, etc. Fungi can more easily infect such vulnerable hosts. Increased usage of antibiotics and rise of immunosuppressive infections have increased incidences of opportunistic fungal infections. Fungi may cause various infections ranging from superficial to systemic mycoses (Table 3.1).

Fungal pathogens are true pathogens if they cause infection irrespective of host immune infection. These demonstrate dimorphic characteristics with geographic restriction, infection by inhalation, and pyogenic/granulomatous host response. Their progression is similar to bacterial infection in tuberculosis. Most fungal infections are caused by opportunistic pathogens. Opportunistic fungi are omnipresent and include yeasts and molds. Their routes of transmission are various openings or skin cuts on host body, and their survival depends on the host response.

Fungal sample collection is the first step toward identification of causative fungi. Collection of the biological sample should be done in appropriate vessels and transport containers. Subsequently, the sample must be labeled and dated immediately,

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and complete requisition forms must be filled. Typical sample types and their initial processing are mentioned in Table 3.2.

In all cases samples should be collected in sterile conditions and taken to laboratory as early as possible. *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* do not survive well in frozen or iced specimens. Hence sample should not be frozen unless necessary.

Table 3.1 Categories of fungal infections and important immune responses against them

S. no.	Types of infection	Target organ or organ system	Major character of fungi	Immune response
1	Superficial	Skin	Opportunistic	Caused by hypersensitivity reactions to fungi normally found on skin
2	Cutaneous mycoses	Skin, eyes, sinuses, oropharynx, external ears, vagina	Opportunistic	Immunodeficiency, hormonal imbalance
3	Subcutaneous	Localized primary infections of subcutaneous tissue	Opportunistic	Can cause the development of cysts and granulomas
				Provoke an innate immune response—eosinophilia
				formation of granulomas
4	Systemic or deep mycoses	All organs' infection	Opportunistic and true fungal pathogens	Immunodeficiency
				Causes the formation of granulomas
				Can necrotize and become calcified

Table 3.2 Sample collection and initial processing for fungal identification and disease diagnosis

S. no.	Specimen	Vessel/container	Remarks
1	Sputum	Screw capped	Decontaminate before culture
2	Bronchoscopy fluid	Screw capped	Sterile conditions should be maintained
3	Cerebrospinal fluids	Screw capped,	Can be stored at room temperature if immediate transfer to lab is not possible
4	Urine	Screw capped vials	Ice boxes or cool pack should be used to maintain temperature at ~4 °C
5	Blood	Biphasic agar broth bottles	Keep in cool conditions
6	Tissue biopsy	Sterile container	Do not freeze the sample unless absolutely necessary. Frozen samples must be rehydrated prior to culturing

3.2 Laboratory Testing for Fungal Identification

Before immunodiagnosis, it is good practice to culture or examine the probable types of fungi. The identification of colonies should be based on basic characteristic of culture morphology. Yeast colonies are visible with smooth, creamy, viscous, or pasty appearance. Dematiaceous mold colonies exhibit dark, gray to black mycelium growth and reverse of the colony is black. Molds have a distinct border and are white on the surface. Slow-growing dimorphic species of molds have a cobweb-like aerial mycelium. A mold colony is mounted in a drop of Lactophenol/cotton blue stain on a glass slide and examined microscopically. The specimen should be directly mounted in 40% and 10% KOH for skin and nail specimens, respectively. This dissolves epithelial cells and enhances visibility of fungus.

3.3 Microscopic and Macroscopic Examination

There are two types of culture media for fungal cultures. One is nonselective, such as brain heart infusion, and is highly useful for all clinically relevant fungi. The other is selective. Sabouraud's dextrose agar is insufficiently rich to recover certain fastidious pathogenic species, particularly dimorphic fungi. Such fungi can be grown on potato flake agar (PFA), inhibitory mold agar (IMA), or a combination of Sabouraud's dextrose agar with heart infusion (SABHI) agar. Czapek's agar can be used for the subculture of *Aspergillus* species if colony morphology is an important identifying criterion for a given unknown isolate. For more fastidious dimorphic fungi such as *Blastomyces dermatitidis* and *Histoplasma capsulatum*, an enriched agar like IMA or SABHI is used, and in particular for *Histoplasma capsulatum*, media supplemented with 5–10% sheep blood is recommended. *Cryptococcus neoformans* and *Aspergillus fumigatus* may be partially or totally inhibited by cycloheximide; therefore a nonselective media must always be used in parallel. Fungal staining can be done by India ink. It gives a dark background around highlighted hyaline yeast cells and capsular material with halo zone. It is used to examine specimens suspected of containing *Cryptococcus neoformans*. Smears of *Candida*, *Malassezia*, and *Sporothrix* may be stained poorly with Gram staining but should not be relied upon to demonstrate yeast or other dimorphic fungi. Gram stain can visualize the filaments of *Nocardia* and *Actinomyces* which may produce clinical signs resembling mycotic infection. Kinyoun Acid Fast Stain is also useful for *Nocardia* (*Nocardia asteroides*).

Fungal infections of birds and animals are undiagnosed clinically in most cases until a postmortem examination is performed. Biopsies of the lungs present characteristic white or gray nodules (plaques) which can be visualized macroscopically. Therefore, presence of mycosis is first recognized after histopathologic examination (Carrasco et al. 1993) in such hosts.

3.4 Immunology of Fungal Infection

Opportunistic fungi cause more fungal infections than true fungal pathogens. There are few vaccines or preventions against these fungi which may cause serious ailments of local skin and mucosa. In some cases it may lead to organ failure and be fatal to host. Fungal spores and cells are long lived, and if conditions are nonfavorable, they wait for optimum conditions. Their attack can be recognized by both innate and adaptive immune system. During innate activation, one can observe a cascade of cell signaling, the release of cytokines and other molecules that recruit phagocytes and antigen-presenting cells. Further, it leads to antibody production by B cells and later on activation of naive T cells. Here it is important to note that fungal cells are eukaryotic in nature; thus fungal virulence factors can overactivate the immune response. Overly secreted T cells and highly activated inflammatory response may cause damage to host tissue if not treated promptly. Fungal infections are many times responsible for granulomatous inflammation characterized by formation of epithelioid tubercles. Mononuclear leukocytes, specifically histiocytes (macrophages), respond to chemical mediators produced after infection causing granulomatous inflammation. The molecules expressed in immune response and the pattern of granulomatous inflammation raised against fungal infection could be among important tools of clinical differential diagnosis. Pathogen-associated molecular patterns (PAMPs) are dominated by component polysaccharides of the fungal cell wall and their cognate receptors, i.e., pattern recognition receptors (PRRs), C-type lectin (CTL), and nod-like receptor (NLR). Dendritic cells and macrophages bind to the components of fungal cell walls using pattern recognition receptors (PRRs) on their surface. C-type lectin receptors (CLRs, e.g., Dectin-1) are particularly important PRRs in antifungal immunity, although several other PRRs are also involved including Toll-like receptors (e.g., TLR2) (Fig. 3.1).

The cell wall of fungi is two layered. Outer wall molecules are either mild proinflammatory or immunologically less active. They include chitin which can provoke overexpression of eosinophilia leading to asthma. Chitin is a simple polysaccharide found in all fungi studied to date. It is made by β(1,4)-linked homopolymer of

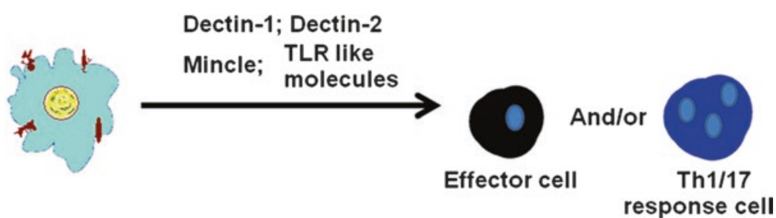

Fig. 3.1 A dendritic cell having antifungal PRRs (Dectin, Mincle, and TLR molecules) which recognize fungal antigen and generate signals to produce Th1/17 adaptive immune responses and activation of innate effector cells

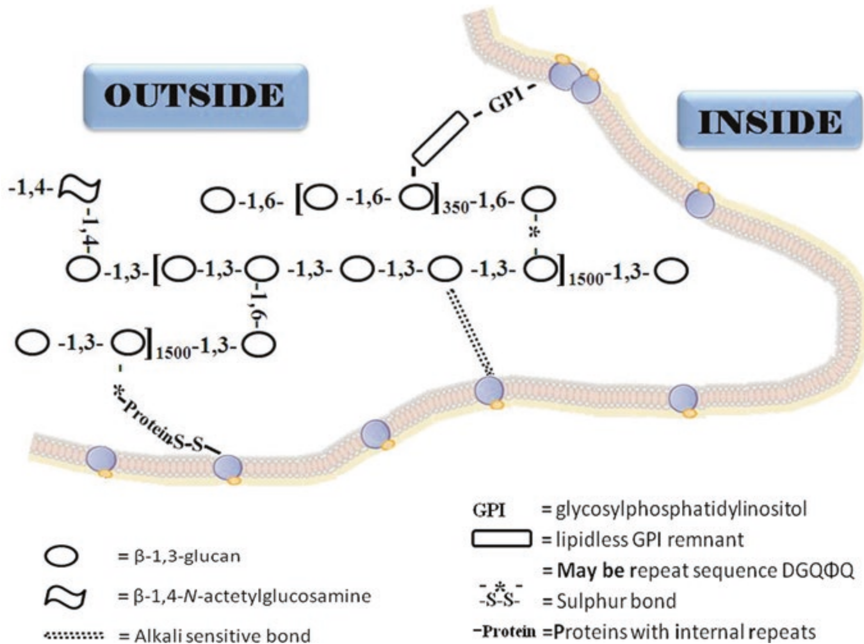

Fig. 3.2 Major cell wall components and their linkage

N-acetylglucosamine (GlcNAc) that plays a significant role in the activation and attenuation of immune responses. In many pathogenic species, major proteins of cell walls are attached through a remnant of their glycosylphosphatidylinositol (GPI) to β(1,3)-glucan via a branched β(1,6)-glucan linker (Fig. 3.2). The links between β-1,3-glucan and β-1,6-glucan or PIR proteins are still uncharacterized. The inner cell wall is predominantly rich in proinflammatory β-1,3-glucan. To date, no β-1,6-glucan synthase protein has been identified in any fungal species (Ziman et al. 1996). Genetic screening indicates that proteins necessary to keep normal levels of cell wall β-1,6-glucan are localized along the secretory pathway; thus, β-1,6-glucan synthesis may involve intracellular events (Shahinian and Bussey 2000; Lesage and Bussey 2006).

Fungal antigens and PAMPs stimulate PRRs and attached molecules (FcRγ) to activate phagocytosis, production of reactive oxygen species (ROS), and cells of adaptive immunity. At present exact mechanism of adaptive immunity is not known, while literature presents an understanding of CD_4^+ T cell-based Th_1 and Th_{17} antifungal immune response. It is considered that PRRs stimulate cytokines responsible for CD_4^+ T cells signaling for Th_1 and/or Th_{17} pathway (Medzhitov 2009; Gordon 2002; Savina and Amigorena 2007; Banchereau and Steinman 1998). The polysaccharide β-1,3-glucan is a marker to identify fungal pathogens. It is also responsible for inflammation by stimulating Th_{17} immune responses and activation of the

NLRP3 inflammasome. Here NLRP3 stands for nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 OR Nod-like receptor protein-3. C-type lectin receptors (CLRs) are the major group of receptors for clearance of fungal antigens. Dectin-1, Dectin-2, and macrophage-inducible C-type lectin (Mincle) are all CLRs that share common signaling mechanism via spleen tyrosine kinase (syk). There is a cascade of kinases that recruit syk to the activated receptor via signaling molecule caspase recruitment domain-containing protein 9 (CARD9). CARD9 is the most important molecule for activating antifungal Th_{17} antifungal immune responses. If an immune cell has the PRRs to bind fungi but does not have CARD9, then the receptors will also be unable to generate signal to evoke immunity. Thus, one can observe high susceptibility of fungal infection in CARD9-deficient mice or human subjects (Glocker et al. 2009). Major effector cells in the granuloma are T lymphocytes of the helper and cytotoxic subsets; activated macrophages, including epithelioid and giant cells; activated polymorphonuclear leukocytes, both neutrophils and eosinophils; NK cells; B lymphocytes; and plasma cells.

3.5 Immunological Techniques for Fungal Diseases

Physicians need an early and correct diagnosis for proper management of infection. Fungal infections are diagnosed in the laboratory with appropriate identifications based primarily on the assessment of colony morphology and microscopic features. Molecular techniques like ribotyping, DNA fingerprinting, and nucleic acid probe assays are costlier tools to identify early infection in suspected cases. Microbiological, biochemical, and immunological or serological tests offer hope for diagnosis at a cheaper price. Serological studies are required in some instances to establish differential diagnosis without costlier and time-consuming culture methods. Automated serological testing is another boost up for diagnosis of fungal infections.

Fungal taxonomists have reported a large number of pathogenic fungi for necessary identification by clinical mycologists. Investigators have to develop and apply novel and superior methods over conventional tools. At present, for management of a suspected infection, isolation of the causative agent from a relevant clinical specimen and its unequivocal physical identification in clinical specimens and/or in histopathological setting of tissue invasion should be done. These methods call for skilled personnel with relatively high levels of specific mycology training. Recent decades show a rapid growth in the field of immunology permitting the development of several new techniques, which are gradually being adopted by researchers interested in the diagnosis of fungal diseases. Molecular methods and antigen detection are also promising techniques for fungal diagnosis.

3.6 Immunofluorescence

Fluorescence is the phenomenon whereby a substance emits electromagnetic radiation especially visible light. Theoretically, some substances can fluoresce if exposed to radiation energy. It persists till the incident or stimulating radiation continues to fall on the substance. There are several cell organelles which can fluoresce. The study of fungal infections of hair using fluorescence was reported for the first time in 1925 (Margarot and Deveze 1925). Gordon (1958) used immunofluorescence technique for the diagnosis of *Candida albicans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* in pure cultures.

Spores of fungi are able to fluoresce after following a typical process given by Gao et al. (2013). Sample spores are placed on a glass slide and mixed with a drop of 2% poly-L-lysine aqueous solution. A suspension is prepared by adding more water and then left to dry. Dried slides are washed twice in phosphate-buffered saline (PBS), pH 7.4–8.0, for 5 minutes each time. Subsequently the slides are treated with a suitable monoclonal antibody and incubated at 4 °C overnight. The next morning, the slides are incubated at 25 °C for 10 minutes and washed thrice with PBS (5 minutes each). Following this the slides are incubated in a solution of goat anti-mouse monoclonal antibody labeled with fluorescent dye [(Phycoerythrin (PE), Cyanine dyes (Cy3 & Cy5), Fluorescein isothiocyanate (FITC), etc.)] at 37 °C for 90 minutes and then washed thrice in PBS (5 minutes each). Finally, the slides are treated with glycerol buffer (0·05 M Na_2CO_3, pH 8·0, 50% (W/V)), and dye excitation is read using a fluoroscope. Fluorescence can be read according to selected fluorescent dye. It is normally near UV wavelength of 320–400 nm. UV light in dermatology is used predominantly in diagnostic areas involving pigmentary disorders, cutaneous infections, and the porphyrias (Asawanonda and Taylor 1999).

3.7 Immunoperoxidase Staining

Fungal species are identified in pathology laboratories by their specific morphology and microscopic features. Morphological similarity of different fungal hyphae found in host tissues can lead to wrong identification by histopathologists (Mendoza et al. 2004). Specific and sensitive methods like immunoperoxidase staining can be used to resolve the problem. The tissue sample in question should be implanted in a paraffin block and dipped in formalin and cut in fine slice. These slices are then treated with xylene to remove paraffin (deparaffinization) and rehydrated in ethanol. Washing is required at each step using phosphate-buffered saline (PBS; pH 7.4). Deparaffinized tissue is then rehydrated and treated with TE buffer (pH 9.0) for 40 minutes and incubated at 95 °C in a water bath. The slides are further treated with 1:4 solution of 3%H_2O_2 in methanol for 30 minutes. At this step nonspecific binding should be prevented by 3% horse serum albumin in phosphate-buffered saline

(blocking buffer). Now, the slide is incubated with rabbit antifungal serum (diluted in blocking buffer) at room temperature for 1 hour in a moist chamber. This is followed by washing twice with PBS for 5 minutes. Tissue sections are subsequently incubated at room temperature with undiluted mouse anti-rabbit antibodies that are conjugated with horseradish peroxidase for 30 minutes. Washing is repeated as mentioned above, and finally slides are treated with a substrate for horseradish peroxidase (like diaminobenzidine) solution prepared in Tris- or phosphate-buffered saline (pH 7.4) for color reaction. Tissue sections should also be counterstained (hematoxylin) to produce distinctly brown-stained fungal elements. These stained tissues can be visualized under a light microscope (Keeratijarut et al. 2009).

3.8 Counter-Immunoelectrophoresis (CIE)

CIE can be an early diagnosis test for fungal infection. It is dependent on precipitation reaction of antigen and antibody. A soluble antigen combines with specific antibodies to form an antigen-antibody complex which is large enough to precipitate. The diffusion of antigen and antibody in gel is accelerated by electric current, and precipitation can be visualized within a few hours. Various antigens are extracted from sonicated fungal cell suspensions. First of all veronal-buffered saline (VBS) should be prepared. VBS is a useful buffer for decay accelerating assays. It is prepared from three separate solutions. The first solution is made up of 1.02 M NaCl and 13 mM sodium barbitone. The second solution is made up of 62.5 mM barbitone in hot distilled water. And the third is made up of 2.18 M $MgCl_2$ and 440 mM $CaCl_2$ in distilled water. The first and second solutions are mixed in 2.8:1 ratio and left to cool at room temperature. Now the third solution is added at 0.36% v/v. Finally, the pH is adjusted to 7.3–8.2 using 1 M HCl. A 5x stock solution can be stored for 4 weeks. The working solution can be prepared by diluting stock (1:5) with distilled water. A coat of 1% agar in 1x veronal-buffered saline, pH 8.2, is layered on a glass plate. Then wells of 3 mm are punched in two parallel rows about 1.5 cm apart. The antigen is loaded into wells toward the cathode and test sera into wells toward the anode. Then the plate is kept in an electrophoresis tank under refrigerated conditions. Whatman paper No.1 should be used as bridge between gel and buffer. Finally a current of 10 mA per plate (of 8 × 8 mm) is applied for 90 min with increasing voltage. The precipitin bands can be visualized with amido Schwarz staining and differentiated with 2 percent acetic acid (Fig. 3.3). Thorough washings with distilled water can remove artifacts.

In comparison, sensitivity of CIE (up to 77%) is almost equal or slightly higher than immunodiffusion (ID) with slightly greater specificity (at least 95%). The complete execution time of CIE and ID are also almost equal (de Camargo 2008) if we include washing of plate (24 hours) and then drying (24 hours) in the procedure of CIE. The expenses of CIE are higher than ID due to use of power supply and agarose gel made up in buffer.

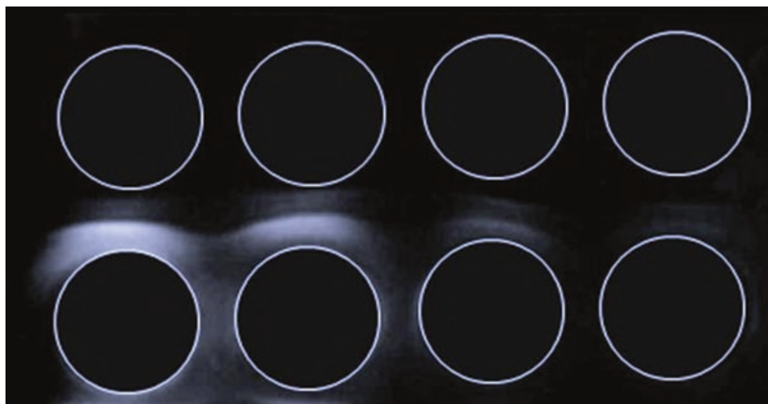

Fig. 3.3 Precipitin lines visualized after staining. The well without precipitin line is negative

3.9 Immunodiffusion (ID)

The specificity and sensitivity of ID test depend on the nature of antigen. The ID test gives 84.3% sensitivity and 98.9% specificity with Ag7 in diagnosis of paracoccidioidomycosis (PCM) (Mendes-Giannini et al. 1994). However, there can be 2–3% false-negative results with gp43 antigen (Del Negro et al. 1995). It is noticed that PCM diagnosis can be done easily by the finding of budding yeast cells in the sputum after treatment with 30% KOH. There may be cases with a depressed immune system during severe PCM, without sufficient antibodies to precipitate in ID test. The paracoccidioidin skin test may also be negative. In such a scenario, a positive ID test can be obtained after 1–2 months as immunity is restored during this period (de Camargo 2008). There is another theory regarding false-negative test that states a linkage of predominant mannose-rich oligosaccharide moiety with Fd part of only one of the Fab arms of the antibody molecule which changes the structure making functionally univalent IgG asymmetric antibodies. This altered IgG is unable to precipitate (Itano 1999).

Similar to CIE, a 1% solution of agarose in phosphate-buffered saline is layered onto a glass plate, and at least two wells are punched 6 mm apart from each other. Plates may also be punched for a central well surrounded by six wells. The central well is loaded with either known antigen or antibody and others with different serum samples. The loaded plate is incubated in a moist chamber at room temperature (20–25 °C) overnight. The next day, it is washed with 5% sodium citrate for at least 1 hour and then dipped in saline for 24–48 hours. After drying, it is dipped for staining in 0.15% Coomassie Brilliant Blue in ethanol-acetic acid-water (4:2:4) for 3–5 min. Excess stain can be removed in a solvent mixture of ethanol-acetic acid-water (4:2:4). Precipitin line/band appears when a minimal concentration of detectable antigen is present (Fig. 3.4). The immunodiffusion (ID) test is a simple and cheaper serological diagnosis technique; still a requirement of trained and qualified technicians is a prerequisite (Silveira-Gomes et al. 2011).

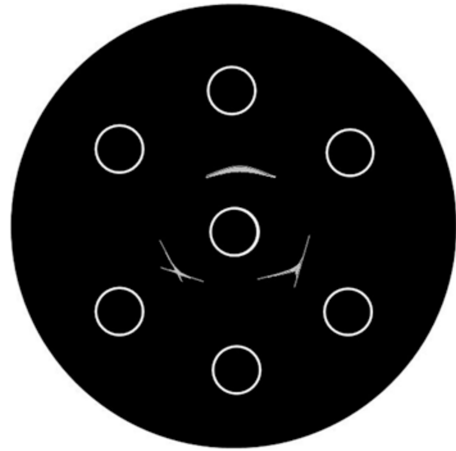

Fig. 3.4 Precipitin lines/bands visualized after staining antigen and antibody complex precipitation. The appearance of lines/bands depends on the specificity and cross-reactivity of antigens. The wells which do not have any lines/bands due to no reactivity between antigen and antibody are negative

3.10 Immunoblotting

The biomolecule separation technique under an electrical gradient was developed in 1970, by Ulrich Laemmli using polyacrylamide gels (Kistler et al. 1984). This separation is based on molecular weight (MW) and charges of molecules. Afterward, Towbin et al. (1979) explain the use of this technique to transfer proteins from gels to nitrocellulose membranes (Towbin et al. 1979). Later on Burnette coined the term Western blot (WB) for the technique using sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) to nitrocellulose membrane (Burnette 1981). The Western blot (WB) is now also known as immunoblotting that serves as an analytical technique to identify specific interaction between antigens and antibodies of fungi. The fungal cells from a known culture of pathogens are sonicated or freeze-thawed in liquid nitrogen to obtain crude extract of proteins. Then electrophoretically separated protein is transferred onto a polyvinylidene difluoride (PVDF) membrane or nitrocellulose membrane (NC). The antigen on PVDF or NC allows to react with serum samples. If the serum contains antibodies for antigen, they will form a complex which can be visualized by means of enzyme-linked secondary antibodies and substrate specific to enzymes (Fig. 3.5). The procedure is highly reproducible for recognized antibodies in patient's sera (Kumar et al. 2010).

3.11 Latex Particle Agglutination

LA test is a useful tool for the diagnosis of various diseases, including fungal infections (Martins et al. 1995; Restrepo and Moncada 1978). The LA test requires antigen-coated latex particles to form complex with antibodies in serum or other body fluids. A suspension of latex particle is mixed in a solution of antigens of

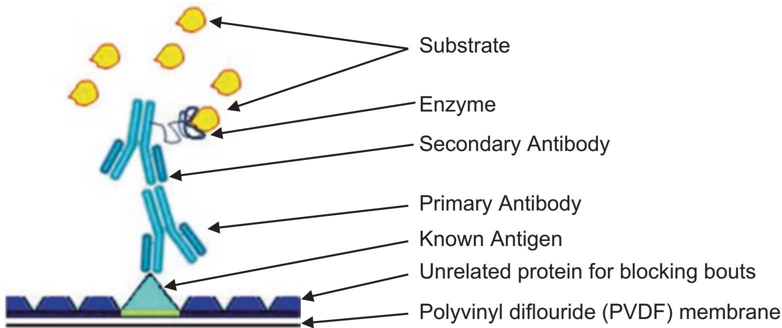

Fig. 3.5 Antigen and antibody complex will be visualized after immunostaining. The serum without antibody against fungi will not present bands on PVDF membrane

desired concentration. Latex polystyrene bead (diameter, 0.8 μm) suspension may be prepared in carbonate-bicarbonate-buffered saline solution (pH 9.2). The bead suspension is mixed with pooled antigens at 4 °C overnight. The next day, they are dipped in 0.1%–1.0% (wt/vol.) bovine serum albumin (BSA) in phosphate-buffered saline. The optimal solution for use is one that produces a clear agglutination. Inactivated serum and sensitized beads are placed on a dark slide. After antigen-antibody agglutination, the slide is gently agitated in an agitator for 5 minutes. Samples are positive if the agglutination is observed. Some laboratories have defined degree of agglutination of the particles as 1–4+. The grading of agglutination is defined as negative when the suspension has a fine granular background or a milky aspect with the absence of agglutination. A one plus (1+) suspension shows small clumping against a cloudy background, a two plus (2+) suspension exhibits small to moderately sized clumps against a slightly cloudy background, a three plus (3+) suspension presents moderately sized and large clumps against a clear background, and a four plus (4+) suspension has large clumps with a very clear background (Silveira-Gomes et al. 2011). The sensitivity of latex agglutination test is higher than fungal culture and direct microscopy with a slightly less specificity. A combination of two tests can be used as confirmatory diagnosis tool for fungal infection. Therefore, latex agglutination test could be an early diagnosis method to detect capsular polysaccharide fungal antigen (Wang et al. 2015).

3.12 Tube Precipitin and Complement Fixation Tests

Tube precipitin and complement fixation tests are very specific and non-cross-reactive diagnostic and prognostic methods. These tests are not seropositive to viral, rickettsial, bacterial, or most other mycotic infections (Kaufman 1973). They can give up to 90% positive results in early cases of coccidioidomycosis (Smith et al. 1950). In comparison, latex particle agglutination test is able to diagnose more

number of cases with more sensitivity than the tube precipitin test, while tube precipitin and CF tests have the advantage of quick and reliable results within few minutes (Huppert et al. 1968).

3.13 Enzyme-Linked Immunosorbent Assay (ELISA)

Solid-phase immunoassays, especially enzyme-linked immunosorbent assays (ELISA), are a popular technique in clinical laboratories for quantization by titration of antibodies. Non-culture-able or hard-to-culture microbial infection can also be diagnosed by immunological methods like ELISA. These assays utilize colorimetric reaction between substrate and an enzyme tagged with antigen-antibody complex. ELISA is also a sensitive and reliable method. It ensures accurate results in the form of optical density (OD) of colored solution. It is cheaper in comparison to other culture methods. One can define ELISA positive if specific IgG or IgM antibody titer in serum samples is higher than samples taken from non-infected animal.

Generally, ELISA is performed in 96-well polystyrene plates. Half the height of well should coat with known antigen of fungi. Then an unrelated protein should be used to coating full height of well. Unrelated protein prevents the antibodies from binding with free space (bouts) on polystyrene plates. Now, serum sample is filled in well up to half mark. An antigen-antibody complex will be formed if serum has antibodies. Now, a detector antibody reagent bound to an enzyme indicator could be chosen to react with this antigen-antibody complex. Finally, a substrate gives color on reaction with the enzyme bound to the detector antibody. One should remember that each step needs washing of wells with appropriate buffer of 7.4–8.0 pH except at the substrate step. There will be higher optical density observed based on the amount of detector antibody bound to known antigen. The optical density can be estimated by using colorimeter known as ELISA reader. The ELISA system has a very high sensitivity, i.e., 0.001–0.0001, due to specific reaction between enzyme and substrate. Besides, an enzyme molecule can use with many substrate molecules. Pons et al. (1972) were the first to introduce ELISA for detection of anti-*Penicillium brasiliensis* antibodies. ELISA has since been the basis of many other publications for detection of serum antibodies to *P. brasiliensis* (Mendes-Giannini et al. 1984, 1990; Camargo et al. 1984; Puccia and Travassos 1991). These assays give reliable and reproducible results.

3.14 Antigens Useful for Diagnosis

The immune diagnostic antigens of fungi may be proteins, polysaccharides, and glycoproteins. In the last decades, various new immunodiagnostic techniques based on these antigens have been developed for identification of fungal diseases. Much

of those immune assays utilize polyclonal/monoclonal antibodies or antigens which can be easily quantified with high sensitivity and have resulted in methods with more sensitivity and specificity.

Protein and DNA markers are useful to check survival or infectious burden in model animals employing radiographic imaging assays, e.g., aspergillosis in rabbits (Latge 1999; Clemons and Stevens 2005; Walsh et al. 1995; Patterson 2005). Research laboratories have applied PCR methods and assays for antigenemia (galactomannan and glucan) to study disease progression and disease diagnosis. Rabbit studies with aspergillosis have shown that galactomannan is useful in diagnostic methods (Verweij 2005; Segal et al. 2005). The titer value of cryptococcal polysaccharide in serum in the presence or absence of treatment establishes a correlation of the concentrations, fungal load, and disease severity in humans as well as animals (Goldman et al. 1994; Wong et al. 1990). Clinical importance of an assay is directly dependent upon observation of animal data and clinical symptoms.

A particular advantage of testing for antigen is the possibility that antigen can be shed from a local site of infection to a body fluid such as blood or urine. As a consequence, it is possible to avoid highly invasive sample collection. Circulating or urinary antigen functions as a surrogate for the actual presence of the microbe. Also, it is possible to use antigen-detection platforms that can be inexpensive, rapid, and capable of use by personnel with limited training. Historically, four types of antigens were employed in detecting complete immune spectrum generated by fungal parasites. In the case of *Histoplasma capsulatum* and *Coccidioides immitis*, these four groups are antigenically active cellular components, i.e., (1) suspension of intact yeast-form cells; (2) soluble mycelial filtrate antigen; (3) suspension of ground yeast phase antigens from either *B. dermatitidis* or *H. capsulatum* cells; and (4) soluble, mycelial filtrate antigen from *C. immitis* (Harrell et al. 1970).

It should always be kept in mind that overdiagnosis of a disease will lead to unnecessary treatment, while underdiagnosis could allow the spread of disease. The ideal diagnostic test should be able to detect all infected samples (100% sensitivity) and indicate absence of pathogen in healthy individuals (100% specificity). Sensitivity and specificity can be determined by comparison with true negative and true positive obtained in another reliable, well-established (gold standard) test (Kumar et al. 2014). Fungal antigens are employed in invasive as well as allergic fungal infection. The abovementioned tests can be selected for diagnosis based on galactomannan (GM) and β-glucan polysaccharides and other protein antigens. Galactomannan (GM) is a highly immunogenic antigen which is present in most *Aspergillus* and *Candida*. A regular evaluation of galactomannan (GM) in the course of antifungal therapy gives a perfectness status of treatment. Observation of GM in serum sample can be affected by multiple factors. Sensitivity of GM-based assays depends on the site of infection and usage of certain antibiotics (e.g., ampicillin, amoxicillin, amoxicillin-clavulanate) which may give false-positive results. Mannan-negative results of the tests do not exclude infection of *Candida*. (1 → 3)-β-D-Glucan (or glucan) is found in fungi, yeast, algae, bacteria, and plants but is not present (or low) in *Cryptococcus* species, zygomycetes, and humans. It is used for diagnosis of candidiasis or fusariosis. Glucan and GM may be used to

confirm diagnosis of these fungi. However, false-positive results may be observed in up to 60% of bacteremic patients.

Aspergillus antibodies can only be detected in allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, and chronic cavitary pulmonary aspergillosis (CCPA) patients. However, detection of four major glycoprotein components, Ag 5 (30 kDa), Ag 7 (150–200 kDa), Ag 3 (24 kDa), and Ag 13 (70 kDa), is more reliable for patients with ABPA and aspergilloma. The presence of anti-*Aspergillus* antibodies in healthy individuals could give false-positive results (Sarma 2002). High level of precipitating antibodies does not prove the presence of ongoing disease. Candidiasis can be diagnosed based on detection of antigen (β-glucan (ELISA) and mannan (ELISA)) and antibody (IgA, IgG—ELISA, immunodiffusion). *Cryptococcosis* (etiologic agent *C. neoformans*) diagnosis is possible on detection of capsular polysaccharide (glucuronoxylomannan) antigen. False-positive results may be caused by rheumatoid factor or cross-reactive organisms (*Trichosporon asahii*) (Yarzabal et al. (1977). An explicit exoantigen found in human paracoccidioidomycosis serum sample has been observed to give a reaction in mixed immunodiffusion tests with sensitivity of up to 97.1% and specificity of 100%. This major antigen was a 43-kDa glycoprotein. For a while, some specific proteins like 20, 21, 94, and 100 kDa were expressed during progression of disease; however, 43-kDa glycoprotein was present throughout (de Camargo et al. 1988). Therefore, the 43-kDa glycoprotein could be an ideal reference antigen for immunodiffusion test (Puccia et al. 1986). The CF antigen, 110 kDa native protein, is a heat-labile chitinase enzyme useful for ID test in cystic fibrosis due to *Aspergillus niger*. It has shown low-titer nonspecific reactions with histoplasmosis cases (Kaufman and Clark 1974). Coccidioidin may be used significantly in complement fixation test to detect *Coccidioides immitis* infection, but it has been known to exhibit nonspecific reactions with *Histoplasma capsulatum* and *Blastomyces dermatitidis* infection due to cross-reactive antigens (Kaufman and Clark 1974).

Immunodiffusion tube precipitin (IDTP) antigen from *Coccidioides immitis* is a polysaccharide antigen of 120 kDa (Zimmer and Pappagianis 1989). *Histoplasma capsulatum* has three exoantigen H, M, and C proteins, which give reaction up to 100% specificity with histoplasmin in CF or ID test. The sensitivity may vary (81–96%) based on sample collected (Kaufman 1988).

Paracoccidioidomycosis (PCM) was detected by Camargo et al. (1989), using a *P. brasiliensis* yeast exoantigen employed in Western blot and anti-*P. brasiliensis* IgG. This exoantigen has four major components of 70, 52, 43, and 20–21 kDa. Among these, 43 kDa glycoprotein is suggested as most promising antigen. The cross-reactivity of antigen gp43 and gp70 can be minimized by serum dilution, treatment with sodium metaperiodate, or first absorbing with *Histoplasma capsulatum* yeast cells and then with a mycelia filtrate. Both of these antigens are considerable markers for human PCM. If *P. brasiliensis* yeast filtrate is utilized, then sera should be absorbed with dead cells of *Candida albicans* to abolish cross-reactivity. ELISA has high sensitivity and specificity (95% and 93.4%, respectively), for a dilution of 1:400 with absorbed serum (Taborda and Camargo 1994). These antigens are also used for in-field dot immunobinding assay (Taborda and Camargo

1994) for detection of PCM. The amount of gp43, an immunodominant antigen coated in the nitrocellulose membrane could be up to 400 ng/dot. The gp43 had also been employed in ELISA and immunoprecipitation with 125I-labeled antigen against PCM sera. Most of the sera were recognized by peptide epitopes of the gp43, and some were recognized by carbohydrate epitopes (Taborda and Camargo 1994). Gp43 is fixed on the Mab anti-gp43 in capture ELISA, and considering the end point of reactivity, the sensitivity and specificity of the capture ELISA was 100% and 96.7%, respectively (Puccia and Travassos 1991).

de Camargo et al. 1988 applied affinity chromatography on Sepharose 4B-immunoglobulin G complex made with immunoglobulin G from patients with paracoccidioidomycosis, with subsequent analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and scanning densitometry to find an antigenic preparation useful for PCM diagnosis. The exoantigen from seventh day culture, named Ag7, was useful for immunodiffusion tests. This antigenic preparation was obtained with the strain B-339, of *P. brasiliensis* culture. The seventh day cultures of approx. 8×10^8 cells should be harvested as it is supposed to be in exponential phase and most cells are viable. A dead cell in the culture may release problematic proteases. Therefore, the methodology should be perfect to produce Ag7. The protein content should be about 300–500 mg per ml and can be tested by Bradford method (Bradford 1976). The researchers also mentioned that Ag7 would be a crude mixture of a doublet at 20–21 kilodaltons (kDa) and molecules of 43 and 52 kDa. Among these, gp43 is secreted throughout the 30 days culture, but the concentration was 80–90% of the preparation at seven days (de Camargo et al. 1988). Recombinant gp43 isoforms as N-mannosylated proteins were expressed in the yeast *Pichia pastoris* and showed good specificity for detection of PCM in sera (Carvalho et al. 2008).

Aspergillosis is a reference fungal disease to study fugal antigens. D-Galactofuranose (Galf) is an important antigenic epitope in glycoconjugates in cell wall of pathogenic fungi. Mammalian cells do not synthesize Galf, so it is considered a potent antigen for identification of fungal diseases. A monoclonal antibody for Galf is also available commercially for aspergillosis (Marino et al. 2017). The galactofuranose-specific monoclonal antibody (mAb476)-based lateral flow dipstick assay can recognize antigens of *Aspergillus fumigatus* in pulmonary disease in urine sample of animals. The sensitivity and specificity of the assay was 92% and 80%, respectively (Marr et al. 2018).

Fava Netto (1972) and Negroni (1972) reported problems associated with antigen and disagreement regarding the sensitivity of serological tests. Further, extensive and diverse literature reviews done by Restrepo (1984), Mendes-Giannini et al. (1994), de Camargo and de Franco (2000), Kozel and Wickes (2014), Teles and Seixas (2015), etc. to explore significance of serological approach and the current knowledge about immunodiagnosis reveal that antigen-based immunodiagnosis is more effective than serological evaluation of dimorphic fungi.

Despite many years of antigenic preparations being identified and employed in serodiagnosis of fungal infections, most antigens lack an optimized formulation. All over the world, laboratories have included sonic extracts from yeast forms,

concentrated filtrates, and lyophilized filtrates in such assays. There are lab to lab variations in culture media and growth conditions like incubation time, growth temperature, size of initial inoculum, shaken or stationary cultures, etc. for antigen preparation. Thus, antigens may vary in activity and quality even from a same lot within the same laboratory. The fungal biomarkers (1–3)-β-D-glucan and galactomannan have been widely used to diagnose the fungal infection, as both are major part of fungal cell walls. However, host response and progression of fungal infection will not always be the same in animals or human beings. A good diagnostic test will be needed to meet the diverse requirements of different fungal infections and the resources available for testing. It should identify early fungal infections and onset of disease. It should be ideal for marking patient near point of care at strong negative predictive value to manage patients at high risk. It should be able to perform accurately even by non-trained staff with minimum number of steps. The test should be cheap or done with low-cost instruments. The findings must be simpler to understand and should not require a complicated statistical analysis. The result should also be obtained easily with noninvasive techniques.

3.15 Conclusion

The ideal test would reduce the cost of patient care and reduce development of antibiotic resistance. Kit-based methods are found to be expensive which makes them difficult to use in monitoring disease progression. Immunodiagnosis is a useful tool for rapid testing of fungal disease. Results may be obtained within a few minutes and on-site without the need of cultures. However, the test should be able to improve outcome of severe patients on advanced medical care settings. Continued screening of novel antigens will allow researchers to develop new diagnostic methods to follow the progression of disease. In conclusion, we suggest that novel diagnostic methods should be applicable on a global level with limited infrastructure to avoid over- and under-treatment.

3.16 Future Prospects

Fungal diseases of human and animals have long been known to mankind, and some pose a serious threat especially to humans in the current scenario where modern sedentary lifestyles, polluted indoor and outdoor environment, and insufficient exposure to clean air and sun promote an immunosuppressive ambience. The arrival of HIV and subsequent AIDS epidemic on the scene has only made the situation more alarming. Existing and available rapid tests for point-of-care diagnosis of important fungal diseases could enable the limitations of current laboratory methods for detection and identification of medically important fungi to be surpassed, both in low-income countries and for first-line diagnosis (screening) in richer

countries. Platforms utilizing molecular biology techniques have been challenging established immunodiagnostic procedures along with other conventional devices and techniques. Molecular biology-based techniques offer multiple benefits including a shortened assay time as well as enhanced sensitivity. This ensures a more accurate and quicker diagnosis and early treatment subsequently. Many such "in-house" tests have been developed, but without standardization and sometimes even validation, their potential remains untapped. Future research should encompass not only novel bio-recognition techniques but also new bio-analytical platforms with the ultimate goal of fully automated testing where possible. With rapid advancements being made in this context, it is of the utmost importance to stay abreast of newer techniques to allow for an exhaustive assessment of those which hold the promise of being more cost effective as well as support the pace of the otherwise tepid developmental status of novel diagnostic procedures for fungal pathogens at present.

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Chapter 4
Aspergillosis in Birds and Mammals: Considerations for Veterinary Medicine

Lisa A. Tell, Julia D. Burco, Leslie Woods, and Karl V. Clemons

4.1 Introduction

Aspergillosis is a disease caused by infection with the saprophytic, ubiquitous fungi of the genus *Aspergillus*. Although *Aspergillus* spp. are common fungi, usually residing in decaying organic matter such as soil, ensilage, and moldy crops or feed, these fungi are also pathogens causing a range of disease syndromes in a number of mammal, avian, and invertebrate species. Although birds appear to be the most susceptible to infection, this disease affects a range of animal species from sea fans to elephants (Seyedmousavi et al. 2015; Tell 2005). Characteristics of some members of the genus *Aspergillus* include ubiquitous geographical distribution, thermophilic nature, pigmented conidia, and production of mycotoxins, such as gliotoxin or aflatoxin. These characteristics lend these organisms to being very successful saprobes, as well as opportunistic and primary pathogens. In general, aspergillosis in veterinary medicine is a disease that impacts a variety of animal classes and species. Definitive diagnosis can be a challenge, and treatment success is rare.

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4.2 Epidemiology

Aspergillosis is a non-communicable fungal disease, usually caused by environmental exposure to high numbers of conidia (spores) of the organism. Animals infected with this disease are often ones that are stressed or have an immunocompromised status, which can result in unique disease presentations in various animal subgroups. In many animal species, infection is a result of inhalation of the small 2–4 um conidia into the respiratory tract. Disease also can develop, albeit less frequently, as a result of ingestion (i.e., bovine alimentary mycosis and mycotoxicosis such as tremorgenic neuromycotoxicosis) or direct contact with the fungus (i.e., local cutaneous or udder infections in cattle).

4.2.1 Aspergillosis in Mammals

Frequently in mammals, as is the case with human infections, an underlying local or systemic immune dysfunction predisposes an individual animal to aspergillosis. In canine, and less often, feline pets, *Aspergillus* spp. may result in nasal aspergillosis and sinusitis. Long-nosed dolichocephalic canine breeds, such as the dachshund and the German shepherd, seem to be predisposed to this disease (Pomrantz et al. 2007). In addition, there is evidence that local immune dysfunction plays a role in the pathogenesis of canine nasal infections (Peeters and Clercx 2007). In cattle, *A. fumigatus* typically enters through the enteric or respiratory tracts and results in bovine mycotic abortion, mastitis, or systemic aspergillosis (McCausland et al. 1987). Equine infections most often manifest as guttural pouch mycoses (Borges and Watanabe 2011). Table 4.1 summarizes reported disease conditions in animals infected with *Aspergillus* spp.

4.2.2 Aspergillosis in Birds

Although aspergillosis results in severe disease in a number of different animal species, birds are by far the most susceptible group to infection. In contrast to humans and many mammals, *Aspergillus* spp. can act as a primary pathogen in birds. Reasons for increased susceptibility are still being investigated, but several unique differences in avian anatomy and life histories provide convincing arguments. First, birds do not have a diaphragm, which is an important anatomic feature that allows mammals to forcibly expel small particles from their airways. Birds also lack an epiglottis, which in mammals, physically blocks foreign material from entering the lower airways (Tell 2005). In addition, birds have an extensive air sac system that extends throughout most of their body and even into their bones. These air sacs are comprised of thin layers of connective tissue sandwiched by single-celled layers of

Table 4.1 Aspergillosis in animals: common clinical signs, disease syndromes, and etiologic agents

Species	Clinical signs	Disease/ syndrome(s)	Etiologic agent(s)
Avian			
Penguins/ other seabirds Waterfowl Raptors Psittacine Poultry	Dyspnea, swelling, lethargy, nasal discharge, change in vocalization, altered buoyancy, increased haul-out time, neurologic signs (with spinal involvement), anorexia or sudden death	Disseminated and localized infection; "brooder pneumonia" in poultry	*Aspergillus fumigatus* (most common), followed by *A. flavus*, and less frequently *A. niger*, *A. glaucus*, *A. nidulans*, and *A. terreus*
	Neurologic signs	Mycotoxicosis	Ingestion of moldy grain usually infected with *A. flavus*
Equine			
	Epistaxis, mucopurulent nasal discharge, dysphagia, laryngeal paralysis, Horner's syndrome	Guttural pouch mycosis	*A. fumigatus, A. versicolor, A. nidulans, A. niger*
	Weight loss, dyspnea, lethargy	Disseminated aspergillosis	*A. fumigatus* (most common)
	Corneal cloudiness	Fungal keratitis	*A. flavus, A. niger, A. fumigatus, A. oryzae*
Bovine			
	Neurologic signs	Tremorogenic neurotoxicosis	Usually caused by ingestion of sprouted grains such as barley (*A. clavatus*)
	Abortion/placentitis	Mycotic abortion	*A. fumigatus, A. terreus, A. nidulans, A. flavus.* Usually acquired through gastrointestinal tract
	Mastitis	Mastitis	*A. fumigatus* other. Usually results from contact with contaminated environment
Canine/ feline			
Dogs	Mucopurulent nasal discharge (usually starts with unilateral and then bilateral after destruction of nasal septum), epistaxis, facial pain	Canine nasal aspergillosis	*A. fumigatus, A. terreus, A. versicolor.* Usually seen in mesaticephalic and dolichocephalic breeds (i.e., dachshunds, German shepherds)
Cats	Mucopurulent nasal discharge (usually starts with unilateral and then bilateral after destruction of nasal septum), epistaxis, facial pain, and/or respiratory signs	Feline nasal aspergillosis/ disseminated aspergillosis	*A. fumigatus*

(continued)

Table 4.1 (continued)

Species	Clinical signs	Disease/ syndrome(s)	Etiologic agent(s)
Marine			
Dolphins	Respiratory signs (pneumonia)	Pulmonary aspergillosis, middle ear infection	*A. fumigatus* (most common)
Sea fans	Purple discoloration/tissue	Aspergillosis	*A. sydowii*

squamous epithelium with minimal numbers of immune surveillance cells (King and McClelland 1984). This unique warm, highly oxygenated air sac microenvironment allows the vegetative (hyphal) and the sporulating (or asexual reproductive) form of *Aspergillus* to grow, whereas in mammalian tissues the vegetative (hyphal) form is nearly always the only form present. Finally, in mammals, pseudostratified ciliated epithelium exists in a majority of the airways and provides an important clearance mechanism. However, ciliated epithelium is sparsely distributed in the respiratory tracts of birds. In the case of the air sac membranes, the ciliated epithelium is primarily located at the ostia, which are the proximal openings to the air sacs (King and McClelland 1984), but the majority of the remaining surface area of the air sac membranes is devoid of this clearance mechanism.

In addition to these anatomic differences that contribute to increased susceptibility of aspergillosis in birds, there are also unique cellular and metabolic variations that provide an excellent environment for *Aspergillus* spp. to invade. Birds' average body temperatures range from 38 to 45 °C, which provides an excellent environment for a thermophilic fungus such as *A. fumigatus* to grow. On the cellular level, avian heterophils kill conidia using hydrolases, lysosomes, and cationic proteins instead of oxidative mechanisms and myeloperoxidase, which is frequently used for fungal killing by macrophages and neutrophils in mammals (Tell 2005). As previously mentioned, air sac and lung macrophages, also known as free avian respiratory macrophages (FARM), in addition to bronchus-associated lymphoid tissue (BALT), are scarce and are not easily found until activated by irritation or infection (Reese et al. 2006). The combination of these factors makes it difficult for the immune system to respond to the inhalation of fungal conidia into the respiratory tract.

4.2.3 Susceptibility of Various Birds

4.2.3.1 Free-Ranging Birds

In free-ranging birds, sporadic cases of individual aspergillosis and epornitics have occurred in migrating waterfowl (e.g., mallards, geese). These infections are often related to changing environmental conditions that provide sufficient moisture and humidity to result in moldy grain or crops (Parker 2011). These migratory birds

subsequently consume the affected feed and die from overwhelming *A. fumigatus* exposure or from aflatoxicosis, caused by aflatoxins produced by *A. flavus* that can also be found in moldy grain. Outbreaks have also been documented in free-ranging gulls and raptors (Beernaert et al. 2010).

4.2.3.2 Domestic (Poultry) Birds

Aspergillosis is of significant economic importance and is a major management concern in poultry operations. In poultry, aspergillosis is often referred to as brooder pneumonia, most commonly affecting young chicks or turkey poults (Zafra et al. 2008; Arné et al. 2011). Infection with *A. fumigatus* occurs via inhalation of large numbers of fungal spores present in the environment because of contaminated bedding, feed, litter, etc. Aflatoxicosis, due to *A. flavus*, results when birds consume aflatoxin-contaminated feed and is another common finding in poultry (Dalvi 1986).

4.2.3.3 Captive Birds (Companion, Rehabilitated, and Zoological Collection)

4.2.3.3.1 Companion Birds

Companion psittacines, such as the African gray parrot (*Psittacus erithacus*), blue-fronted amazon parrot (*Amazona aestiva*), and pionus parrot (*Pionus* spp.), are highly susceptible to aspergillosis (Tsai et al. 1992). The reason for this species susceptibility is unknown and does not seem to be related to larger percentages of these birds being kept as pets compared to other psittacines.

4.2.3.3.2 Rehabilitation/Zoological Collection Birds

Raptors brought into captivity for rehabilitation purposes or those used in falconry are extremely susceptible to aspergillosis. Goshawks (*Accipiter gentilis*), gyrfalcons (*Falco rusticolus*), and immature red-tailed hawks (*Buteo jamaicensis*) are species that frequently acquire aspergillosis in captivity (Deem 2003). Seabirds are another avian group that are highly susceptible to infection, especially when brought into captivity for rehabilitation prior to release back into the wild or those kept in captivity, long term, in zoological parks, or in aquaria. Penguins, alcids, loons, grebes, cormorants, and sea ducks are among the groups of seabirds where aspergillosis has been extremely difficult to manage in captivity (Burco et al. 2012b; Flach et al. 1990). One reason for an increased susceptibility of seabirds to infection may be because of a low natural exposure to *Aspergillus* spp. in the wild (Burco et al. 2014). In addition, birds recovered after oil spill events are exposed to caustic materials that requires them to undergo a protracted rehabilitation process to regain

waterproofing, and these animals are highly stressed and immunocompromised (Leighton 1993; Briggs et al. 1996).

4.3 Etiology

Although greater than 260 species of *Aspergillus* have been identified (Samson and Varga 2009), less than 20 species are responsible for causing disease in animals. In general, the most common etiologic agent in mammal and bird infections is *Aspergillus fumigatus* (Tables 4.1 and 4.2) (Seyedmousavi et al. 2015).

4.4 Clinical Signs

Clinical signs of aspergillosis in birds and mammals are related to the site of infection. In avian patients, one may observe a change in vocalization, decreased activity and food consumption, acute or chronic weight loss, abnormal balance or buoyancy, and labored breathing. Unfortunately, once clinical signs are observed, progression of the disease is usually rapid and treatment is often challenging or ineffective. In both mammals and birds with disseminated aspergillosis, clinical signs may be nonspecific. Clinical signs for mammals with aspergillosis include lethargy, inappetence, pyrexia, or other symptoms related to localized infection (Table 4.1).

Small animals, such as cats and dogs, can also develop localized or systemic aspergillosis, although this occurs less frequently in mammals than in birds. Dogs more frequently contract sinonasal aspergillosis (SNA) with rare cases of systemic infection also occurring. Young-to-middle-aged mesaticephalic and dolichocephalic (the head is longer compared to the width of the head) canine breeds are most commonly affected. Clinical signs are usually represented by one or a combination of the following: unilateral or bilateral mucopurulent nasal discharge, nasal or sinus pain, nasal depigmentation, sneezing, or facial deformity (Peeters and Clercx 2007). Cats have been less frequently documented with aspergillosis. If a cat is unfortunate enough to become ill with this disease, sinoorbital (SNO) and sinonasal infection is the typical presentation, with only occasional systemic infection. Infected cats usually present with sneezing and nasal discharge (within the last six months), and/or exophthalmos, and ulceration or swelling of the hard palate when there is SNO involvement (Barrs et al. 2012).

Horses most frequently develop guttural pouch mycosis which results in one or a combination of the following clinical signs: mucopurulent nasal discharge, epistaxis (usually unilateral), and neurologic abnormalities such as dysphagia, Horner's syndrome, laryngeal hemiplegia, or tongue atrophy. All of these clinical signs are related to anatomical structures present within the equine guttural pouch, which include the vagosympathetic trunk, cranial cervical ganglion, cranial laryngeal nerve, pharyngeal branch of the vagus nerve, mandibular nerve, choroid plexus

Table 4.2 Compilation of *Aspergillus* spp. recovered from various sample sites from different animals[a]

Animal category	Animal type	Sample site/type	Isolated *Aspergillus* spp.[b]
Avian (28)	*Avian (2)*	Air sac (cranial)	*Aspergillus fumigatus*
		Nasal flush	*Aspergillus terreus*
	Flamingo	Trachea	*Aspergillus flavus* species complex
	Golden weaver	Air sac	*Aspergillus fumigatus*
	Hawk	No information	*Aspergillus fumigatus*
	Loon (2)	Coelom (2)	*Aspergillus fumigatus* (2)
	Owl (3)	Skin	*Aspergillus niger* species complex
		Nodule (2)	*Aspergillus fumigatus* (2)
	Parrot (3)	Air sac	*Aspergillus fumigatus* species complex
		Beak	*Aspergillus terreus*
		Nasal flush	*Aspergillus terreus* species complex
	Penguin (12)	Air sac (4)	*Aspergillus fumigatus* (12)
		Gastric (2)	
		Lung (4)	
		Trachea (1) No information (1)	
	Puffin (3)	Air sac (3)	*Aspergillus fumigatus* (3)
	Raven	Lung	*Aspergillus fumigatus*
	Spoonbill	Air sac	*Aspergillus fumigatus* species complex
Canine (64)	Canine (64)	Abdomen (2)	*Aspergillus deflectus* (2)
		Airway biopsy (2)	*Aspergillus fumigatus* (2)
		Bone (4)	*Aspergillus terreus* (4)
		Ear	*Aspergillus terreus* species complex
		Endotracheal wash	*Aspergillus fumigatus*
		Frontal sinus	*Aspergillus fumigatus*
		Heart valve	*Aspergillus carneus*
		Kidney	*Aspergillus terreus*
		Liver	*Aspergillus versicolor* species complex
		Lung	*Aspergillus lentulus*
		Lymph node(8)	*Aspergillus deflectus*
			Aspergillus terreus
			Aspergillus glaucus group(4)
			Aspergillus niger species complex (2)
		Mass (mediastinal)	*Aspergillus niger* species complex
		Nasal (24)	*Aspergillus fumigatus* (22)
			Aspergillus niger species complex (2)

(continued)

Table 4.2 (continued)

Animal category	Animal type	Sample site/type	Isolated *Aspergillus* spp.[b]
		Pancreas	*Aspergillus deflectus*
		Peritoneal fluid	*Aspergillus alabamensis*
		Thoracic cavity	*Aspergillus fumigatus*
		Urine (13)	*Aspergillus deflectus* (3)
			Aspergillus terreus (8)
			Aspergillus terreus species complex
			Aspergillus flavipes
Cervid	Deer (tufted)	Tracheal wash	*Aspergillus flavus* species complex
	Elk	Lung mass	*Aspergillus fumigatus*
Equine	Equine (9)	Cornea (4)	*Aspergillus flavus* (4)
		Guttural pouch	*Aspergillus fumigatus* species complex
		Lung	*Aspergillus fumigatus*
		Tracheal wash (3)	*Aspergillus fumigatus* (2)
			Aspergillus fumigatus species complex
Feline	Feline (7)	Nasal (4)	*Aspergillus fumigatus* (6)
		Orbital mass (2)	
		Skin (biopsy from chin)	*Aspergillus ochraceus* species complex (section *Circumdati*)
Marine invertebrate	Marine sponge (17)	Marine sponge (17)	*Aspergillus niger* species complex (4)
			Aspergillus ochraceus species complex (section *Circumdati*)(3)
			Aspergillus phoenicis
			Aspergillus restrictus group (2)
			Aspergillus sydowii (3)
			Aspergillus tamari
			Aspergillus versicolor species complex
			Aspergillus flavus (2)
Marine mammal	Dolphin (29)	Blowhole (17)	*Aspergillus flavus* (3)
			Aspergillus fumigatus (13)
			Aspergillus versicolor species complex
		Bronchial (3)	*Aspergillus fumigatus* (3)
		Gastric sample	*Aspergillus niger* species complex
		Nasal (2)	*Aspergillus niger* species complex
		No information (4)	*Aspergillus fumigatus* (4)
		Trachea (2)	*Aspergillus fumigatus* (2)

(continued)

Table 4.2 (continued)

Animal category	Animal type	Sample site/type	Isolated *Aspergillus* spp.[b]
	Manatee (6)	Skin (6)	*Aspergillus niger* species complex (6)
	Marine mammal (5)	Blowhole (5)	*Aspergillus fumigatus* (4)
			Aspergillus terreus
	Whale(6)	Blowhole (4)	*Aspergillus fumigatus* (6)
		Lung	
		Lesion	
Primate	Orangutan	Bronchoalveolar lavage	*Aspergillus fumigatus*
Reptile	Sea turtle (Kemp's ridley)	Bone	*Aspergillus niger* species complex
Rodent	Sentinel mole rat (2)	Skin	*Aspergillus flavus* (2)

[a]Data were provided by the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center, San Antonio, TX. The clinical isolates were submitted between the years of 2001–2012

[b]Identification of isolates was based on macroscopic and microscopic morphology, and in some instances, identification was confirmed by molecular techniques

nerve, facial nerve, auriculotemporal nerve, glossopharyngeal nerve, vagus nerve, accessory nerve, hypoglossal nerve, common carotid artery, linguofacial trunk, external carotid artery, internal carotid artery, occipital artery, maxillary artery, superficial temporal artery, and lingual artery (Borges and Watanabe 2011). *Aspergillus* spp. have also frequently been cultured from the cornea in cases of equine keratitis, where there is often a whitish corneal infiltrate and associated inflammation. Systemic pulmonary infection (pneumonia) in horses often presents with anorexia, depression, and respiratory signs following a bout of severe enteritis (Sweeney and Habecker 1999).

In cattle, severe neurologic signs are often seen with ingestion of moldy grain and beer residues contaminated with neuromycotoxins (a.k.a. tremorgenic metabolites) from *A. clavatus*. In contaminated foodstuffs, patulin is the most frequently isolated mold toxin; however, other neuromycotoxins are produced by *A. clavatus*. Clinical signs include posterior ataxia and neurologic deficits such as dragging, knuckling, or high-stepping of the hind legs, stiff gait, flaccid paralysis, muscular tremors, and progressive paresis (Hollinger and Ekperigin 1999). Surprisingly these animals usually maintain their appetite, but have a decrease in milk production (McKenzie et al. 2004).

In marine mammals, aspergillosis is usually localized to the respiratory tract creating a fungal pneumonia, but has also been documented to cause localized disease such as a middle ear infection (Prahl et al. 2011; St. Leger 2010). Another marine organism particularly impacted with aspergillosis in the last few decades is the sea fan. Sea fans throughout the Caribbean ocean have been affected and usually

present with purple discoloration of the tissues and tissue loss from infection with *A. sydowii* (Kim et al. 2006).

4.5 Antemortem Diagnostics

Early diagnosis of aspergillosis remains one of the most frustrating aspects of this disease. The range of diagnostic options is variable depending on the veterinary clinic, rehabilitation, or zoological facilities. In birds there are a few initial diagnostic tests used as screening tools. Physical examination is one diagnostic tool, which includes general auscultation of the lung quadrants and general observations of respiratory stridor. Preliminary blood work such as packed cell volume (PCV), total solids (TS), and white blood cell (WBC) count with a differential can provide some clues into general inflammatory conditions. Based on previous studies and clinical observations, an elevated total protein and heterophilia, with or without monocytosis, are suggestive of a chronic inflammatory condition and require further diagnostics for confirmation.

Other diagnostics that have routinely been used in avian medicine in attempts to diagnose aspergillosis include radiography, tracheal lavage, endoscopy, polymerase chain reaction (PCR), protein electrophoresis, and multiple enzyme-linked immunosorbent assays (ELISA). Abnormal findings on radiographic images include linear opacities along the air sac membrane borders or large nodular lesions at the later stages of disease. In general, radiographic imaging is not a useful diagnostic screening tool unless the infection is severe. Tracheal wash or lavage, followed by cytospin, is a useful way to obtain cells that can be used to identify what type, if any, of inflammation is present. In addition, fungal hyphae may be observed on the cytologic smear. Tracheal wash fluid can also be used for culture and antigen detection. Endoscopy provides utility in visually identifying and potentially treating localized granulomas. In addition, biopsy samples can be harvested during an endoscopic examination. Plasma protein electrophoresis has been another useful tool in detecting inflammation, although it is not specific for aspergillosis. A number of ELISAs for aspergillosis have been developed for use with bird specimens, but these have shown variable sensitivities and specificities. Other promising antigen serologic assays originally developed for use in detecting human invasive aspergillosis (IA) are now being evaluated in avian species. These include the beta-D-glucan (Fungitell®; Associates of Cape Cod) assay and the galactomannan (Platelia®; Bio-Rad) assays. Both detect circulating fungal cell wall antigen in serum or plasma samples. The Platelia® test is a sandwich ELISA, which has also shown utility in diagnosing mycotic infections using bronchiolar lavage fluid or urine samples. These assays are promising for diagnosing aspergillosis using avian samples since there is no reliance on species-specific secondary antibodies. Neither the Platelia® nor the Fungitell® assay is specific for *Aspergillus* spp.; therefore, other clinically significant fungal infections can complicate the diagnosis. A study performed on multiple avian species demonstrated a significant rise in β-D-glucan plasma

concentrations especially in advanced or disseminated forms of the disease and may be a useful adjunct for diagnosis (Burco et al. 2012a). Sensitivity of the galactomannan assay has been shown to be poor in falcons, which makes it a poor choice for a screening tool in this group of birds (Arca-Ruibal et al. 2006). Improved sensitivity but poor specificity was observed in psittacines (Cray et al. 2009b). The sensitivity and specificity of diagnosis was improved in birds when combined with protein electrophoresis (Cray et al. 2009a).

In mammals, antemortem diagnosis of aspergillosis also has been challenging and has not had any major advances recently. Usually a combination of procedures needs to be performed for an accurate and definitive diagnosis. In canine and feline patients, a diagnosis of nasal aspergillosis usually starts with nonspecific imaging techniques such as radiography, computed tomography (CT), or magnetic resonance imaging (MRI). MRI and CT are usually more optimal for making a diagnosis of SNA than radiography. This is due to less superimposition of bony structures and the complexity of the nasal turbinates that are difficult to evaluate on radiographic images (Saunders et al. 2003). Imaging is usually followed by rhinoscopic examination to visualize the extent of damage, to visualize fungal plaques, and/or to perform a nasal flush or biopsy for cytology, culture, and/or histopathology. Rhinoscopy should not be performed prior to imaging since hemorrhage and fluid from a nasal flush can alter imaging results. In obtaining samples from rhinoscopy, squash preparation smears have been shown to be the most sensitive for detecting fungal elements when compared to other techniques such as mucosal brushing, blind swab, or direct smear (De Lorenzi et al. 2006; Schultz et al. 2008). Clinical pathology (hematology) findings suggestive of disease include hyperglobulinemia, hypercalcemia, and leukocytosis (Schultz et al. 2008). Adjunct serological tests have also been used to aid in diagnosis, which include counter-immunoelectrophoresis, and other antibody and antigen enzyme-linked immunosorbent assays. An ELISA that detects *Aspergillus* galactomannan antigen in urine and serum has recently been demonstrated to show promise in dogs (Garcia et al. 2012). Limitations include nonspecificity for other systemic fungal infections or treatment with plasmalyte.

In horses, diagnosis of guttural pouch mycosis is usually made using endoscopy, followed with a swab, flush, or biopsy of the infection site to confirm etiology. In cattle, immunohistochemistry (IHC) or simple histopathology is frequently used on placental tissue to identify hyphae. Similarly, fungal mastitis is confirmed by culture and histopathology/IHC of tissues. Other manifestations of disease may be difficult to confirm antemortem (intestinal and tremorogenic neurotoxicosis) and are frequently based on history, clinical signs, necropsy findings of a cow that has died in the herd, or detection of mycotoxin in feed samples.

Some of the newer diagnostic techniques being developed for use in human infections, which may also show promise in animal infections, include the use of a breath test to identify specific volatile organic compounds that are specific for infection with *Aspergillus* spp. (Chambers et al. 2012), multiple PCR platforms (both RNA and DNA), and detection of fungal cell wall components via antigen ELISAs that have been developed for commercial use.

4.6 Culture and Identification

Aspergillus spp. isolates can be obtained from clinical patients by taking swab samples in a sterile manner from gross lesions. The swabs can be transported in Ames bacterial transport medium or anaerobic bacteria transport medium (although not ideal) to an appropriate diagnostic facility or streaked directly onto a Sabouraud dextrose agar or inhibitory mold agar (IMA) slants or agar plates. Czapek-Dox agar is a medium that allows for further differentiation by providing a good evaluation of morphological characteristics and variations. Tracheal or bronchiolar lavage samples may also be plated directly or transported in a sterile vial (and kept cool) to a microbiology laboratory for culture and identification. For most *Aspergillus* spp., growth takes a minimum of 24–48 hours incubated at 35–37 °C. Gross morphologic characteristics of surface and background color followed by microscopic characteristics of conidiation including vesicle serration, organization and coverage of the conidiophore head by phialides or metula, and pigmentation and surface texture of conidia are used for initial identification. A scotch tape mount can be performed by gently pressing the tape to the edge of the sporulating colony and then placed on a glass slide with a drop of lactophenol cotton blue or lacto-fuchsin to evaluate conidia and conidiophore phialide characteristics in more detail (McClenny 2005). The photographic images in Figure 4.1a–d illustrate examples of colonial morphology and microscopic morphology, both of which are important in the identification of these organisms (Fig. 4.1a).

Further confirmation of the identification can be performed by molecular techniques. These include the use of species-specific PCR, partial DNA sequencing of the internal transcribed spacer (ITS) region of rDNA, or specific genes for β-tubulin, and calmodulin, or the use of the Luminex xMAP technology. Identification of isolates of *Aspergillus* to the species level is important in that different species have differing susceptibilities to the currently available antifungal drugs. For example, most isolates of *A. terreus* are resistant to amphotericin B, whereas other species such as *A. fumigatus* or *A. flavus* are susceptible. Differences in azole resistance have been reported to occur in the cryptic species of the *A. niger* group (Howard et al. 2011), further demonstrating the potential importance of correct identification to subsequent therapeutic success.

4.7 Postmortem Evaluation

The lesions of aspergillosis depend on the site of infection. Infection in the nasal cavity or paranasal sinuses in dogs may present as greenish-black plaques of fungal mats surrounded by hyperemic and edematous mucosa or as mucosal hemorrhage with purulent or caseous exudate with or without destruction of turbinate bone and bone remodeling (Songer and Post 2005). Aspergillosis of the guttural pouch in horses may occur as a diphtheritic membrane on the mucosa, and fungal hyphae can

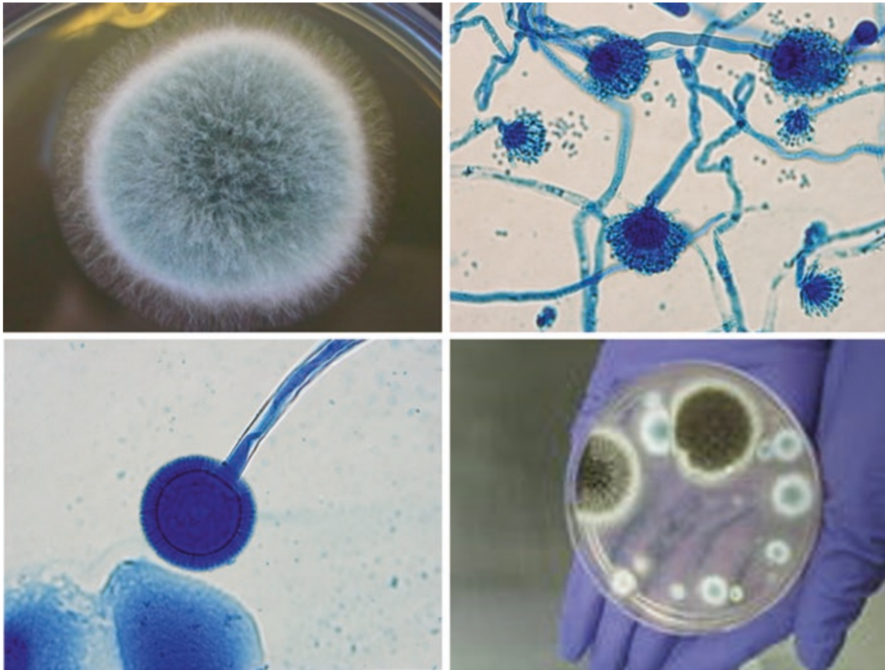

Fig. 4.1 (**a**) *Aspergillus fumigatus* culture (4-day growth) on malt extract agar, (**b**) *A. fumigatus* under light microscopy (40 X) stained with lactophenol cotton blue, (**c**) *A. niger* under light microscopy, and (**d**) air environmental sampling on Sabouraud dextrose agar showing two large *A. niger* colonies (dark color) and a number of *A. fumigatus* colonies (bluish-green color)

invade the internal carotid artery and vein resulting in fatal hemorrhage into the pouch (Songer and Post 2005). Avian species with respiratory aspergillosis may have miliary, elastic to firm yellow, white or tan nodules of variable sizes in the lungs and on the air sacs, or lungs may be gray and wet without the presence of nodules. Fungal mycelium with caseonecrotic material may occlude the lumen of the syrinx, adherent to necrotic mucosa (Barton et al. 1992). Lesions in the brain may be white to yellow, well-circumscribed foci in the cerebellum or cerebrum (Richard et al. 1984). Air sac membranes, or cavitations that communicate with airways, might be lined by white or green fungal mats (Fig. 4.2). Ascites is a frequent sequela to pulmonary aspergillosis in chickens, likely secondary to heart failure (Julian and Goryo 1990). *Aspergillus glaucus* infection of the integument may present in pigeons as scaly, yellow spots on the skin with dry, easily broken feathers (Lahaye 1928). Mycotic plaques can also develop in the oropharynx such as the tongue, palate, and larynx as reported in canaries (DeJong 1912). Bovine and equine mycotic placentitis is characterized by leathery, thickened allantois-chorion with superficial necrosis and enlarged, necrotic placentomes and sticky brown exudate covering the surface of the thickened, leathery areas of the chorion, respectively. Aborted fetuses are typically fresh and may have elevated irregular gray plaques on the skin resembling ichthyosis.

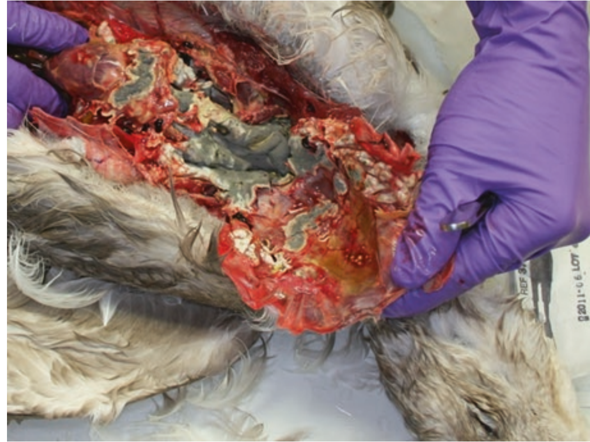

Fig. 4.2 Cervical and thoracic air sacs of a herring gull (*Larus smithsonianus*) affected with disseminated aspergillosis (note the green coloration indicating the presence of sporulating conidiophores). (Photographic image by Julia Burco)

Characteristic microscopic features of *Aspergillus* spp. in tissue sections include slender septate hyphae of variable length with parallel walls, 3–6 micrometers in diameter, and dichotomous branching with two equal hyphal branches of the same diameter as the original segment forming acute angles (Songer and Post 2005). Morphology of fungal hyphae is typically uniform in acute invasive lesions. In chronic lesions, however, in addition to the aforementioned morphology, hyphae may be globose, distorted, and up to 12 microns wide in diameter (Chandler et al. 1980). Reproductive structures (conidiophores) do not typically develop in tissues, except in those tissues under high oxygen tension such as bronchi and nasal passages or avian air sacs. In these tissues, aerial mycelia bearing conidiophores project from luminal mucosal surfaces exposed to air (Figs. 4.3 and 4.4). Stains that accentuate glycogen in the cell wall demonstrate *Aspergillus* spp. in tissue sections and include periodic acid-Schiff (PAS), Gomori methenamine silver (GMS), and Gridley stains. Immunohistochemical methods using *Aspergillus* antigen-specific antibodies provide definitive identification of *Aspergillus* spp. in tissue sections, fluorescent antibody in fresh/frozen tissues, and immunohistochemistry in formalin-fixed, paraffin-embedded tissues.

In general, for both birds and mammals, light microscopic lesions in affected tissues are characterized by hemorrhage, necrosis and infarction, and granulomatous inflammation (Chandler et al. 1980). Necrosis and hemorrhage with polymorphonuclear and mononuclear leukocytes are seen in acute stages of infection. Colonies of fungi are large and indiscriminately infiltrate adjacent tissues and invade walls of arteries leading to thrombosis and infarction. Necrosis is attributable to ischemia associated with thrombosis. Inflammation progresses to granulomatous inflammation. Classic granulomas form with central caseous (or liquefactive) necrotic cores in which organisms are sometimes found that are surrounded by a zone of epithelioid macrophages and foreign body multinucleated giant cells (Langerhans giant cells) with variable numbers of lymphocytes and plasma cells. Granulomas are surrounded by a collar of fibrovascular connective tissue. Fibrosis is progressive and can be severe. Small colonies of irregularly dispersed hyphae are

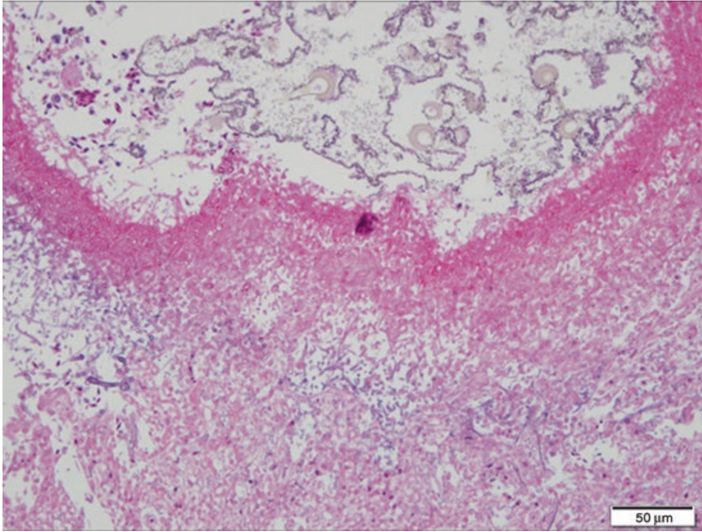

Fig. 4.3 Histologic image of a lung section from a parrot with pulmonary aspergillosis. Pulmonary parenchyma in the lower half of the image is diffusely necrotic and infiltrated with fungal hyphae. Necrotic airway mucosa is lined by fungal hyphae and sporulating conidiophores which extend into the airway lumen. H&E stain. (Histopathology photo by Leslie Woods)

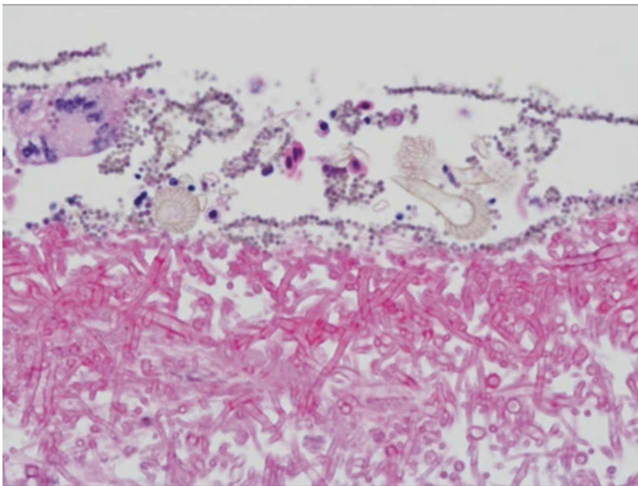

Fig. 4.4 Higher magnification of Fig. 4.3 demonstrating the dense array of *Aspergillus* sp. hyphae (lower half) with overlying pigmented sporulating conidiophores within the airway lumen. Hyphae are 3–6 microns in diameter and septate with dichotomous branching. H&E stain. (Histopathology photo by Leslie Woods)

typically seen within granulomas or with superficial infections. If cavities communicate with well-aerated airways, conidiophores can develop. The presence of sporulating conidiophores with conidia is unique, particularly to birds, as most mammalian lesions are created by tissue invasion by branching hyphae (Figs. 4.2, 4.3, and 4.4).

4.8 Treatment

Treatment of aspergillosis in veterinary medicine is challenging. Owing to the insidious nature of this disease and the fact that many of the antifungal agents used in veterinary medicine are fungistatic in nature, long-term therapy is required. Occasional reports of successful treatment of aspergillosis in veterinary medicine are scattered throughout the literature; however, such reports are the exception rather than the rule.

Before treatment of aspergillosis is initiated, financial and emotional commitments to long-term therapy should be discussed with the owner and considered. Both emotional and financial commitment by the owner/caretaker is essential for successful outcome, and in many cases despite that, treatment failure is more common than not.

In veterinary medicine, currently the most challenging aspect in treating aspergillosis is not necessarily drug resistance, but penetration of the therapeutic agent to the site of infection. Thus, localized treatment is often employed and ranges from local infusion of the sinuses with antifungal agents (e.g., dogs with nasosinal infections) (Pomrantz and Johnson 2010) to nebulization delivery of antifungal agents to the avian respiratory tract (Beernaert et al. 2009). In addition to localized treatment, systemic therapy is often utilized. When accessible, surgical excision of the fungal granulomas can be helpful. In most cases of aspergillosis, whether localized or disseminated, systemic long-term therapy with fungistatic agents is often indicated. Initial treatment of aspergillosis might involve the fungicidal agent, amphotericin B; however, this therapeutic regime is not commonly employed in veterinary medicine due to the need for direct vascular access for a somewhat extended period of time. Thus, fungistatic agents are more commonly used in veterinary medicine for treating aspergillosis in animals since they can be administered per os. Historically, the older generation azoles (ketoconazole and itraconazole) are used more commonly to treat aspergillosis in animals; it should be noted that fluconazole is not useful against aspergillosis. However, more recently practitioners are utilizing the newer generation azoles (voriconazole and posaconazole). Dosage guideline recommendations for a variety of antifungals in specific animals have been made by Seyedmousavi et al. 2015.

The pharmacokinetic parameters for VRC have been published for various laboratory animal species, ungulates, and birds. In mice, rats, and dogs, voriconazole (VRC) exhibits nonlinear pharmacokinetics with capacity-limited clearance and

autoinduction of VRC metabolism by cytochrome P450 enzymes after multiple dosing. It is suspected that similarly complex pharmacokinetics occur in other animal species. VRC has been reported to be associated with adverse clinical signs in cats, and to date no pharmacokinetic studies in feline species have been published. Fungal identification (Table 4.2) and MIC data (Table 4.3) for *Aspergillus* spp. isolated from specific groups of animals are very limited. For the MIC data, grouping of the data by *Aspergillus* spp. and reporting MIC ranges for the antifungal agents is necessary since insufficient data are available for individual animal groups to determine whether differences in antifungal susceptibility of different *Aspergillus* spp. exist when recovered from different animals.

4.9 Prevention

4.9.1 Monitoring and Epidemiology

Aspergillosis is a disease that in some cases (e.g., birds in rehabilitation settings) might be controlled via environmental management. Very few epidemiologic studies have been performed in free-ranging or captive settings that help address issues of disease ecology and interactions between birds and their local environment. Molecular genotyping is a useful tool that can be employed for understanding epidemiologic relationships between clinical and environmental isolates, which can then be used in outbreak investigations or in identifying hot spots of contamination. A subset of these environmental isolates collected through air, surface, and/or water sampling can be banked for use in genetic comparisons to clinical isolates collected from the same hospital environment. A number of human studies, and now avian studies, have tried to address this question using various molecular genotyping techniques.

The major genotyping techniques that have been used in genetic epidemiology studies include restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis, sequence-specific DNA primer (SSDP) analysis, polymorphic microsatellite marker (PMM) analysis, and multi-locus sequence typing (MLST). Microsatellite analysis and MLST sequencing are newer exact typing methods that are repeatable and can be shared easily between laboratories. On the genome front, multiple *Aspergillus* spp. (including *A. fumigatus, A. nidulans*, *A. oryzae*, and *A. niger)* have been sequenced in full, which will help identify targets for new antifungal drug therapy as well as better understand the biology of the organism. Sequencing has also opened the door for microarray analysis, an extremely useful new tool in diagnostic and ecological investigations of this pathogen. Another important discovery was proof of sexuality (O'Gorman et al. 2009; Varga et al. 2014). Genetic diversity identified within the *A. fumigatus* complex suggested sexual reproduction occurred, but the sexual stages had remained elusive.

Table 4.3 Antifungal medication minimum inhibitory concentration (MIC) ranges for *Aspergillus* spp. isolated from various animal species[a]

	MIC (μg.ml^{-1})							
Aspergillus spp.[b]	AMB	CAS	MICA	ITRA	KETO	POS	VORI	TERB
Aspergillus deflectus ($n = 5$)	1.0–4.0 ($n = 2$)[c]	0.03–0.03 ($n = 3$)[c]	ND	4.0–16.0 ($n = 3$)[c]	ND	4.0–8.0 ($n = 2$)[c]	1.0–8.0 ($n = 3$)[c]	0.008 ($n = 1$)[c]
Aspergillus flavus ($n = 5$)	2.0 ($n = 1$)[c]	ND	ND	0.25–0.5 ($n = 3$)[c]	0.5 ($n = 1$)[c]	0.125–0.25 ($n = 2$)[c]	0.25–0.5 ($n = 3$)[c]	0.06–0.5 ($n = 2$)[c]
Aspergillus flavus species complex ($n = 3$)	4.0 ($n = 1$)[c]	ND	ND	0.25–0.5 ($n = 2$)[c]	ND	0.125–0.125 ($n = 2$)[c]	0.25–1.0 ($n = 3$)[c]	ND
Aspergillus fumigatus ($n = 49$)	0.05–4.0 ($n = 11$)[c]	0.125–0.5 ($n = 3$)[c]	0.015 ($n = 1$)[c]	0.06–4.0 ($n = 42$)[c]	4.0–8.0 ($n = 4$)[c]	0.06–0.50 ($n = 22$)[c]	0.25–2.0 ($n = 34$)[c]	0.03–2.0 ($n = 17$)[c]
Aspergillus fumigatus species complex ($n = 4$)	0.5 ($n = 1$)[c]	ND	ND	0.5–1.0 ($n = 2$)[c]	2.0–8.0 ($n = 2$)[c]	0.125 ($n = 1$)[c]	0.25–0.5 ($n = 2$)[c]	0.5 ($n = 1$)[c]
Aspergillus glaucus group ($n = 2$)	ND	ND	ND	0.125–0.25 ($n = 2$)[c]	ND	ND	ND	0.06–0.125 ($n = 2$)[c]
Aspergillus niger species complex ($n = 11$)	0.125–0.5 ($n = 2$)[c]	0.06–0.5 ($n = 2$)[c]	0.015 ($n = 1$)[c]	0.5-8.0 ($n = 10$)[c]	8.0–16.0 ($n = 2$)[c]	0.25–0.25 ($n = 6$)[c]	0.06–2.0 ($n = 9$)[c]	0.06–0.5 ($n = 4$)[c]
Aspergillus terreus ($n = 10$)	2.0–2.0 ($n = 3$)[c]	0.03–0.25 ($n = 4$)[c]	0.015 ($n = 1$)[c]	0.06–0.5 ($n = 8$)[c]	ND	0.06–0.125 ($n = 5$)[c]	0.125–1.0 ($n = 7$)[c]	0.015–0.06 ($n = 4$)[c]
Aspergillus terreus species complex ($n = 3$)	ND	ND	ND	0.25 ($n = 1$)[c]	0.5 ($n = 1$)[c]	0.125 ($n = 1$)[c]	0.25–0.5 ($n = 2$)[c]	0.25 ($n = 1$)[c]
Aspergillus ustus ($n = 1$)	ND	ND	ND	0.5 ($n = 1$)[c]	ND	0.125 ($n = 1$)[c]	0.5 ($n = 1$)[c]	0.03 ($n = 1$)[c]
Aspergillus versicolor species complex ($n = 1$)	ND	ND	ND	0.125 ($n = 1$)[c]	ND	ND	0.125 ($n = 1$)[c]	ND

Abbreviations: *AMB* amphotericin B, *CAS* caspofungin, *MICA* micafungin, *ITRA* itraconazole, *KETO* ketoconazole, *POS* posaconazole, *VORI* voriconazole, *TERB* terbinafine, *ND* no data

[a]Data were provided by the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center, San Antonio, TX. The clinical isolates were submitted between the years of 2001 and 2012

[b]Identification was as described in Table 4.2

[c]*n* number of isolates evaluated

In addition to understanding genetic relationships between various clinical and environmental isolates, quantification of environmental fungal burden is an important aspect of understanding the pathogenesis of aspergillosis, especially since clinical infection is thought to be a result of a combination of exposure dose, environment, and host factors. Evaluation of fungal load in the native environment of susceptible bird species and comparing it with fungal burdens in the captive setting could be instrumental in making management recommendations. A variety of studies have examined fungal load in captive avian environments such as zoological institutions, aquaria, rehabilitation centers, and poultry houses. In general, there appears to be a higher fungal load in these environments than in the native habitats of the birds, although fluctuations in fungal load throughout the year have not been directly correlated with an increased incidence of aspergillosis. In the human arena, a number of studies have been performed evaluating *Aspergillus* spp. load in the hospital setting in order to identify risk factors or hot spots of environmental contamination. Similar studies have been performed looking at microenvironments within the avian rehabilitation setting and natural avian microenvironments (Burco et al. 2012b).

Indoor fungal burdens can be reduced through the use of HEPA filtration units, directed room air flow, and high-frequency air exchanges (10–15 exchanges/hour), which have been demonstrated to reduce ambient fungal counts in human hospital settings (Cornet et al. 1999). It is also important to remove organic debris, such as bedding, feces, or food (especially grain or mash) and minimize plants for decorative or interactive purposes for animals temporarily or permanently in a captive setting.

4.9.2 Vaccination

Innate immunity plays a significant role in preventing invasive disease and thus is a major area of study. Alveolar or free avian respiratory macrophages as well as respiratory epithelial cells represent the first line of defense in responding to inhaled conidia via phagocytosis (Reese et al. 2006; Balloy and Chignard 2009). Neutrophils in mammals or heterophils in birds are recruited for killing once swelling of the conidia occur and are especially important in eliminating the hyphal forms of the fungus through oxidative and non-oxidative means. Toll-like receptors (TLRs) and other pattern recognition receptors (e.g., mannose receptor or dectin-1) on the surface of these immune cells play an important role in recognizing pathogens. For *A. fumigatus*, TLR2 and TLR4 are important in recognition and elaboration of inflammatory mediators (Balloy and Chignard 2009). Adaptive immunity may also be triggered through CD4+ T cells, but is not always helpful and often comes too late for acute invasive disease. In birds, immunology studies prove difficult due to the need for many species-specific reagents. Consequently, there has been a trend in comparative immunology to evaluate more functional immune assays that are easy to use and can be compared across species, such as whole blood microbiocidal and phagocytosis activity, natural antibody levels, acute phase proteins, and the

production of reactive oxygen species. With the increase in knowledge about the immunological response to fungi, *Aspergillus* research has focused more on creating an effective vaccine. Several experimental studies have shown vaccine protection in turkey and murine models, and progress in vaccines against aspergillosis is being made (Stevens 2004; Stevens et al. 2011). However, no commercial vaccine is as yet available.

4.9.3 Environmental Control

Environmental control of aspergillosis is a major factor in limiting the disease in animals. In general, focusing efforts on minimizing environmental conditions for the growth of *Aspergillus* spp. around susceptible animals is important in reducing the development of disease. In human medicine, the Centers for Disease Control and Prevention (CDC) and the Healthcare Infection Control Practices Advisory Committee (HICPAC) have created guidelines to minimize the occurrence of infection in hospital settings (Weber et al. 2009). Similar guidelines could also be created for high-risk situations for animals.

4.10 Conclusions and Future Directions

Given to the ubiquitous nature of *Aspergillus,* the diversity of animal species affected, and the ability of animals to succumb to primary and secondary infection, aspergillosis continues to be a significant disease in veterinary medicine. Infection usually results in invasion of fungal hyphae into surrounding tissues and vasculature, and occasionally sporulation of conidiophores (i.e., air sac membranes of birds). Clinical signs can also be a result of mycotoxins produced by various *Aspergillus* spp. Presentation of disease is usually related to site of infection and can be quite severe (including acute mortality) in some animals. Antemortem diagnosis has largely relied on nonspecific imaging modalities and inflammatory markers, but has recently been combined with PCR-based techniques and serology to achieve a more accurate diagnosis. Treatment with antifungals is often prolonged and has mixed success. Consequently, prevention through minimizing fungal exposure is an important strategy in captive animals, and especially those that are highly susceptible to infection.

Several areas of research relevant to aspergillosis in animals are important for the future. There are a number of exciting recent advances and discoveries specific to the study of *Aspergillus*. Studies on the pathogenesis of aspergillosis in specific animals and birds will be instrumental in understanding the disease process further and moving research forward in veterinary medicine. Continued work on the role of cryptic species of *Aspergillus* in animal disease is important given differences in their susceptibility to antifungals. Thus, further studies on the epidemiology of dis-

ease in different animals should be pursued, as should continuing efforts on the advancement in molecular identification of medically important isolates using molecular biology techniques (i.e., microsphere-based Luminex assays, real-time PCR, and microsatellite analysis), as this knowledge will help direct treatment and management more quickly and effectively. In addition, more work is needed on rapid diagnostic tests that are affordable and useful in animals. Point-of-care tests, such as the newer lateral flow assays, need to be evaluated as diagnostic assays are useful not only in humans but also in birds and animals. The efforts of antifungal drug discovery for use in humans should also be applied in aspergillosis in veterinary medicine as differences in metabolism and pharmacodynamics of antifungals may be different. Thus, efficacy studies done in animals, other than rodents, are needed to determine the most effective therapies, including dosage, frequency, and duration of treatment. Lastly, increased knowledge and research on the immune response to *Aspergillus* infections indicate that vaccination may be another useful tool for the prevention of the disease to be employed in the future. Work on vaccines needs methods of evaluating protective efficacy in animals and like work with antifungals, the development of clinical trials. All of these areas are important for reducing the impact of aspergillosis in veterinary medicine.

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Chapter 5
Fungal Diseases of Animals: Symptoms and Their Cure by Natural Products

Hilal Ahmad Ganaie

5.1 Introduction

The medicinal plants were used as herbal formulations in crude forms like extracts, teas, powders and even in plasters during the breakage of bones. The traditional way by which these plants were used can still be found in communities, passed down through natural history, and still prevails to be a successful form of medication, despite the advent of modern medicine. According to Bakkali et al. (2008), medicinal and aromatic plants (MAPs) are *chemical goldmines* not only with characteristic aroma but also with medicinal properties because of the diverse range of secondary metabolites. The use of aromatic medicinal plants has been increasing steadily with notable use in pharmaceutical, cosmetic and food industries (Christaki et al. 2012).

In modern system of medicine, the antibiotics have played a great role in control and treatment of various dreadful diseases. According to Atta-ur-Atta-ur-Rahman Choudhary and Thomsen (2001), an antibiotic can be defined as any chemical substance, synthetic or natural, that either kills pathogenic microbial organisms like bacteria, viruses, fungi, protozoa, etc. or retards their growth. With the discovery and extensive consumption of synthetic antibiotics, some resistant strains of microbes like multidrug-resistant strains of *Mycobacterium tuberculosis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, penicillin-resistant *Streptococcus pneumoniae* (PRSP), methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) have emerged and are a threat to the successful treatment of different dreadful diseases associated with these microbes. Thus, it is a strong challenge to the scientist community to search for alternatives to curb these dreadful diseases. Nowadays, this has diverted the attention of the scientist to discover some compounds from the natural wealth that will

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check the risk of antibiotic resistance development. This natural wealth includes plant extracts, volatile oils, drug combinations and their active compounds, which act on bacterial cell wall and plasma membrane, making them permeable and in turn results in loss of ions and membrane potential, crumpling of proton pump and ATP pool exhaustion (Tally 1999; Gibbons 2004; Bakkali et al. 2008).

5.2 Mechanism of Antibiotic Action

For the effectiveness of any antibiotic against bacteria, the following three conditions must be fulfilled:

(i) Existence of susceptible antibiotic target site in the bacterial cell.
(ii) Sufficient antibiotic dose must reach the target site.
(iii) Before reaching the target site, antibiotic drug must not be modified or deactivated (Russell and Chopra 1990; Sutcliffe et al. 1999).

Antibiotics are often classified based on their mechanism of action and are broadly categorized into following groups (Table 5.1).

Table 5.1 Classification of antibiotics based on mechanism of action

Mechanisms of action	Drugs
Cell wall synthesis interference	β-Lactams: penicillins, cephalosporins, carbapenems, monobactams
Inhibit cross-linking of peptidoglycan by inactivating transpeptidases (PBPs)	
Bind to terminal D-ala-D-ala and prevent incorporation into growing peptidoglycan	Glycopeptides: vancomycin, teicoplanin
Inhibition of protein synthesis	Chloramphenicol, clindamycin, macrolides, quinupristin-dalfopristin, linezolid, mupirocin
Bind to 50S ribosomal subunit	
Bind to 30S ribosomal subunit	
Bind to isoleucyl-tRNA synthetase	
	Aminoglycosides, tetracyclines
Nucleic acid synthesis interference	Fluoroquinolones
Inhibit DNA synthesis	Rifampin
Inhibit RNA synthesis	
Inhibition of metabolic pathway	Sulfonamides, folic acid analogues
Inhibition of tetrahydrofolic acid production (cofactor for nucleotide synthesis)	
Bacterial membrane disruption	Polymyxins, daptomycin
Sulfonamides, folic acid analogues	

Source: Fred 2006

5.3 Mechanism of Antibiotic Resistance

Bacteria may become resistant to antibacterial drugs in a number of different ways. Figure 5.1 represents the mechanism of antibiotic resistance in bacterial strains in terms of biochemical and genetic aspects.

The attack of drugs is resisted by many bacteria by inactivating the drugs as a result of which they produce enzymes that transform or degrade the drug itself prior to reaching the target site mainly in three ways, e.g. β-lactamases which hydrolyze the β-lactam rings of penicillin and cephalosporin antibiotics; transferase enzymes which usually inactivate antibiotics by substitution of chemical groups like acetyl, phosphoryl or adenylyl that modify the antibiotics (Houang et al. 2003; Schwarz et al. 2004) and Tet X enzyme which induces oxidation of tetracycline antibiotics causing drug inactivation by redox processes (Yang et al. 2004).

The second major mechanism of antibiotic resistance is the modification of antibiotic target site. As a result, the antibiotic is not able to bind properly. The bacterial resistance to streptomycin occurs due to the modification in S12 protein of 30 S ribosomal subunit rendering the ribosome insensitive to the drug. Erythromycin resistance is caused by mutation in L4 and L12 proteins of 50 S ribosomal subunit.

Efflux pumps are proteinaceous transporters that are localized in the cytoplasmic membrane of all kinds of cells. The drug efflux pumps drive out the antibiotic from

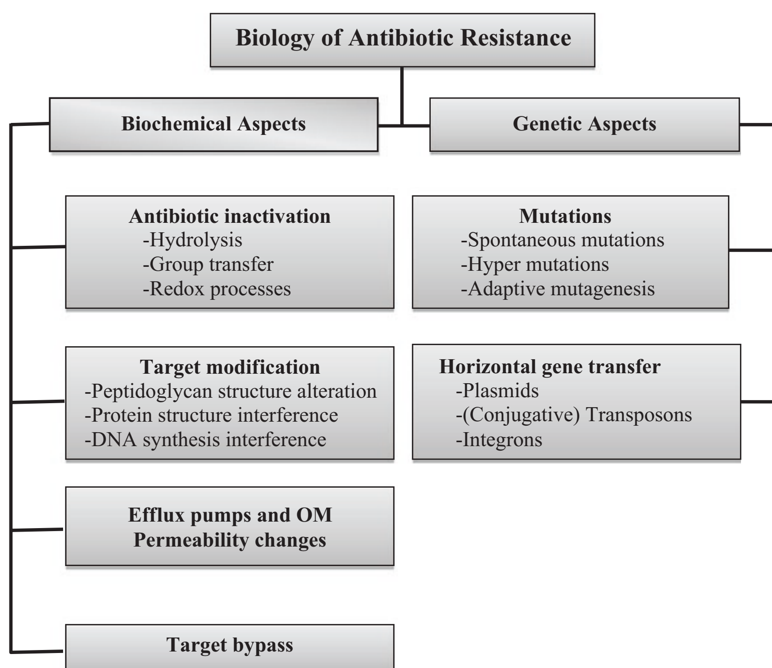

Fig. 5.1 Mechanism of antibiotic resistance in bacteria

the cell. These pumps are non-specific and can pump different drugs out of the cell. These pumps are also called multidrug-resistant pumps (Webber and Piddock 2003). Many pathogenic bacteria like *S. aureus*, *E. coli*, *P. aeruginosa* and *Mycobacterium smegmatis* possess drug/proton (H^+) antiporter import protons in the cell and simultaneously export drug out from the cell.

All the characteristics that an organism possesses are controlled by the genetic material of that very organism. The adaptation of an organism to a particular trait is also linked to its genetic material. In bacteria, various genetic loci have been identified that are associated with antibiotic resistance (Dong et al. 2015). Resistance in bacteria can be of two types: inherent or inborn and acquired. The acquired antibiotic resistance results from the bacterial gene mutation or insertion of foreign resistant gene in the bacteria. The bacteria acquire antibiotic resistance mainly by two ways:

(i) Gene mutations at different chromosomal loci
(ii) Through the acquirement of resistant genes from other microorganisms (horizontal gene transfer)

Bacteria have drug-resistant genes on chromosomal as well as plasmid DNA. The R plasmids of drug-resistant bacterial strains have one or more resistant genes (Sarkar et al. 2015). These plasmids usually code the enzymes that destroy or modify the antibiotics, e.g. penicillin hydrolysis, acetylation of chloramphenicol, etc. The horizontal gene transfer involves the transfer of gene from one bacterium to another by conjugation, transformation and transduction leading to antibiotic resistance. The drug-resistant genes acquired by the recipient are found in clusters and are transferred together; hence, horizontal transfer of resistance genes is a mechanism for the dissemination of multiple drug resistance and is facilitated by existence of specific DNA structures known as integrons (Rowe-Magnus and Mazel 1999; Ploy et al. 2000; Palmer et al. 2010; Ravi et al. 2015). In recent years, medicinal plants have been reported throughout the world as a source of antimicrobial agents (Bouhdid et al. 2010; Fadli et al. 2012; Agyare et al. 2016). Thus, the need of the hour is to discover new antimicrobial substances from natural sources, and for the achievement of this goal, all possible approaches should be explored.

5.4 Methodology for Carrying Out Antibacterial Activity

5.4.1 Cleaning of Glassware

The presence of undesirable substances may affect the growth of bacterial culture; therefore, cleaning of glassware is an important part of methodology. The glassware are kept in labolene for 2 hours to remove oil, grease and other organic matter from it and then washed vigorously under a jet of tap water. This is followed by a second

rinse with distilled water. The cleaned glassware are then placed in a hot air oven at high temperature for sterilization.

5.4.2 Sterilization

In order to have contamination-free experiments from all organisms including bacteria, fungi and viruses, all glassware, culture media and equipment have to be sterilized. The process of sterilization can be done through four methods:

5.4.2.1 Dry Heat Sterilization

Dry heat sterilization is achieved by hot air oven equipped with a thermostat. All the glassware, metal instruments and equipment were sterilized keeping in hot air oven at 160 °C for 1 hour. Dry heat sterilization is not an efficient method of sterilization as some bacteria in desiccated vegetative state or as spores can survive; therefore, moist heat sterilization method is followed.

5.4.2.2 Moist Heat Sterilization

As per the guidelines of APHA (1998), moist heat sterilization is the most effective and reliable method for sterilization. Both the culture media and glassware are kept in an autoclave for 15 minutes at 121 °C and at 15 lb./inch^2 pressure. This method also has a setback as it cannot be used for materials damaged by moisture or culture media containing compounds decomposed at high temperature. For this purpose, other methods of sterilization are followed.

5.4.2.3 Direct Flaming

Inoculating loops, inoculating wires, forceps, needles and spreaders are sterilized by the method of direct flaming. In this method, a burner is used to sterilize the above-mentioned objects by exposing them to flame until they become red hot.

5.4.2.4 Ultraviolet (UV) Sterilization

In order to kill the microbes in the inoculating chamber (laminar air flow cabinet), the UV radiation treatment was given for 5 minutes prior to the start of experiment.

5.4.3 Preparation of Culture Media

The first and the foremost thing in the preparation of culture media is to check the composition of the medium because it should support the growth of a wide range of bacterial strains in a reproducible way. Mueller-Hinton agar or broth satisfies the above criteria and is used for the evaluation of antibacterial activity. The culture media is prepared by mixing 38 grams of commercially available Mueller-Hinton agar in 1 litre of distilled water in an Erlenmeyer flask and heated. After dissolving completely, the agar from the flask is poured in the boiling tubes, each containing 20–25 mL of agar. The agar tubes are tightly plugged with cotton and sterilized by autoclaving at 121 °C and 15 lbs. pressure for 20–25 min. Immediately after autoclaving, the media is allowed to cool to 40–45 °C under laminar hood. The media are then poured into 90 mm disposable flat-bottomed Petri dishes, allowed to solidify and later used for susceptibility assay.

5.5 Evaluation of Antibacterial Activity by Agar Well Diffusion Method

Well-established agar well diffusion method as adopted by Perez et al. (1990) is mostly used to evaluate the antibacterial activities of natural products against bacterial strains. As mentioned earlier, the bacterial strains were subcultured on Mueller-Hinton agar slants before checking antibacterial susceptibility. From the freshly prepared bacterial suspension, 100 μl of 0.5 McFarland standard inoculum from each bacterial strain is inoculated on molten Mueller-Hinton agar in culture tubes. The media in the tubes are then homogenized by rubbing between the hands and poured into 90 mm sterile Petri plates. The plates are then allowed to solidify under laminar air flow. The wells are dug using a 5 mm standard cork borer. Three different concentrations (40, 70 and 100 μl corresponding to 4, 7 and 10 mg/mL of plant extract) of 100 mg/mL of each extract dissolved in respective solvents are added to respective wells. Gentamicin (30 μg/disc) is used as positive control and DMSO solvent as negative control. The plates are then sealed and incubated at 37 ± 1 °C temperature for 24 h. The antibacterial activity is calculated by measuring the zone of inhibitions using standard scale (Norrell and Messley 1997).

5.6 Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial compound that will inhibit the visible growth of a microorganism after overnight incubation. The MIC is studied for extracts which showed susceptibility in

agar well diffusion method. MIC is determined by broth dilution assay (Barry 1976). The required ranges of concentration of each extract (6–0.12 mg/mL) and standard antibiotic are prepared by serial twofold dilution in Muller-Hinton broth. The fresh inoculum of each bacterial strain is made from 12 h broth cultures, and the bacterial concentrations were adjusted to standard turbidity level of 0.5 McFarland. The test samples are stirred in standard test tubes and are inoculated with 50 μl of bacterial suspension containing 2 × 105 CFU/mL. The tubes are then incubated in shaker incubator at 37 ± 1 °C for 24 hours. The lowest concentration of the test sample at which bacteria does not show any growth is considered as the MIC value (Delaquis et al. 2002).

5.7 Fungal Diseases of Animals

Some important fungal diseases of animals are discussed below:

5.7.1 *Aspergillosis (***Aspergillus fumigatus** *and Other Species)*

Aspergillosis is a primary respiratory and occasionally generalized infection caused by species of *Aspergillus*. It is worldwide in distribution and has been recorded in almost domestic animals and birds as well as many wild species. The disease is characterized by the formation of yellowish caseous nodules or plaques and has been known to affect almost every organ of the body. Diagnosis is by the demonstration of hyphae in the lesions and by the culture of the pathogen. Iodide therapy has been used with some success in human infections, but few animal cases have been diagnosed before death. The removal of the source of the fungus spores appears to be the only satisfactory method of control.

Aspergillosis has been recorded in almost every species of domesticated animal and bird and in a great many free-living and captive wild creatures. It was one of the first of the mycoses of animals to be described and helped to focus attention on the microbial causes of disease at a time when bacteria were still microscopical curiosities. From the time of its discovery in a jay by Mayer and Emmert in 1815, until the end of the nineteenth century, a great many papers appeared on the disease, including a number of monographic treatments, and as a result, much of our present knowledge of the disease was already known by 1900. Relatively few reports have appeared since that time, but within the past few years, renewed interest has led to the confirmation of much of the earlier work and has provided a better understanding of the role of *Aspergillus fumigatus* and other fungi in respiratory diseases in both man and animals. There is no recent general review of aspergillosis, and the outstanding works of Renon (1897) and Lucet (1897) have remained the standard texts for 60 years.

The striking post-mortem picture presented by avian aspergillosis was no doubt responsible for its early recognition so that by the end of the nineteenth century, some 60 contributions had been made on the disease of birds alone. At first there was little attempt to identify the “green mould” seen in the air sac and bronchial lesions (Owen 1832), and many of the early accounts are difficult to interpret for this reason. There was also much confusion between the pathogenic and saprophytic species of *Aspergillus*. Progress in the study of the disease was also slowed down by controversy over the aetiological significance of the fungi found sporulating on the lesions, and although the primary pathogenicity of most of these is now well established, the epidemiology and development of the disease still require further investigation.

There are extensive reviews of avian aspergillosis by Lesbouyrles (1952), Urbain and Guillot (1938) and Verge (1927), in which may be found detailed accounts of the species of birds affected and of the pathology, diagnosis, mycology and therapy of the disease.

5.7.1.1 Treatment and Control

The treatment of avian aspergillosis is influenced by the lack of clinical diagnosis, for the symptoms are not specific and generally the peak of mortality has been passed before any therapeutic measures can be instituted. Because of this, reports of successful treatment or even spontaneous recovery are doubtful.

Various methods have been used in the direct treatment of individual birds. They include the inhalation of tar vapours (Otte 1928), chlorine or sulphur dust, but so far, no information is available concerning the use of iodide therapy which has been successfully employed in the experimental disease in rabbits and also in human cases.

By far the most effective method of control is by removing the source of infection, that is, the mouldy litter or food; this should be burnt and the premises adequately cleaned up, perhaps using a fungicidal solution. Another method of prevention is described by Urbain and Guillot (1938), who regularly fed the penguins in the Paris zoo with 5–6 mg of potassium iodide daily for eight consecutive days in each month, each dose being inserted into a fish. By this technique, they found that the incidence of aspergillosis in these birds was greatly reduced. Good sanitation and careful control of feeding stuffs and litter remain the best methods of prevention in domestic birds.

Except in man, aspergillosis is not a well-known disease of mammals, although it has been recognized with increasing frequency in the past few years and the literature now consists of approximately a hundred papers dating from the brief mention of the disease in a deer by Rousseau and Serrurier (1841). It is probable that the most important aspect of mammalian aspergillosis is in connection with mycotic abortion in which infection of the placenta of cows is thought to be a sequel to subclinical pulmonary aspergillosis. The aspergilli may also contribute to allergic types of respiratory troubles such as pneumonitis and asthma, especially in young animals.

There is no recent review of mammalian aspergillosis, but Renon (1897) gave a comprehensive survey of the cases up to that time.

The serological study of aspergillosis is unfortunately not nearly so advanced as in histoplasmosis and coccidioidomycosis. Reliable serological diagnostic methods would be particularly useful in large animals in which radiography is difficult and in which subclinical infection appears to be more common than at present supposed.

Complement fixation was found by Boe et al. (1939) to be the most sensitive method for the detection of antibodies. They obtained titres of 1:1000–1:1600 irrespective of whether spores or the powdered mycelium was used as the antigen. The agglutination and precipitin tests performed were as unsuccessful as those performed by Macaigne and Nicaud (1927a). Matsumoto (1929) obtained a titre of 1:10 for *A. fumigatus* and an inhibition of haemolysis by *A. amstelodami* of 1:320. Tests for skin sensitivity have been successfully used in man by Macaigne and Nicaud (1927b), Hyde et al. (1956) and others, but urgently require further study.

Research on production of toxins has mostly been carried out using *A. fumigatus* but often with conflicting results. Leber (1882), Ceni and Besta (1905) and Bodin and Lenormand (1912) all considered that they had evidence of toxin production, but this was contrary to the findings of Kotliar (1894) and Obici (1898). Henrici (1939) seems to have clearly demonstrated the presence of an endotoxin in *A. fumigatus*, and Salvin (1952) later confirmed this observation. Henrici found that a thermolabile substance could be extracted by cutting up, squeezing and centrifuging the mycelium, and that this affected all experimental animals when injected by subcutaneous, intraperitoneal and intravenous routes, producing nervous symptoms and proving fatal in quite small doses. Many of the properties of the toxin indicated that it was a toxalbumin, but it was apparently a non-protein substance.

5.8 Moniliasis

Moniliasis is a general term covering diseases caused by mycelial yeasts of the genus *Candida*, especially by *C. albicans*. Infection is usually restricted to the alimentary canal, but generalization may occur. Among animals, moniliasis of poultry (especially turkeys) is of particular importance. *C. albicans*, though at times undoubtedly a primary pathogen, is frequently found in association with apparently normal animals.

Oral moniliasis in infants (or thrush) caused by *Candida albicans* has been well known for more than a century. The thrush fungus and other species of *Candida* have also been frequently recorded from the lungs, skin, nails, vagina, etc. in man, and there is a vast and very confused literature. Much of the confusion has been due to claims of a pathogenic association or inadequate evidence and perhaps even more due to inadequate data on the fungi. No clarification is possible unless the mycological approach is satisfactory and so, for moniliasis, as for ringworm, the general pattern adopted in this survey will be varied and the mycology will be considered first.

5.9 Cryptococcosis

Cryptococcosis is a chronic and often fatal infection with no clear clinical pattern. In small animals, the central nervous system is frequently infected. In cattle, there may be mastitis and pneumonia. Diagnosis is made by demonstrating capsulated yeast cells in tissues, spinal fluid, pus or other exudate and confirmed by culture of the pathogen. No reliable treatment is known.

Neither in man nor in animals is the clinical picture of *Cryptococcus neoformans* infection well defined. *C. neoformans* shows a predilection for the central nervous system in man and commonly induces fatal meningitis which, as Cox and Tolhurst (1946) pointed out, can readily be confused with tuberculous and other forms of subacute and chronic meningitis. In some cases, the lungs are the site of the primary infection and more rarely the skin involved. Inoculation of experimental animals, especially mice, almost invariably results in severe infection of the central nervous system, but spontaneous infections in animals are more varied. Natural infection of the central nervous system is not uncommon (Mc Grath 1954), but, as in man, the lungs or skin may be involved, and there are records of bovine *Cryptococcal mastitis*. Most of the records for animals, as for man, have been from the USA, but the widespread incidence of cryptococcosis throughout the world suggests that this may be due merely to the condition being more often correctly diagnosed in North America than elsewhere.

5.9.1 Treatment

There is no known treatment. In the major outbreak of C*ryptococcal mastitis* referred to above, the infected animals had to be destroyed. Simon (1955) recorded that ethyl vanillate, polymyxin B and neomycin all inhibited the growth in vitro of *C. neoformans* from cattle, whilst Segretain et al. (1956) treated affected cows successfully with cycloheximide (Acti-dione).

5.10 Epizootic Lymphangitis (*Histoplasma farciminosum*)

This is a chronic infection of horses and other equidae resulting in a cording of the subcutaneous lymphatics, especially of the limbs and neck, and the development of a series of granulating abscesses, the discharge from which contains yeastlike cells of the pathogen. Infection may also lead to pneumonia and conjunctivitis. Subcutaneous infections respond to surgery and various fungicides. A policy of slaughter in the early stages of an outbreak may be advisable.

5.10.1 Symptomology

The clinical picture of epizootic lymphangitis has been described many times (Pallin 1904; Curasson 1942; Plunkett 1949). Typically there is infection of the subcutaneous lymphatic vessels particularly of the limbs and neck. The infected vessels become dilated at intervals and form lines of hard abscesses (the so-called cording of the lymphatics). These abscesses eventually rupture, forming characteristic ulcers from which the discharge of blood-stained pus continuing for some weeks until normal healing occurs. By this time, further lesions may be followed in different stages of development. Lesions may also occur on the mucous membrane of the nostrils and may extend inwards along the *septum nasi* to the pharynx, larynx and trachea. Bennett (1931) described a primary interstitial pneumonia in mules and horses in the Sudan, which was not associated with skin lesions. In sections of the affected lung tissue, an organism closely resembling *H. farciminosum* was seen, but as no cultural examinations were made, its identity is in doubt.

Singh (1956), in India, described in detail the ocular involvement in mules (in 176 of a series of 188 cases). This resulted first in a watery discharge from one or both eyes, some swelling of the eyelids, and later in the development on the conjunctiva and (or alternatively) on the membrana nictitans of papules which became ulcerating button-like growths, the eye becoming closed by a diffuse swelling of the eyelids.

The course of typical lymphatic infections was well described by Bullen (1950–51) from a study of experimental infections. He found that when pus was rubbed into a superficial wound, healing proceeded without any clinical signs of infection. The primary granulating ulcer at the point of inoculation underwent alternating periods of discharge and quiescence until, after 6–8 weeks, it had attained a diameter of an inch. The lesion then healed spontaneously and at the end of 2–3 months only a scar remained. During this series of events, infection had spread along the lymphatic vessels and additional abscesses showing a similar developmental pattern resulted.

5.11 Cure of Fungal Diseases by Natural Products

Many plants have been used to treat various microbial diseases of animals. Comparing the antibacterial activities of *Melissa officinalis* and *Ajuga bracteosa,* the former showed good antibacterial activity against the tested bacterial strains at all the concentrations, but *Melissa officinalis* showed higher activity than *Ajuga bracteosa*. Among the different extracts of *Melissa officinalis,* methanolic extract showed highest activity against all the tested bacterial strains at all the concentrations, i.e. 40 μL, 70 μL and 100 μL containing 4, 7 and 10 mg of the plant extract, respectively. Thus, the findings of our study provide evidence in support of earlier studies claiming that methanol is the most reliable and better solvent for extraction

of antimicrobial agents from herbs (Ahmad et al. 1998; Lin et al. 1999). The methanolic extract showed least activity against *Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, Salmonella typhi* and *Escherichia coli.* The least activity of *Melissa officinalis* against above-mentioned Gram-negative bacteria was also noted (Canadanovic-Brunet et al. 2008). Our results also corroborate the findings of various previous studies on different extracts of *Melissa officinalis* which showed different levels of antimicrobial activity (Iauk et al. 2003; Erturk 2006; Uzun et al. 2004; Dastmalchi et al. 2008; Stanojevic et al. 2010; Rostami et al. 2012).

Among the different extracts of *Ajuga bracteosa*, methanolic extract showed highest activity against Gram-positive bacteria, *Proteus vulgaris* and *Pseudomonas aeruginosa*, at 40 μL, 70 μL and 100 μL prepared from 100 mg/mL of extract in respective solvent. The methanolic extract also showed least activity against Gram-negative bacteria.

According to Ahmad (2013), the methanolic extract of *Ajuga parviflora* showed highest activity against *Citrobacter* and *Pseudomonas aeruginosa* with zone of inhibition of 12 mm. The genus *Ajuga* possesses various phytoconstituents like withanolids, triglycerides and flavonoids which have broad spectrum of biological, pharmacological and medicinal properties including antibacterial properties (Israili and Lyoussi 2009; Yildirim et al. 2013).

Gram-positive bacterial strains were found to be more sensitive than Gram-negative bacterial strains. Each extract acted differently and showed variation in the antibacterial activity against each bacterial strain, which might be due to cell wall structural differences between the bacterial species (Feng et al. 2000; Gaunt et al. 2005). Gram-negative bacterial strains were less susceptible to the plant extracts, which is due to lipopolysaccharide layer in the outer membrane that protects the penetration of hydrophobic antimicrobial agents. Therefore, higher concentrations of antibiotic agents are required as compared to that of Gram-positive bacteria. The harmless commensal human gut bacteria transfer resistance genes between themselves and turn into drug-resistant pathogens (Ochman et al. 2000; Manges et al. 2001).

Various bacterial isolates are practically resistant to all existing antibiotics. Multidrug resistance has been confirmed in *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Shigella dysenteriae, Salmonella typhimurium, Acinetobacter baumannii, Stenotrophomonas maltophilia, Enterococcus faecium, Burkholderia* and *Xanthomonas* (Betina 1994). Plant secondary metabolites exert antimicrobial activity by increasing the inner membrane permeability and decreasing the ATP production through loss of membrane potential (Tsuchiya and Iinuma 2000; Rios and Recio 2005; Cos et al. 2006). These secondary metabolites also inhibit the DNA gyrase which is involved in nucleotide synthesis in bacteria. Tannins are known to inhibit protein synthesis by forming irreversible complexes with proline-rich proteins. Such active compounds from plants are very useful against infectious diseases. The plant extracts being active against both clinical and laboratory isolates are also an indication that it can be a source of very potent antibiotic substances that can be used against multidrug-resistant bacterial strains.

The ethanolic and aqueous leaf extracts of *Bridelia ferruginea* were investigated by Adetutu et al. (2011) for evaluation of antibacterial activity against bacterial species causing a number of infectious diseases: *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.* The ethanolic extract was further studied to assess antioxidant potential and its effect on growth of human dermal fibroblasts (FS5). Both ethanolic and aqueous leaf extracts showed weak antibacterial activity against all bacterial pathogens with an MIC value of more than 470 μg/mL. Ethanolic extract showed significant increased growth in FS5 fibroblasts at the concentration of 5 μg/mL and above which the extract showed toxicity. Ethanolic extract showed significant protection of FS5 cells against hydrogen peroxide-induced damage, and the results were comparable to catalase (82% at 250 μg/mL concentration). The ethanolic leaf extract also showed high DPPH free radical inhibition with $IC_{50}$of 12.5 μg/mL compared to ascorbic acid (7.3 μg/mL).

Shokeen et al. (2009) evaluated the antibacterial activity of (50%) ethanolic extracts from different parts of 16 medicinal plants against different clinical and WHO strains of *Neisseria gonorrhoeae* including multidrug-resistant (MDR) strains. They found that the extracts exhibited better inhibition of MDR strains and differential inhibition against *Neisseria gonorrhoeae*. Among all the extracts tested against *Neisseria gonorrhoeae*, 60% showed higher activity, 20% showed moderate activity and 20% showed weak activity.

Chomnawang et al. (2009) studied the antimicrobial activity of 17 Thai medicinal plants against methicillin-resistant *Staphylococcus aureus* by using disc diffusion method and broth dilution assay. They found that the extracts *of Barleria lupulina, Psidium guajava, Garcinia mangostana, Tagetes erecta, Hibiscus sabdariffa, Senna alata, Eupatorium odoratum* and *Lawsonia inermis* inhibited the growth of standard *Staphylococcus aureus* bacterial strain. Among these, *Garcinia mangostana* extract was found as most potent with highest zone of inhibition (11.3 ± 0.60) and MIC value of 39 μg/mL.

Essential oil was extracted from leaves, flowers, stem and whole aerial part of *Tamarix boveana* by hydrodistillation method (Saidana et al. 2008). After extraction, the oils were further studied for chemical composition and evaluation of antimicrobial activity. A total of 62 components were identified from whole aerial parts. The major components identified were hexadecanoic acid (18.14%), docosane (13.34%), germacrene D (7.68%), fenchyl acetate (7.34%) and benzyl benzoate (4.11%). The essential oil did not show any activity against fungal strains but showed significant antibacterial activity against all bacterial strains except *Pseudomonas aeruginosa*.

5.12 Conclusion and Future Prospects

Kashmir Himalaya has been imparted with a rich wealth of floral diversity by nature. Most of the ethno-pharmacologically important plants are endemic to this region. The medicinal values of plants are attributed to the presence of active compounds,

synthesized via different biosynthetic pathways. The basic health care of nearly 75–80% of global population relies on herbal medicine. This dependence has resulted from their better cultural acceptability, their being economical, their better compatibility with the body and their usage free from side effects.

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Chapter 6
Mycotic Infections in Bovines: Recent Trends and Insights on Pathogenicity After Post-Industrial Temperature Rise

Kamal Jaiswal, Awanish Kumar Singh, and Suman Mishra

6.1 Introduction

According to the Intergovernmental Panel on Climate Change (IPCC 2007), climate change can be defined as a variation in climate over a period of time, whether in average weather or extreme events. Increasing temperatures, decreasing rainfall reliability, and increasing frequency and severity of extreme climatic events are the main outcome of variation in climate change. Climate change after the post-industrialization period has altered the molecular biology of pathogens, vectors (if any), farming practices and land use, and environmental and zoological factors. It has established new microenvironments and microclimates and influenced the occurrence, distribution, and prevalence of livestock diseases under the changing ecological conditions. The implication is the drier subtropical regions warming more than the moister tropics (Alcamo et al. 2007; Speranzaa 2010). Climate variability, by affecting the environmental conditions, has the consequence of impacting pasture growth and quality, availability of water resources, and, thus, the distribution of livestock diseases (Gale et al. 2008). Plant, animal, and human epidemics are influenced climatically (Bosch et al. 2007; Thomson et al. 2006; Wint et al. 2002).

Fungi are an organism that have the capacity to spread in many different habitats around the world and can affect living and non-living creatures. The fungi, which are thought to number around 140,000 on Earth, play an important role in the global ecosystem. Fungi help the ecosystem by promoting nutrient conversion, the formation of soil, and decomposition of organic matter (Bal et al. 2017). They live as symbiotic, parasitic (mostly plant pathogens), and saprotrophic (Kauserud et al. 2005; Durugbo et al. 2013; Sevindik et al. 2017), in which

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parasitic fungi are the second largest group, of whose members do a lot of serious damage. Their (fungi) spores can spread through meteorological activities and can cause harm to living and non-living organisms in the areas in which they spread (Garcia et al. 2012).

Veterinary mycology refers to the branch dealing with the detection and identification of veterinary fungi, pathophysiology and diagnosis of fungal infections, the pharmacodynamics of antifungals in animals, epidemiology of animal mycoses, eradication, and promotion of vaccine development against invasive veterinary fungal infections. It is different from human mycology in mainly two different aspects. First, the phenomenon of pathological or iatrogenic immunosuppression has affected an ever-increasing number of people after 1980, which has led to the emergence of fungal infections mainly caused by opportunistic fungi previously not or only rarely involved in human mycoses (Enoch et al. 2017). These new phenomena promoted the discovery of multiple ranges of antimycotic drugs, many of which are prohibitively expensive for veterinary use. This phenomenon has not been seen in animals since the gamut of fungi associated with veterinary mycoses has remained largely the same, with differences primarily from factors such as improved diagnostic methods and raised awareness (Elad 2011). The second aspect that separates veterinary from human mycology is the cost-effectiveness. The price and cost associated with diagnosis, identification, etiology, and cure are major hindrances in the eradication of fungal infections in animals, especially cattle.

As bovines are warm-blooded animals, "A major factor protecting warm-blooded animals from fungal disease is the difference in temperature between the body temperature and the outside environment. As fungi adapt to living at higher temperatures after post-industrial temperature rise, animals may begin to lose some of that natural protection" (Casadevall 2010). For a heat-tolerant fungus, the animal body might become pastureland. Casadevall and colleagues have demonstrated that some fungi have already started to adapt to warmer temperatures. When they analyzed temperature susceptibility data for several strains of yeast (a type of fungus) gathered since 1985, they found an increase in the temperature tolerance in the past 20 years in some fungal strains. The findings, say the researchers, are a "clear warning that global warming may cause fungi to become significantly more thermally resistant," though it is not yet possible to predict which species may become new threats.

Dufour et al. (2008) conducted an experiment to study the impact of climatic changes on the vector, host reservoir, characteristics, and epidemiology of the parasite. This study was an attempt to analyze susceptibility to the emergence and development of contagious disease in France as a result of global warming and found that climate change affects the incidence of livestock diseases transmitted by direct contact due to changes in the frequency and duration of animal contacts. Changes in the degree of mixing of cattle will affect the prevalence of some diseases (Fig. 6.1).

Fig. 6.1 The proportion of plant and animal disease alerts recorded by the Program for Monitoring Emerging Diseases attributable to fungi has risen in recent years. (Diagram courtesy of Nature Publishing Group)

6.2 Mycotic Diseases in Cattle

6.2.1 Mycotic Abortion

Bovine mycotic abortions (BMAs) take place mostly in the third trimester of pregnancy and are mostly sporadic. They are the major cause of mycotic abortions in some countries (Hugh-Jones and Austwick 1967). Bovines are most sensitive for mycotic abortion in comparison with ovines, camels, and caprines (Dehkordi et al. 2012).

6.2.1.1 Pathogen

One of the most common causes of mycotic reproductive failure, abortions due to *Aspergillus fumigatus*, has a tendency to occur in the second and third trimesters (McCausland et al. 1987). It is a storage fungus and grows well in conditions of relatively high moisture and temperature. Temperature rise, as well as extreme rain in some places, may promote the fungal growth, as this fungus proliferates in decomposing hay, badly preserved silage and soil, and produces a non-airborne, pathogenic spore (Cordes et al. 1964). The spores confine to the uterine caruncle and induce inflammation-induced abortion after 2–5 weeks of proliferation. The placenta often has swollen, necrotic cotyledons and the intercotyledonary membrane may be diffusely thickened, wrinkled, and leather-like. Occasionally, aborted fetuses have characteristic fungal plaques, 1–10 cm in diameter involving the skin around the eyelids, neck, dorsum, and thorax (Cordes and Shortridge 1968). This is due to fungal proliferation in the amnion, penetration of the epidermis, and the fetal inflammatory reaction, as well as hyperkeratosis. Mycotic abortion can be diagnosed by culture, and histologically from placental changes, with visible fungal hyphae confirming infection (Hill et al. 1971).

Mortierella wolfii is sporadically reported in Australian cattle and often reported in the North Island of New Zealand (Gabor 2003). The appearance of *M. wolfii* infection in pregnant cows is the same as *A. fumigatus*, except that abortion is followed by fatal pneumonia 4–5 days later in about 20% of cases (Cordes et al. 1972). Inoculation of spores occurs through inhalation from contaminated silage, and pass into arterial circulation via the pulmonary vascular bed (Curtis et al. 2017). Pathogenesis is also the same as *A. fumigatus* and, after hematogenous spread, fungal growth at the uterine caruncle causes widespread tissue necrosis and inflammation, leading to placentitis and abortion. In a nutshell, the placenta appears thickened, edematous, and necrotic, and other lesions appear consistent with mycotic abortion described above.

6.2.1.2 Symptoms

Symptoms comprise modifications in the placenta, which becomes thick and tough, may have puffy cotyledons and necrotic spots, with raised, hyperkeratotic plaques (Glover et al. 2011) on the fetus' skin, although the frequency of this symptom is doubtful. A tentative clinical diagnosis may be made on the appearance of the placenta and particularly the cotyledons, and also on the presence of skin lesions on the fetus.

6.2.1.3 Pathogenesis

The pathogenesis of mycotic abortions has significant implication for the analysis of the diagnostic results. Examining the fetus alone does not reveal the abortive agent in cattle, as the contact between the fetal and the maternal part of the cattle placenta is not contiguous but take places in contact organs called placentomes, composed of the fetal part (the cotyledon) and the maternal part (the caruncle), and, also, there is no direct contact between the fetal and maternal blood vessels. Thus, infectious agents that spread hematogenously do not do so by the maternal to fetal blood but through the placentome and cause infection. If the pathological process is too severe to lead to an early abortion, as happens in about 70% of cases, the agent will not diagnose in the fetal organs, but if the abortion is delayed, the microorganism will spread through the amniotic fluid first to the fetus' stomach (abomasum), then to the lungs, and, finally, to the other organs (Sheridan et al. 1985).

Laboratory diagnosis is based on the identification of the fungus by culturing the sample from the placenta and/or the fetus. Since the culture of *Aspergillus* spp. may be the result of contamination and can contradict the result, it is vital to assess tissue invasion by histopathology (Jensen et al. 1991). Contaminant overgrowth is another hindrance in the mycological identification of the fungus causing the abortion in a comparison between three diagnostic methods. Better conformity was found between immunofluorescence and histopathological or mycological identifi-

cation (kappa D 0.4 and 0.48, respectively). Jensen et al. (1993) also assessed the presence of galactomannan in the sera and urine of calves infected artificially and intravenously with *A. fumigatus* in cows with mycotic placentitis and abortion (confirmed by histopathology and culture), cows that aborted for other reasons, cows that did not abort but had other infections, and healthy cows at the slaughterhouse by inhibition enzyme-linked immunosorbent assay (ELISA). The results showed that the test was not specific enough to be considered a dependable means to diagnose mycotic abortions in cattle. Additionally, the clinical and microbiological diagnosis and possibilities of serological diagnosis of mycotic abortions in cattle was examined, but there is no considerable result in the diagnostic approaches (Corbel et al. 1973; Wiseman et al. 1984; Jensen et al. 1991, 1993)

6.3 Cryptococcosis

6.3.1 Disease Name

Cryptococcosis.

6.3.2 Syn. (synonym)

Torulosis, European blastomycosis, Busse–Buschke disease.

6.3.3 Pathogen

Cryptococcus neoformans.

6.3.4 Symptoms

Cryptococcosis is a chronic and often fatal infection in most of the cases, with no clear clinical pattern. In small animals, the central nervous system is often infected. In cattle, there may be mastitis, pneumonia, encephalitis, and cryptococcal meningitis. On the other hand, cattle are likely to develop cryptococcal mastitis as a result of ascending infection of the mammary gland via the teat canal. Affected cows have anorexia, reduced milk flow, swelling and hardness of affected quarters, and enlarged supramammary lymph nodes. The milk becomes viscid, mucoid, and gray-white, or it may be watery with flakes. The clinical signs included multifocal

neurological deficits manifested by hypermetria, ataxia, depression, circling, impaired vision, head pressing, low head carriage, wide-based stance, and falling to the side or backwards. Diagnosis is made by demonstrating capsulated yeast cells in tissues, spinal fluid, pus, or other exudate and confirmed by culture of the pathogen. No reliable treatment is known.

6.3.5 Cryptococcosis in Bovines

Cryptococcus infections were mostly reported in association with mastitis. Klein (1901) was the first to isolate a yeast from a case of mastitis and reported it to be identical to strains of *C. neoformans* of human and plant origin. Almost 50 years later, *C. neoformans* was diagnosed as the causative agent of severe outbreaks of mastitis in cattle (Carter and Young 1950; Stuart 1951; Emmons 1952; Hulse 1952). Pounden et al. (1952) studied the clinical aspects of an outbreak, in which they found 106 cows affected in a herd of 235 cows. They observed that *Cryptococcus* has been isolated from samples without any visible change either in the gland or milk, and the cases with visible signs varied from mild and brief swelling of one or more quarters of the udder to severe swelling of the affected glands. Cryptococcal mastitis was also detected in sporadic cases by El-Ghany et al. (1978), Rahman et al. (1983), and Moawad (1991). On the other hand, Rippon (1982) emphasized the worldwide presence of cryptococcal mastitis in dairy cows. Among the cryptococci, *C. neoformans* is a common pathogen, but other cryptococcal species express putative virulence factors, such as polysaccharide capsule and melanization (Petter et al. 2001). As the temperature is increasing day by day, acquisition of thermotolerance by other species in the post-industrial temperature rise era may increase their potential to become pathogenic. One such case can be attributed to *C. laurentii*, which normally does not grow at 37 °C, but thermotolerant strains have increasingly been associated with disease in extremely immunosuppressed hosts (Khawcharoenporn et al. 2007) and it may also affect the prevalence of cryptococcosis in bovines.

6.3.6 Pathogen

In cows, *C. neoformans* was the most commonly recorded species (El-Far et al. 1987; Saleh 2005; Abou-Elmagd et al. 2011). *Cryptococcus neoformans* as a causative agent for mastitis in buffaloes was found by Jand and Dhillon (1975) and Pal and Mehrotra (1983). Other species, such as *C. laurentii*, *C. flavus*, *C. lactativorus*, *C. albidus*, *C. luteolus*, *C. uniguttulatus*, and *C. terreus*, were also reported in several studies (El-Far et al. 1987; Costa et al. 1993; Klimaite et al. 2003; Türkyılmaz and Kaynarca 2010; Wawron et al. 2010; Fadda et al. 2013; Zhou et al. 2013; Akodouch et al. 2014).

Table 6.1 Cryptococcosis in bovines (Refai et al. 2017)

Species	References
Cryptococcal mastitis	
C. neoformans	Carter and Young (1950); Stuart (1951); Emmons (1952); Hulse (1952); Pounden et al. (1952); Innes et al. (1952); Simon et al. (1953); Redaelli (1957); Menhnert et al. (1964); Galli (1965); Monga and Kalra (1971); Jand and Dhillon (1975); Sipka and Petrović (1975); El-Ghany et al. (1978); Rippon (1982); Rahman et al. (1983); Pal and Mehrotra (1983); El-Far et al. (1987); Kotb (1990); Moawad (1991); Pal (1991); Pengov (2002); Moshref (2004); Saleh (2005); Asfour et al. (2009); Türkyılmaz and Kaynarca (2010); Abou-Elmagd et al. (2011); Saleh et al. (2011); Sharma et al. (2012); Hassan et al. (2013); Al-Ameed (2013)
C. albidus	El-Far et al. (1987); Costa et al. (1993)
C. laurentii	Wawron et al. (2010); Costa et al. (1993); Klimaite et al. (2003); Türkyılmaz and Kaynarca (2010); Zhou et al. (2013)
C. flavus	Costa et al. (1993)
C. luteolus	Costa et al. (1993)
C. uniguttulatus	Zhou et al. (2013)
C. lactativorus	Al-Khalidi et al. (2012)
C. terreus	Akodouch et al. (2014)
Cryptococcus species	Jand and Dhillon (1975); Kirk and Bartlett (1986); Costa et al. (1993)
Cryptococcal pneumonia	
C. neoformans	Hassan et al. (2013); Akodouch et al. (2014)
C. laurentii	Pal (2007)
Systemic cryptococcosis	
C. neoformans	Riet-Correa et al. (2011)
Cryptococcus species	Akange et al. (2013)

Cryptococcal pneumonia was occasionally reported in bovines (Pal 2007; Hassan et al. 2013). Bovine systemic cryptococcosis was also rarely diagnosed. Only two reports indicate its presence (Riet-Correa et al. 2011; Akange et al. 2013). Similar findings were observed in the case of abortion due to *Cryptococcus* species (Riet-Correa et al. 2011; Akange et al. 2013) (Table 6.1).

6.3.7 Cattle

Cryptococcus neoformans was first isolated from peach juice in 1894 in San Felice in Italy. Madsen (1942) isolated a *Cryptococcus* sp. from a cow in New York. The most impressive outbreak of bovine cryptococcosis during recent years, however,

was of cryptococcal mastitis described in detail by Pounden et al. (1952), Innes et al. (1952), and Emmons (1952), in which, over the course of 12 months, 106 of 235 cows of a Holstein Friesian herd in Maryland became infected. Fifty-four of the infected animals developed a visible abnormality of the udder or milk and, in severe cases, the udder was greatly enlarged. A similar outbreak involving 50 of a herd of 280 cows was recorded by Simon et al. (1953). Hammer and Engler (1956) recorded *C. neoformans* as the cause of ulcerative endocarditis in a bull.

6.4 Coccidioidomycosis

6.4.1 Syn

Coccidioidal granuloma, oidiomycosis.

6.4.2 Pathogen

Coccidioides immitis. (A partial list of synonyms is given by Conant et al. 1954).

It has been more than a century since coccidioidomycosis was first recognized as a serious disease, and its etiology and epidemiology have been well documented. However, the disease remains an enigma to many, and it often goes undiagnosed, even in endemic areas. As management of this chronic disease remains problematic, new preventive or therapeutic options are needed.

6.4.3 Etiology and Epidemiology

Coccidioidomycosis is a fungal disease found only in the Western Hemisphere. It is caused by two nearly identical species, *C. immitis* and *C. posadasii*, generically referred to as the "Californian" and "non-Californian" species, respectively (Fisher et al. 2002). The fungus grows in a mycelial phase in the soil within a geographically delineated area of the United States known as the Lower Sonoran Life Zone(Maddy 1957). This semi-arid zone encompasses the southern parts of Texas, Arizona, New Mexico, and much of central and southern California. *Coccidioides immitis* is sensitive to climate variability and responds to changes in temperature and precipitation. Previous studies have found a relationship between climatic conditions like temperature, precipitation, humidity, wind, and occurrence of dust storms with growth of *C. immitis* or the distribution of the arthrospores.

Mycelial phase—the growth form in the soil, composed of filamentous hyphae and reproductive spores called arthroconidia.
Arthroconidia—reproductive spores, highly resistant to desication, which are the infectious particles inhaled by man and animals.
Spherules—the parasitic phase of this dimorphic fungus; spherules are round cells of 30–100 μM or more than reproducing the progeny endospores.
Endospores—the progeny units of the parasitic phase, derived from spherules.

6.4.4 Cattle

Infection in cattle was first recorded by Giltner (1918) in a cow from the San Joaquin Valley, California and, since then, more than 3000 cases have been reported in cattle from the same state (Maddy 1954). In cattle, the infection is usually benign. Typically, there is a granulomatous involvement of the bronchial and mediastinal lymph nodes. More rarely, the lungs are attacked (Davis et al. 1937).

6.5 Rhinosporidiosis

6.5.1 Pathogen

The disease is caused by *Rhinosporidium seeberi*, an organism that was earlier known as a fungus but has been regrouped into the class Mesomycetozoa (family Rhinosporideacae). This class consists of several parasitic and saprophytic organisms, most of which infect fish and amphibians; only *R. seeberi* infects mammals (Adl et al. 2005).

Rhinosporidiosis is a non-infectious, sporadic, benign, generally non-fatal, and chronic granulomatous disease of humans and animals (Pal 2007). It is characterized by the development of nasal polyps. Diagnosis is confirmed by finding many large sporangia in the diseased tissues. The standard treatment is surgical excision of the growths.

6.5.2 Geographical Distribution

It is endemic in India (Rao and Narayan 1938) and sporadic in South Africa (Zschokke 1913; Quinlan and De Kock 1926), Uruguay (Saunders 1948), Argentina (Prieto and Pires 1944), the USA (Robinson 1951), and Australia (Albiston and Gorrie 1935).

6.5.3 Symptomology

In animals, the site attacked is the mucous membrane of the nose and the infection is unilateral. According to Rao and Narayan (1938), in cattle and horses, the tumors are rarely larger than 2–3 cm in diameter and may be either pedunculated or sessile. They are lobulated, soft, and pink in color, bleed readily, and are dotted with small white specks (the sporangia). In man, the nasal polyps are similar but often larger, and other sites, including the pharynx, larynx, eye, ear, skin, vagina, and rectum, may be infected.

6.6 Mucormycosis

Species of *Mucor*, *Absidia*, and *Rhizopus*.

6.6.1 Disease Name

Mucormycosis, including mycotic ulceration.

6.6.2 Pathogen

Absidia corymbifera (Cohn) Sacco (Vink 1941). Syn. *A. lichtheimi* (Christiansen 1922). *Mucor javanicus* (Porges et al. 1935). *Mucor pusillus* (Tscherniak 1928). *Mucor racemosus* (Frank 1890). *Mucor rhizopodiformis* (Christiansen 1922).

Host: Cattle (Gleiser 1953).

6.6.3 Geographical Distribution: Europe and North America

Although the pathogenicity of mucoraceous fungi for experimental animals has been known since the work of Lichtheim (1884), spontaneous mucormycosis in animals has rarely been reported, with there being only some 40 contributions appearing in the literature. The early accounts are concerned with the disease in man (Paltauf 1885) and are of limited value owing to their lack of adequate descriptions, so it is impossible to be certain whether the fungi concerned were, in fact, mucoraceous at all. To some extent, the characteristics of the lesions described are a guide, but isolation of fungi was not often accomplished. When the species was identified, it invariably proved to be non-pathogenic, e.g., *Mucor racemosus* (Frank 1890). For

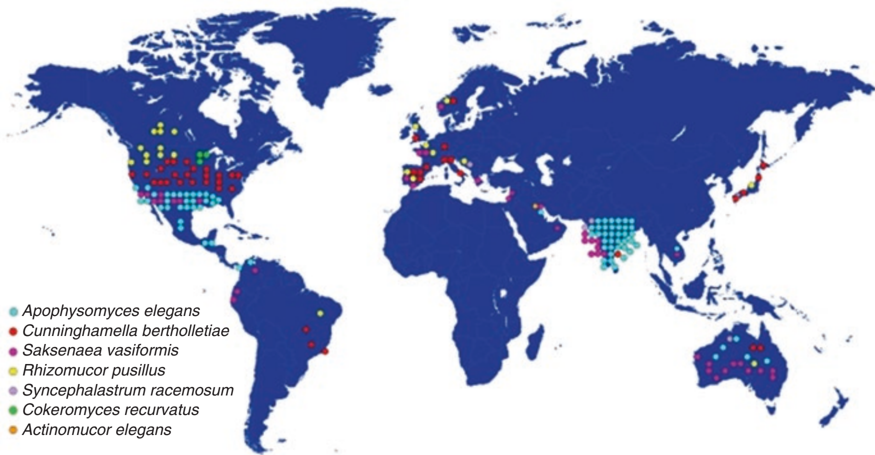

Fig. 6.2 Geographical distribution of reported cases of unusual mucormycosis (Gomes et al. 2011)

this reason, no reliable account of mucormycosis in animals has been located before that of Christiansen (1922), who described two cases in pigs. Subsequently, the disease has been defined in several domestic animals and differentiated from other granulomatous and ulcerative diseases. The diseases are now recognized as having regular occurrence in the Northern Hemisphere, whereas *R. pusillus* (formerly known as *Mucor pusillus*) is thermophilic and, thus, grows in high-temperature environments. To understand the impact of climate change on mucormycosis, comprehensive epidemiological surveillance, better methods of detection, and multidisciplinary collaboration will be required (Fig. 6.2).

6.6.4 Symptomatology

Mucormycosis is chiefly known as a granulomatous disease generally involving the lymph nodes. As it rarely produces any signs until either the granulomata become visible as protuberances or the animal wastes away, most of the reported cases in pigs and cattle have been discovered during a routine inspection in abattoirs.

6.6.5 Cattle

Gleiser (1953) first mentioned the occurrence of caseocalcareous lesions in the bronchial and mediastinal lymph nodes of a yearling heifer. Davis et al. (1955) also found similar lesions in 11 cattle during meat inspection. The most frequent lesions

were in the mesenteric lymph nodes and lungs and consisted of a greenish-yellow caseocalcareous mass with a variable amount of scar formation. None of the animals had shown any apparent symptoms of the disease. Abomasal ulceration has been the more frequently reported form of bovine mucormycosis and most likely accounted for a proportion of the early literature on peptic ulcers in animals. Sanchez Botija (1951) first found mucoraceous hyphae in a single ulcer 5–6 cm in diameter in an ox, whilst Gentles and O'sullivan (1957) summarized seven acute cases in 47 young calves, stressing that these represented every case of abomasal ulceration seen in 208 consecutive post-mortem examinations of calves. All except one of the calves died following the onset of severe scouring and one to several ulcers up to 4 cm in diameter were found on each occasion. The early lesions were small, raised, inflamed foci which developed into ulcers with raised hemorrhagic margins and gray, depressed, necrotic central areas.

6.7 The Ringworm Fungi

Dermatophytes infecting cattle.

6.7.1 Pathogen

Trichophyton asteroides: cattle (Davidson et al. 1934; Muende and Webb 1937; Gentles and O'sullivan 1957). *Microsporum* and *Epidermophyton* (Gudding and Lund 1995). *Trichophyton verrucosum*: cattle (Lebasque 1933; Blank 1955, El-Diasty et al. 2013).

6.7.2 Pathogenicity

Ringworm is a highly infectious skin disease of cattle all over the world. It affects the keratinized structures of the skin and hair of animals superficially. The causative agent of this disease is a faction of keratinophilic filamentous fungi called dermatophytes in the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* (Gudding and Lund 1995). *Trichophyton verrucosum* is the most observed etiologic agent of cattle (Akbarmehr 2011; El-Diasty et al. 2013). Infection is caused mainly by contact with arthrospores (asexual spores formed in the parasitic stage) or conidia (sexual or asexual spores formed in the normal environmental stage). Transmission between hosts usually occurs by direct contact with a host (Murray et al. 2005). It was observed that housing animals in close proximity to the presence of infected debris was responsible for the high occurrence of the disease in winter (Al-Ani et al. 2002). However, it appears that the disease is more common in tropical than

temperate climates, and particularly in areas having tropical or subtropical climatic conditions (Radostits and Done 2007). Dermatophytosis is mainly observed in young calves, whose high skin pH and ill-developed immune system are more sensitive to the infection (Radostits et al. 1997).

6.7.3 Symptoms

The characteristic lesion symptoms include patches of hair loss (10–50 mm), desquamation, and crust formation usually confined to the head, dewlap, and sometimes other parts of the body surface. In a study by El-Diasty et al. (2013), they collected skin scrapings and hair samples from beef calves and observed clinical signs of dermatophytosis. The collected samples were examined for fungal elements by microscopy and fungal culture preparation. Fungal culture revealed *T. verrucosum*. A second study was conducted by El-Ashmawy et al. (2015) during September 2013 till April 2014 (autumn and winter months) in a farm containing 250 beef calves (local mixed breeds) aged 6–9 months. Clinical signs of skin lesions appeared in 30 animals after 2 weeks in the form of circumscribed areas of hair loss filled with puffy white scales all over the body. All examined samples were found to be positive for fungal elements (ectothrix spores and endothrix hyphae) by microscopic examination. The fungus grew slowly on SDA, producing white, cottony, and heaped colonies with some submerged growth and yellow pigmentation. Microscopic examination after staining with lactophenol cotton blue revealed septate hyphae with numerous clavate microconidia borne across the hyphae and many chlamydospores arranged in chains (chains of pearls) characteristic of *T. verrucosum*.

The disease causes great economic loss to animal products (wool, meat, etc.) due to skin injuries and many deformities, as reported by Weber (2000). Dermatophytosis has also been considered the most common zoonosis worldwide (Achterman and White 2012; Adeleke et al. 2008).

6.8 Antifungal Immunotherapy: Vaccines

Given their increasing frequency and unacceptably high morbidity and mortality rates, the prevention of invasive fungal infections has become of vital importance (Spellberg 2011; Medici and Del Poeta 2015). Vaccination of high-risk groups is a particularly promising strategy to check invasive fungal infections because easily identifiable risk factors are clearly defined from any such infections (Perlroth et al. 2007; Spellberg 2011). Advances in our understanding of the host defense and pathogenic mechanisms underlying fungal infections have supported the development of effective vaccines to combat these diseases. Invasive fungal infections can be some of the trickiest medical problems to treat. A dearth of antifungal medications is one reason for this, says Edwards: “There just aren’t a lot of drug options

for preventing or treating fungal disease. We have vaccines for parasites, viruses, bacteria but no fungal vaccines." Recently, the feasibility of vaccination of mice with crude antigen preparations from an *Aspergillus* strain, *A. fumigatus*, has been demonstrated in animals that were, afterwards, immunocompromised (Cenci et al. 2000; Ito and Lyons 2002). In these studies, vaccination was found to be effective for survival against both inhaled as well as intravenously administered fungi. Researchers have dedicated studies towards developing robust, durable, and safe fungal vaccines. For most active vaccines studied against invasive fungal infections, the key to protection has been the induction of cell-mediated, proinflammatory, Th1 or Th17 responses, which improve phagocytic killing of the fungus. It is also clear that antigens targeted for vaccination need not be restricted to virulence factors but also focus on niche vaccination of patients in restricted geographical areas. The lack of complete understanding of the market potential for such vaccines and the requisite capital to develop these vaccines are also a matter of concern in this area (Spellberg 2011).

6.9 Future Prospects

Economically sustainable therapeutic techniques and improved diagnostic methods can significantly improve the prognosis of cattle mycotic diseases. Reports of adapting modifications of human kits such as that aimed at galactomannan detection to animals, especially pets, are scarce and should be the matter of more detailed assessment for cattle aspergillosis for clear use recommendations. The role of imaging techniques has also been found to be often unreliable and should be investigated further. While great advances in antifungal therapy have been made in the last several years, cost-effectiveness is still a major hindrance, as the price of the newer drugs is unaffordable for animal use, especially considering the necessity of extended, possibly life-long, treatments. This has restricted their use to a very low number of cases, mostly for scientific researches. As the research in this field is ongoing, the price of these drugs will decrease in the future, but the development of specific antifungal drugs for veterinary use should be promoted in order to achieve the required level in treatment. The discovery of drug-resistant aspergillosis in patients who have never previously taken antifungal medications suggests that drug resistance in some cases could be coming from the environment and not from the previous use of antifungals. Resistance acquired through the environment needs special attention from researchers, dairy owners, and policymakers, as it can pose a new challenge to antifungal therapy and treatment in bovines.

The conventional identification of pathogenic fungi in the clinical microbiology laboratory is not appropriate, as it is based on morphological and physiological tests, which often require three or more days and may be inaccurate. The early, quick, and accurate detection of the pathogenic fungus is critical for timely, suitable dairy management. In recent years, a multiplex polymerase chain reaction (PCR) method was developed to simultaneously identify multiple fungal pathogens in a

single reaction (Luo and Mitchell 2002, which can prove to be a very useful tool for timely detection and, so, curing the animal. It is also the need of the hour to relate weather-based plant disease forecasts to recent climate change models, and, hence, predict the effects of climate change on where, which, and by how much mycotoxins will be changed.

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Chapter 7
Collateral Development of Invasive Pulmonary Aspergillosis (IPA) in Chronic Obstructive Pulmonary Disease (COPD) Patients

Saurabh Kumar and Suchit Swaroop

7.1 Introduction

Aspergillus fungi of class Eurotiomycetes is the most frequently occurring fungi among this class. They are the inhabitants of humid soil. Amongst 250 species reported till date, *Aspergillus fumigatus* causes 90% of human infections. Although *A. fumigatus* is the chief infectious pathogen of human, other examples include *A. lentulus, A. fumigatiaffinis, A. fumisynnematus* and *A. felis* (Balajee and Marr 2006; Barrs et al. 2013). Its pathogenicity lies in the high sporulating capacity, and due to this very reason, the spore concentration ranging in the air increases from 1 to 100 (Streifel et al. 1983; Latgé 2001). These spores later release conidia in the air which are capable enough to reach the alveoli due to its minute diameter of 2–3 mm. The air laden with these spores when inhaled majorly affects lung. With these disease-causing properties of *Aspergillus*, it was found that it may result in severe pathological conditions like chronic obstructive pulmonary disease (COPD), allergic bronchopulmonary aspergillosis and aspergilloma (McNeil et al. 2001). The current chapter deals with the COPD and its association with *Aspergillus* infection.

According to the WHO and the National Heart, Lung, and Blood Institute, COPD is defined as a "disease state characterised by airflow limitation that is not fully reversible". Similarly, a disease called IPA is indicated by the lung parenchyma invasion and necrosis due to *Aspergillus* spp. It is further divided into two classes on the basis of invasion: subacute IPA representing the direct invasion of lung parenchyma by hyphal elements but with a slowly progressively COPD cavitary lung disease and chronic pulmonary aspergillosis which shows radiological signs of tissue destruction, but without evidence of hyphal invasion, with microbiological markers of *Aspergillus* infection. The term tracheobronchitis is used when *Aspergillus*

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organisms invade the tracheobronchial tree. Recent data are obtained through studies performed by autopsy, and an exponential increase has been found over the last 20 years (Groll et al. 1996; Yamazaki et al. 1999; Kontoyiannis and Bodey 2002). To keep this in check, the medications include immunosuppressive and corticosteroid therapies. Due to the adverse effect of these drugs, mortality rate has been increased further (Warnock et al. 2001; Pegues et al. 2001). Later a few clinical studies have shown the association of IPA with that of COPD. Due to the IPA association, doctors prefer steroids above other treatments available. This treatment even worsens the condition of the patient (Rello et al. 1998; Bulpa et al. 2001; Dimopoulos et al. 2003; Meersseman et al. 2004; Ader et al. 2005; Garnacho-Montero et al. 2005). With the colonization of *Aspergillus* spp. in lung airways, possibility of IPA in COPD increases, and thus (Pepys et al. 1959; Yu et al. 1986) it becomes critical to detect it at an early stage. It is specific in severe (Global Initiative for Chronic Obstructive Lung Disease (GOLD)) steroid-dependent stage IV COPD patients. To come up with the better therapeutic targets for any of the disease, we need to focus firstly on the epidemiology of the disease along with the pathophysiology and symptoms commonly occurring in that disease. The underneath topics will deal with the epidemiology, pathophysiology and symptoms associated with the disease in detail.

7.2 Epidemiology

Although the occurrence of COPD in IPA patients is very less and poorly documented, it has been shown through various demographic data that patients with COPD are at greater risk of developing IPA. Assessment of the incidence of IPA in

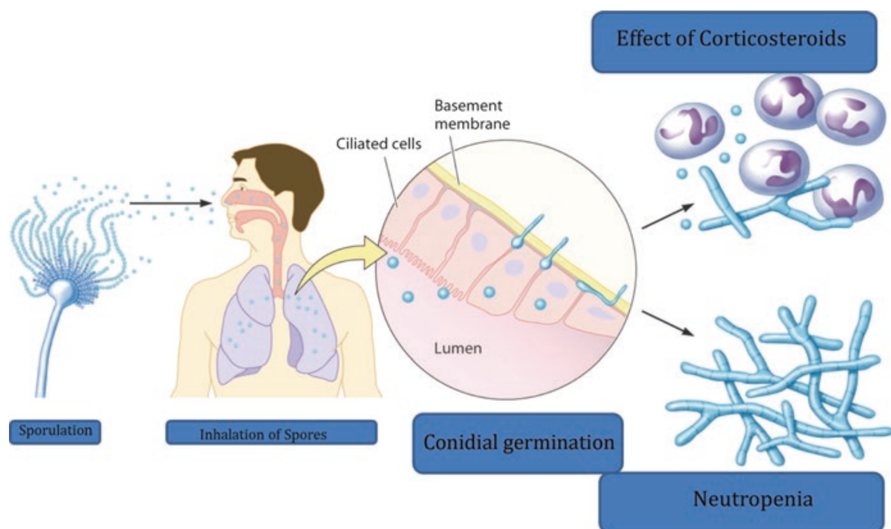

Fig. 7.1 The respective figure shows the pathophysiology and steps of spore invasion in human respiratory tract (Dagenais and Keller 2009)

the population is difficult due to the lack of a consistent case, lack of a particular definition and lack of infection surveillance measures. Above all, the next problem lies in distinguishing the colonization of *Aspergillus* spp. with that of IPA, particularly at an early stage. However, there is growing evidence of data suggesting that COPD patients are at a greater risk of IPA. A review by Lin et al. (2001) of 50 patients shows that COPD was the underlying condition in 26 out of 1,941 (1.3%) patients with aspergillosis. In one large study conducted by Patterson et al. (2000a), 9% of 595 patients with invasive aspergillosis (IA) suffered from pulmonary disease, without a distinct pulmonary disorder. A study performed by Rodrigues et al. (1992) reported that COPD patients contribute to 1% of all cases of IPA in their institution. Steroidal drugs are shown to play a pivotal role in the emergence of IPA. Some authors have also investigated the correlation between the daily dose of corticosteroids and the probability of developing IA. It was suggested that the average dose of 1.25 mg/kg-1/day-1 of prednisone in renal transplant patients is the best method to develop a model for IPA (Gustafson et al. 1983). But the case was a bit different in the haematological patients treated with peripheral blood or bone marrow transplantation. In them, a dosage of 1 mg/kg-1/day-1 of prednisone for 21 days was enough to develop IPA (Grow et al. 2002). On summarising the data it was found that a dosage of 0.2 mg of prednisone per day or a cumulative dosage of 0.7 mg was associated with an increased risk of infections (Stuck et al. 1989). Various data provided by different authors support the patients undergoing corticotherapy for a longer duration of time are at a higher risk of developing COPD with IPA (Rello et al. 1998; Bulpa et al. 2001; Ader et al. 2005; Muquim et al. 2005). Despite the close association between steroids and IPA in COPD patients, it has been reported that some COPD patients may develop IPA without steroid exposure (Ali et al. 2003). Moreover, it has been reported that some infections, in particular viral infection, such as influenza (Urban et al. 1985; Bulpa et al. 1995) or cytomegalovirus (Warnock et al. 2001), may precede IPA, suggesting a role in causation (Ordronneau et al. 1987). Later, we will discuss the pathophysiology of the disease.

7.3 Pathophysiology

Aspergillus spp. is an airborne pathogen with a spore size of 2–3 mm in diameter. It is widely spread in the air, and when a person inhales spore-containing air, these penetrate through the airways to the lungs. Although the majority of conidia are excluded from the lungs by the ciliary action of the bronchial epithelium, some make their way through the respiratory tract to the lungs, hence leading to pathological conditions. In COPD patients, ciliary activity is impaired by tobacco smoke and multiple episodes of infection, as well as repeated epithelial damage. Thus, the impairment in defence mechanisms of the airways facilitates the binding of conidia to the epithelial layer (Latgé 2001). Stages of *Aspergillus* infection are depicted in the diagram below:

The phagocytic system is the next one in line to be affected (Shoham and Levitz 2005). It comprises the alveolar macrophages (AM) that play a prominent role in

destroying the *A. fumigatus* conidia and the neutrophils that kill hyphae and germinating spores (Schaffner et al. 1982; Levitz 1992; Roilides et al. 1993; Balloy et al. 2005). Other immune cells such as natural killer lymphocytes (Morrison et al. 2003) and the innate pulmonary host defences also play a role in protecting individuals against IPA (Walsh et al. 2005; Feldmesser 2005) and thus the COPD.

7.4 Prevention of Infection

Due to difficulty in diagnosis associated with IPA, mortality rate is still high (up to 90%, depending on the underlying disease), thus making prevention of infection very important (Warnock et al. 2001). The avoidance of infections may be taken in the following ways:

- To eliminate obvious environmental sources of *Aspergillus* spp.
- Cleaning the surfaces to avoid dust accumulation. Moreover to prevent outbreaks, environmental protection by enriching solid barriers must be applied during construction (Patterson et al. 2000a, b).
- Environmental protection by impenetrable barriers must be implemented during construction (Oren et al. 2001).

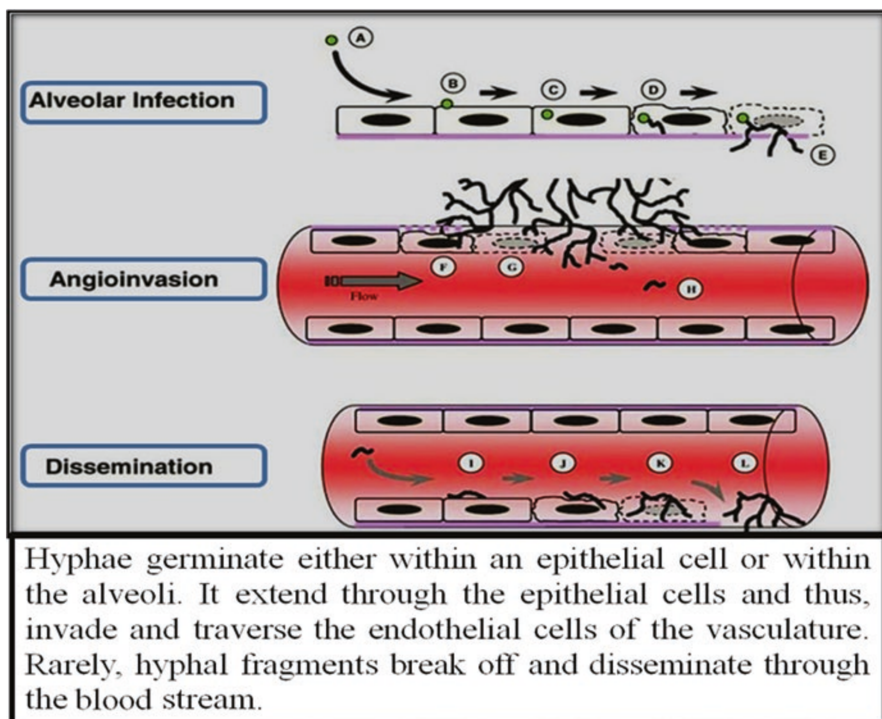

Fig. 7.2 Steps of germination and dissemination of fungal hyphae (Ben-Ami et al 2010)

- COPD patients should wear a high-efficiency mask or avoid exposure.
- Prevention must be focused on the avoidance of high load sources, e.g., constructions, cellars or lofts, composts, etc.
- IPA could also be a water-borne infection (Warris and Verweij 2005), and pillows may contain high loads of *Aspergillus* (Woodcock et al. 2006). Finally, if the patient is receiving corticosteroids, the dose should be decreased as much as possible or even stopped altogether.
- The dose of corticosteroids should be kept in check or should be decreased or stopped if possible.

7.5 Prognosis

Therapy of COPD with a fungal infection includes voriconazole. It was considered to be the first-line treatment of the disease (Caillot et al. 2001). The study was conducted on the guinea pigs with a dosage of 10 mg/kg/day. To turn our target to a better treatment option, the focus was made on amphotericin B at 1 and 1.25 mg/kg/day, respectively (Maertens et al. 2002; Montoya et al. 2003). Another study concluded that the efficacy of high-dose liposomal amphotericin B (at 5 and 10 mg/kg) was superior to that of voriconazole (10 mg/kg) in a mouse model of pulmonary aspergillosis (Comstock et al. 1974). Apart from these two drugs with distinct doses, it became necessary to design a clinical trial with a better formulation.

Due to economic constraints, voriconazole is still the prescribed treatment than amphotericin. Properties of the drug are as follows:

- Synthetic triazole.
- Exerts an inhibitory effect on two kinds of *Aspergillus* by cytochrome P450-dependent enzymes and the fungal respiration chain.
- Triazoles are generally considered as fungistatic.
- The drugs given in association with voriconazole concentrations are erythromycin, indinavir, ranitidine, cimetidine and omeprazole. Drugs that potentially decrease voriconazole concentrations are principally rifampicin and phenytoin.

7.6 Symptoms

Symptoms of the disease are not clearly distinguishable as pulmonary disease shows some common features, but some of the key features include:

- Fever and chills
- A cough that brings up blood (hemoptysis)
- Shortness of breath
- Chest or joint pain
- Headaches or eye symptoms
- Skin lesions

7.7 Conclusion

The pulmonary diseases are attributed to airborne foreign particles. Spores of *Aspergillus* are found to be a newer target to cause a fatal disease like COPD. This chronic disease when not treated at an earlier stage may lead to the development of another asymptomatic condition called invasive pulmonary aspergillosis. The symptoms and diagnosis of these pathogenic conditions are less distinct, and hence it becomes a challenge to cure them at an earlier stage. Due to the continuous advancement of technology, we have achieved success at certain levels. Drugs like voriconazole have been developed which aid in the treatment of the disease. Thus from the above chapter we may conclude that the chronic pulmonary disease may arise due to fungal penetration in lungs.

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Chapter 8
Sporotrichosis: The Emerging Fungal Threats to Animals

Shiv Shanker Gautam, Navneet, and Neelesh Babu

8.1 Introduction

Fungi are the most important group of organisms on our planet. Without fungi, the terrestrial ecosystems cannot exist. By various means, fungi play very significant roles in recycling, plant growth, mycorrhizal associations, food sources, medicines, biocontrol, crop diseases and animal diseases. Pathogenic fungi are those species that occupy and receive nutrients from living organisms (Sharon and Shlezinger 2013). The animal diseases caused by fungi can be placed into two major categories: superficial and invasive mycoses (Casadevall and Pirofski 2003). If we look at the economic importance, fungal diseases take a heavy toll and cause massive economic losses worldwide (Fig. 8.1).

Sporotrichosis is a subcutaneous and very common fungal infection caused by dimorphic fungi *Sporothrix schenckii*. It is also known as rose gardener's disease. The disease may transmit by inoculation of agents on the skin or mucous membrane from the soil, infected plant material, organic matter contaminated from the causal organism, transmission through one animal to another, biting and scratching or, less frequently, inhalation of fungal propagules of the causal organism (Barros et al. 2011). Sporotrichosis has been reported in most domestic mammals, wild animals and humans also (Pereira et al. 2015). In mammals, it occurs by two major sources of infection, traumatic injury in cutaneous and subcutaneous tissues, through which the causal organism enters and causes local lesions (Roets et al. 2010). Sporotrichosis

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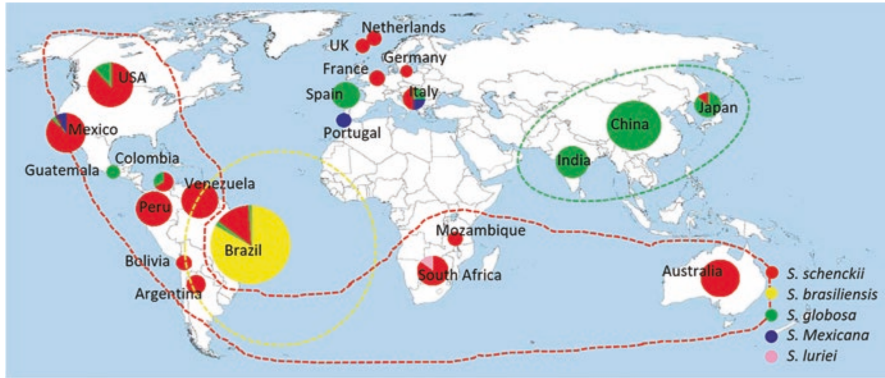

Fig. 8.1 Worldwide distribution of sporotrichosis. The graphics are proportional to the number of isolates identified by DNA sequencing. Main endemic areas indicated by dotted lines. (From Zhang et al. 2015)

shows three types of clinical infection in animals, namely, cutaneous lymphatic form, development of nodules at the site of infection and infection in subcutaneous tissues and lymphatic structures. Among these cutaneous forms is primarily localised infection with the development of nodules which further ulcerate (Reed et al. 1993). Furthermore, the infection could be systemic, i.e. infection could reach other parts of the body if the fungal propagules are inhaled and reach the respiratory tract (Barros et al. 2011).

8.2 Epidemiology

Sporothrix schenckii is distributed all over the world, mainly in the tropical and subtropical regions which have high humidity of 80–100% and mild temperature around 20–27 °C (Barros et al. 2004; Rippon 1982). This fungus lives in soil and on plant matter, such as sphagnum moss, rose bushes and hay (Barros et al. 2011; Chakrabarti et al. 2015). Basically, sporotrichosis is a chronic granulomatous lymphocutaneous infection affecting humans as well as animals, including cats, dogs, horses, camels, fowls, rats, mice, hamsters, chimpanzees, mules, donkeys, goats, cattle, as well as rats, foxes, dolphins and armadillos (Cafarchia et al. 2007; Davis and Worthington 1964; Barros et al. 2004; Jungerman and Schwartzman 1972; Schubach et al. 2006; Pereira et al. 2015). This disease was frequently reported in the United States, Japan, Mexico, China, Brazil, Uruguay, Peru, South Africa and Colombia. Among these, Brazil, China, India and South Africa are hyperendemic (Barros et al. 2004; Kauffman 1999; Zhou et al. 2006; Agarwal et al. 2008; Barros et al. 2011).

8.3 Etiological Agent and Taxonomy

The *Sporothrix* genus was identified by Smith, a mycologist, isolated by Schenck as "Sporotricha" and Haward confirmed its dimorphic form in 1961. Later, the fungus was named as *Sporothrix schenckii* and Guarro et al. (1999) classified the fungus under division Ascomycota, class Pyrenomycetes, order Ophistomatales, family Ophistomataceae.

8.3.1 Classification

Kingdom –Fungi
Division – Ascomycota
Class – Sordariomycetes
Order – Ophiostomatales
Family – Ophiostomataceae
Genus – *Sporothrix*
Species – *schenckii*

8.3.2 Morphology

Sporothrix schenckii is thermally dimorphic, with a hyphal or yeast form. The hyphal form is found in the environment on plants and decaying matter, while the yeast form is the result of transition into a host. The fungal cell wall is complex and rigid, chemically composed of polysaccharides and glycoprotein, which surrounds plasma membrane in both forms of fungi, mycelial as well as yeast form.

8.3.2.1 Hyphal Form

At 25 °C, *S. schenckii* assumes its hyphal form (Morris et al. 2003). Filaments are apparent and colonies moist, leathery to velvety, with fine wrinkled surface. The colour is white initially and may change over time to become cream to dark brown (Barros et al. 2011). Microscopically, hyphae are septate, with diameter approximately 1–2 μm. Conidia are oval in shape and hyaline (Fig. 8.2a).

8.3.2.2 Yeast Form

At 37 °C in laboratory conditions or in host tissue, *S. schenckii* assumes its yeast form (Fig. 8.2b). It appears as smooth white or off-white colonies. Microscopically, yeast cells are 2–6 μm long and have a cigar-shaped morphology (Barros et al.

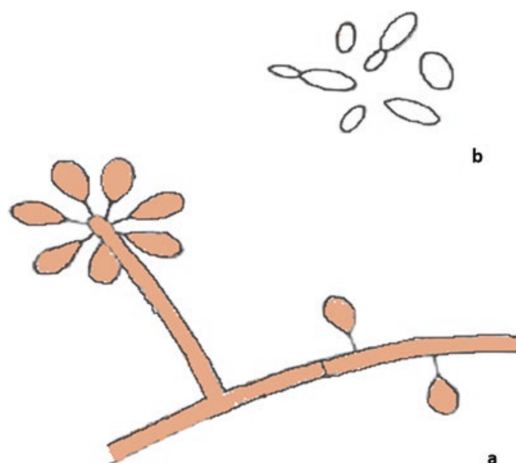

Fig. 8.2 *Sporothrix schenckii*: (**a**) hyphal form at 25 °C, (**b**) yeast form at 37 °C

2011). The genus *Sporothrix* has several other species, including *S. inflata*, *S. humicola*, *S. pallida*, *S. mexicana*, *S. chilensis, S. brasiliensis* and *S. globosa*, among which few of the environmental strains are opportunistic, rarely causing infection by traumatic inoculation (Costa et al. 2017).

8.3.3 Cultural Characteristics

On potato dextrose agar (PDA) medium, it is shown both smooth and wrinkled filamentous colonies, which, after a few days, turn brown-black from creamy white colour (Rippon 1988; Morris 2002). Because of its dimorphic characteristics, it can also be grown like yeast cells in several sizes, which may be oval to round, resembling a cigar. In Sabouraud dextrose agar (SDA) medium, its colonies are smooth, with brownish to cream colour (Larone 2002). Mould to yeast transition can be attained by its mycelia or conidia in enriched culture medium.

The dimorphic character is due to the involvement of protein kinases, known as being calcium/calmodulin complex dependent. The signalling pathway interaction between enzyme phospholipidase and G-protein is necessary to control the dimorphism in its nature (Morris 2002; Valle et al. 2007; Kikuchi et al. 2006).

8.4 Transmission

Sporotrichosis mostly occurs in cats, dogs, horses, cattle, goats, swine, mules, camels, primates, birds, rodents etc. As the propagules of causative fungi are easily available in the environment, it can be transmitted by accidental inoculation of its

propagules on the skin or in mucous membranes. Dead and decaying vegetables, soil, wood, splinters, thrones, hay, moss, insects and other animals are known to be an easy source of the pathogen (CFSPH 2013; CDC 2018). Previously, it was known as rose breeder's disease because agricultural workers are easily infected by the pathogen. Transmission of the pathogen from plants to animals is one of the most important features of the family Ophiostomatales. On the other hand, it is also transmitted from animals to humans as zoonotic transmission through deep scratches and bites from the infected animals, usually by cats. Cats play an important role in the transmission of the pathogen from cats to cats and cats to humans. Sporotrichosis caused by the transmission of the pathogen from cats is termed feline sporotrichosis, whereas if the same infection is caused by dogs, it is termed canine sporotrichosis.

There are several case studies regarding feline transmission because cats are more likely to be close to humans and can easily acquire infection, either directly from the source or through other animals, such as the hunting of the mice which were previously infected from the pathogen. It then becomes the vector for transmission to humans and other animals (cats, dogs etc.) associated with them. Finally, the pathogen can be returned to the atmosphere through faecal matter of the infected animal and forming a cyclic transmission (Rodrigues et al. 2016).

8.5 Pathogenesis

The human infections of *Sporothrix* are associated with scratches by infected animals. The incubation period of the fungus after the infection depends upon the size of the inoculum for the cutaneous infection. On average, it is up to 3 weeks as per previous reports, whereas for respiratory tract infection, it is uncertain (Bonifaz and Tirado-Sanchez 2017). In humans, the severity of the infection depends upon the immune response of the individual. Patients with acquired immunodeficiency syndrome (AIDS) are more prone to the infection. Several other factors are also included, such as diabetes, chronic alcoholism, malignancy etc. (Aronson 1992; Addy 1992). Patients who undergo transplantation surgery, pregnant women and malnourished individuals are also reported (Bonifaz and Vazquez-Gonzalez 2010; Barros et al. 2011; Hernandez et al. 2014). After 2 or 3 weeks of infection, immune response is generated to control the infection. Immune response includes CD4+ T lymphocytes, along with macrophages, dendritic cells and neutrophils. Later, these develop a granulomatous reaction by their infiltration (Hassan et al. 2016). Pulmonary sporotrichosis results in an asymptomatic pneumonic disease, which later becomes systematic, depending upon the immunity of the person (Barros et al. 2011; Aung et al. 2015) (Fig. 8.3).

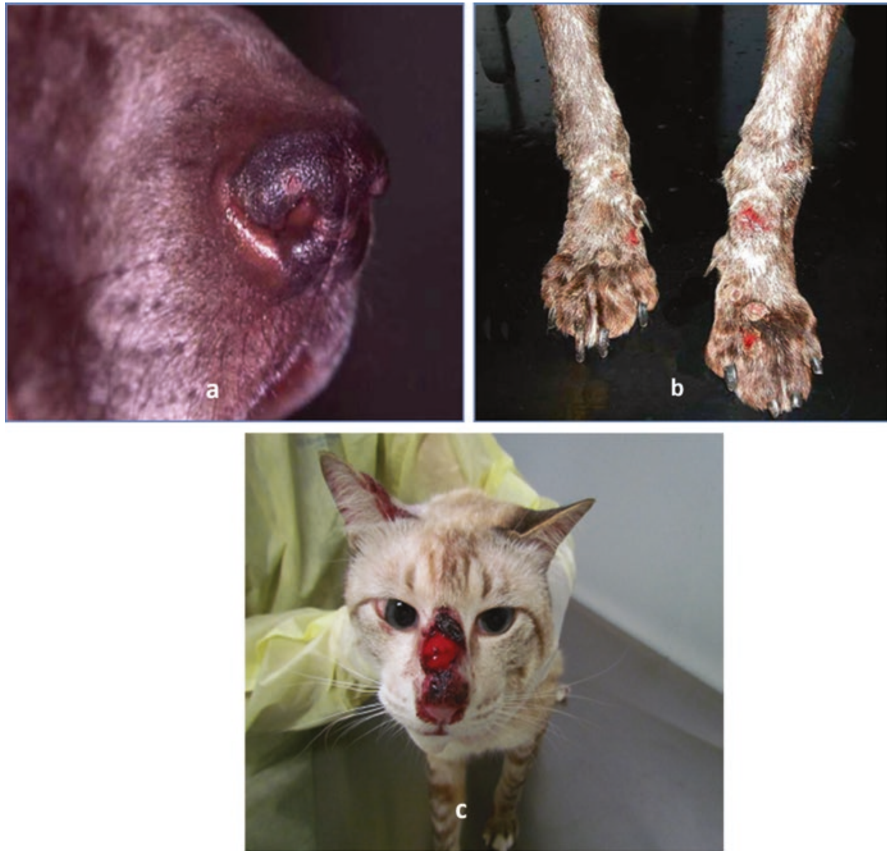

Fig. 8.3 The lymphocutaneous and nasal sporotrichosis: (**a**) erosive lesions on the nares, (**b**) multiple, circular, alopecic lesions on the legs. (From Cafarchia et al. 2007), (**c**) feline sporotrichosis. (From Gremiao et al. 2015)

collected and transferred into SDA or brain heart infusion (BHI) agar and placed at room temperature. Fungal colonies may appear as off-white or dark brown/black hyphal form. The microscopic observation can proceed for morphological study. The yeast form is grown on BHI medium at 37 °C. The serological methods may give effective results in case of humans and dogs (Cafarchia et al. 2007).

8.6.1 Sporotrichin Test

The sporotrichin is a peptide-rhamnomannan-based antigenic complex, relevant with respect to pathogenic fungi. It is primarily used for serological and cutaneous testing. The sporotrichin test is not accepted in some European countries due to the

8.6 Laboratory Diagnosis

The diagnosis of infection is based on cytological examination of exudates, cat sera and histological examination of biopsy specimens, followed by isolation of the fungal organism (Fig. 8.4) (Bonifaz et al. 2018). The animal biopsy sample is

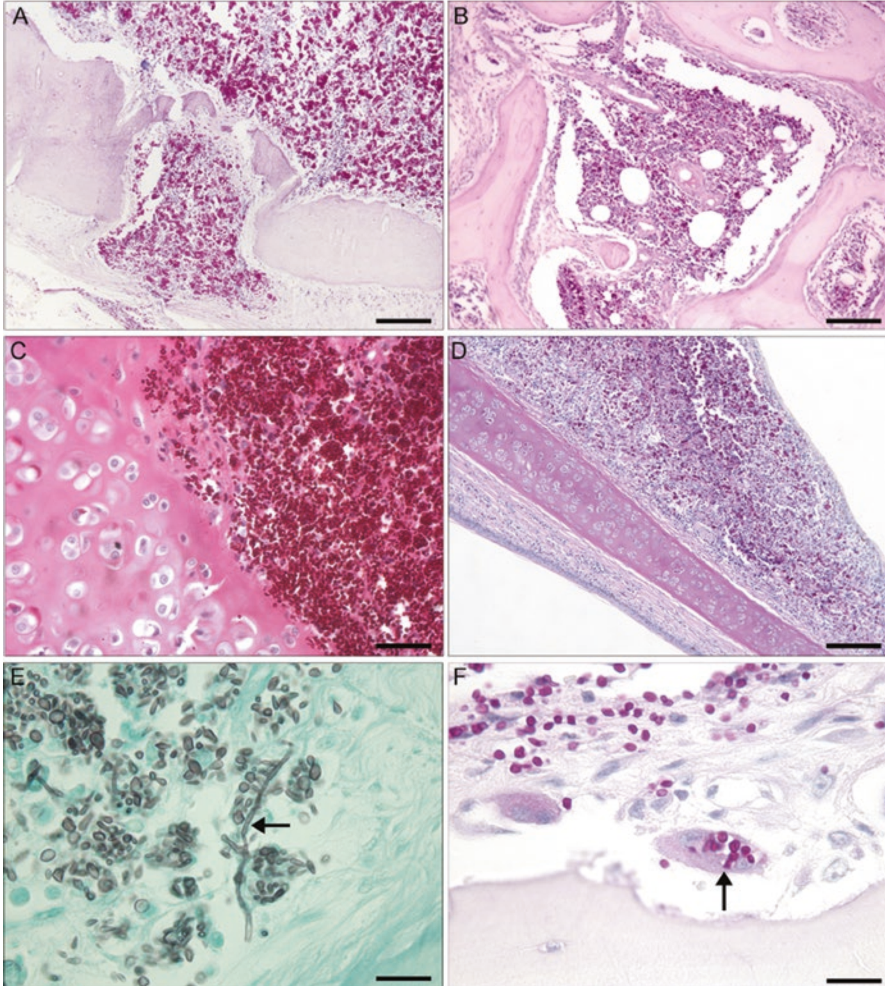

Fig. 8.4 Histology of the nose of a cat with sporotrichosis: (**a**) severe pyogranulomatous rhinitis showing lyses of osseous tissue and several yeast-like forms within macrophages, (**b**) severe pyogranulomatous osteomyelitis showing several yeast-like forms of *Sporothrix* within macrophages in the bone marrow, (**c**) mucosa of vestibule showing several yeast-like forms within macrophages or extracellular invasion and causing necrosis of hyaline cartilage, (**d**) severe pyogranulomatous rhinitis showing unilateral thickening of the mucosa of the vestibule and several yeast-like forms, (**e**) several cigar-shaped or round to oval yeast-like forms and a hypha (*arrow*) in the mucosa of the vestibule, (**f**) cat with no previous history of treatment for sporotrichosis. (From Gremiao et al. 2015)

lack of some standardisation, although some fungal fragments are used in this case (Bonifaz et al. 2018), since no commercial or standard antigen is available. Some recent studies have obtained favourable results for sporotrichin (>90% positive) and suggest that it is an excellent antigen for skin testing and epidemiological study (Ishizaki et al. 1976; Barros et al. 2011; Bonifaz et al. 2018).

8.6.2 Molecular Methods

Molecular diagnostic methods are highly sensitive, rapid and specific tests for the detection of sporotrichosis. To detect *S. schenckii* in clinical samples, ribosomal RNA (rRNA) and the internal transcribed spacer (ITS) gene have been used (Hu et al. 2003; Scheufen et al. 2015). Serum antibody reactivity for *S. schenckii* antigens and fractions of cell-wall peptide-rhamnomannan-based enzyme-linked immunosorbent assay (ELISA) detection showed high specificity and sensitivity for *S. schenckii* isolates (Almeida-Paes et al. 2007; Fernandes et al. 2011).

8.6.3 Immunodiffusion Test

Exoantigens are soluble immunogenic macromolecules produced by fungi early in their development. For the immunodiffusion test, exoantigens of *S. schenckii* is used. It is also a sensitive and accurate method of detection (Fernandes et al. 2011).

8.7 Treatment

For cutaneous infection, itraconazole or terbinafine as the primary treatment with fluconazole is recommended. When infection is severe and spread throughout the body, amphotericin B is prescribed. For complete removal of the infection, the course of treatment may last 3–6 months. Potassium iodine solution for preliminary treatment can be given orally for sporotrichosis. However, long doses may give rise to side effects, including gastrointestinal upset and thyroid imbalance.

The antifungal drugs have quite impressive results to overcome sporotrichosis. Despite the availability of antifungal drugs, the incidence of disease may occur due to drug resistance developed by fungal cells. Vettorato et al. (2018) reported a case study of a patient with lymphocutaneous sporotrichosis in the right upper limb and found high resistance to itraconazole (200 mg/day) and another six antifungals, with the exception of terbinafine. The drug resistance can be due to complex reasons, such as increased efflux of the drug, phenotypic alterations in the drug target site, genomic recombination etc., which minimise the toxic effect of the drug (Srinivasan et al. 2014).

The choice of plant-derived drugs may be an alternative approach against the antifungal drug resistance. Various plants extracts and essential oils have been reported with antifungal activity against *S. schenckii*, including *Origanum majorana* (Waller et al. 2016), *Curcuma longa* (Apisariyakul et al. 1995), *Agave lechuguilla*, *A. lophantha*, *A. picta*, *A. scabra* (Verastegui et al. 2008), *Artemisia ludoviciana*, *Heliopsis longipes*, *Satureja macrostema*, *Tagetes lucida* (Damian-Badillo et al. 2008), *Ophryosporus peruvianus*, *Senecio culcitioides*, *Croton ruizianus*, *Wigandia urens*, *Iryanthera lancifolia*, *Oenothera multicaulis*, *Cestrum auriculatum* (Rojas et al. 2003), *Lobelia pyramidalis* (Joshi et al. 2011), *Glycyrrhiza glabra* (Fatima et al. 2009), *Rosmarinus officinalis* (Luqman et al. 2007), *Psidium acutangulum* (Wen et al. 2011), *Cymbopogon flexuosus*, *C. martini*, *C. winterianus* (Saikia et al. 2001) and *Eucalyptus citriodora* (Akhtar et al. 2014). In the context of sporotrichosis, these natural plant-based drugs can overcome drug resistance and act as antifungal agents.

8.8 Conclusion and Future Prospect

Sporotrichosis is an endemic fungal disease, although it is occasionally seen in small pockets of the world. But in the context of the economy and related to the treatment of domestic animals, more harm is done. The symptoms of sporotrichosis appear late in this situation, and internal organs like the lungs and lymph nodes get infected. The skin may have developed lesions and is characterised by nodules and ulcers. Treatment is delayed in this context and, by then, the animal dies. Therefore, prevention is better compared to treatment of this disease. Contact with infected animals carries a high risk of human infection. Domestic animal owners travelling to endemic areas should be warned and advised to keep them indoors to prevent infection. Individuals must wear gloves when handling skin nodules and ulcers and taking diagnostic samples. The chemotherapeutics have remarkable results but it is noted that there are some incidences of reoccurrence of the disease due to drug resistance. Therefore, plant-based drugs may act as an alternative approach for significantly curing the disease and overcoming drug resistance.

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Chapter 9
Emergence of Invasive Fungal Infection: Diagnosis and Treatment in Humans

Ravindra Kumar and Rajrani Ruhel

9.1 Introduction

Fungi are opportunistic pathogens (endogenous or exogeneous) that are freely present in the environment and cause either no or very mild symptoms in healthy individuals, but manifest a fatal physical morbid state in immunocompromised individuals. Invasive fungal infections are those infections wherein fungal pathogens have invaded and colonized the deep tissues, which results in prolonged illness (Badiee and Hashemizadeh 2014). The Invasive Fungal Infections Cooperative group (IFICG) of the European Organisation for Research and Treatment of Cancer (EORTC) and the Mycology Study Group (MSG) of the National Institute of Allergy and Infectious Diseases (NIAID) define invasive fungal infections as the presence of fungal elements either as mold or yeast in deep tissues or needle aspiration biopsy that is further confirmed by culturing and histopathological examination (Ramana et al. 2013). The innate immune system of healthy individuals combats the invasion from fungal infection and does not acquire invasive fungal infections, except exhibiting mild symptoms. The reason for this is that the skin barrier of healthy individuals and the innate and adaptive immunities provide defense against these infections in most normal situations. In the case of patients with severe burns or severely impaired immune system, the chances of invasive fungal infections increase significantly. Invasive fungal infections can affect any part of the body and result in morbidity as well as enhanced mortality chances in immunocompromised individuals due to weakened immune system in response to these infections. These individuals can also have multiple comorbidities (Hope et al. 2013). The number of

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patients at risk of suffering from invasive fungal infections is increasing worldwide. Research in this area has attracted attention due to: (a) the complex nature of infections; (b) often there being multiple infections occurring at the same time; and (c) its diagnosis and treatment. In addition to the immunocompromised individuals, the patients undergoing immunosuppressive therapy, major surgery, hematopoietic stem cell transplantation (HSCT), and the patients with neoplastic diseases, acquired immune deficiency syndrome (AIDS), chronic pulmonary diseases, etc. form a high-risk group of individuals (Fig. 9.1) (Badiee and Hashemizadeh 2014; Person et al. 2010). Invasive fungal infections have also been increasingly reported in individuals with grafts, prosthetic devices, and also in the patients who underwent any major aggressive surgery or organ transplantation (Person et al. 2010). There are several factors that can increase the chances of invasive fungal infections in individuals. One of the major reasons is the use of broad-spectrum antibiotics and antineoplastic and immunosuppressive agents (Enoch et al. 2006; Ravikumar et al. 2015). The increased predisposition of patients with pancreatitis, human immunodeficiency virus (HIV) infection, burns, and neutropenia to fungal infection are other reasons for increased risk of invasive fungal infections (Ruping et al. 2008). Various reports have demonstrated that the chances of invasive fungal infections are also determined by several factors, which include both genetic as well as environmental conditions (Maskarinec et al. 2016; Benedict and Park 2014).

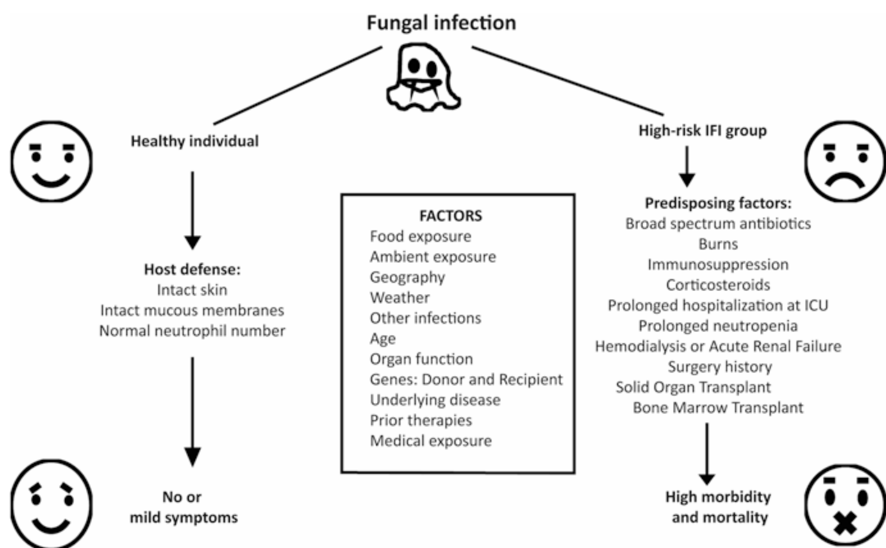

Fig. 9.1 Factors promoting invasive fungal infections and the formation of a high-risk invasive fungal infection group

9.2 Invasive Fungal Infections

The most important fungal pathogens causing invasive fungal infections are yeasts (*Candida* spp., *Cryptococcus* spp., and *Pneumocystis* spp.) and molds (*Aspergillus* spp., *Fusarium* spp., *Scedosporium prolificans*, *Mucor*, *Rhizopus*, *Rhizomucor*, *and Absidia*) (Ramana et al. 2013). Of these, *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis* collectively cause more than 90% of the deaths due to invasive fungal infections globally (Brown et al. 2012). These infections can be transmitted by the inhalation of spores, penetration into the mucosa by commensal organisms, such as *Candida albicans*, and the ingestion of contaminated food (Badiee and Hashemizadeh 2014). The resulting infection might be mild or life-threatening, depending upon various factors, as shown in Fig. 9.1. Invasive fungal infections are associated with high morbidity and mortality rates. For instance, mortality associated with invasive candidiasis varies from 50 to 75%, while it can reach almost 100% in the case of invasive aspergillosis (Pfaller and Diekema 2007). Delay in antifungal therapy to treat invasive fungal infections also increases mortality and, therefore, requires early diagnosis and medical intervention, but both of which are very challenging (Badiee and Hashemizadeh 2014; Morrell et al. 2005). Table 9.1 lists the major invasive fungal infections and shows the statistics for invasive fungal infections worldwide.

In the following sections, the major invasive fungal infections and their diagnosis and treatment will be discussed.

9.2.1 Aspergillosis

Aspergillosis is the most common fungal infection in humans and is caused by a group of filamentous fungi, *Aspergillus* (Hartwick and Batsakis 1991; Schmiedel and Zimmerli 2016). *Aspergillus* is mostly found in soil, food, air, water, and

Table 9.1 Statistics of the most significant invasive fungal infections (Brown et al. 2012)

Disease	Causative agent	Mortality rates in infected populations (%)
Aspergillosis	*Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus flavus*	30–95
Candidiasis	*Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*	46–75
Cryptococcosis	*Cryptococcus neoformans* and *Cryptococcus gattii*	20–70
Mucormycosis	*Rhizopus oryzae*, *Rhizopus microsporus*, and *Rhizomucor pusillus*	30–90
Pneumocystis	*Pneumocystis jirovecii*	20–80

decomposing matter (Anaissie et al. 2002; Tamgadge et al. 2012) and cause a gradual destructive disease of the lungs called chronic pulmonary aspergillosis, which complicates other pulmonary-related conditions or diseases, such as tuberculosis, chronic obstructive pulmonary disease (COPD), and systemic inflammatory disease. There are several species of this genus but *A. fumigatus, A. niger*, and *A. flavus* are the most common causative agents of aspergillosis (Sethi et al. 2012). According to the guidelines of the Infectious Diseases Society of America (IDSA) for the treatment of aspergillosis, there are three major types of aspergillosis: invasive aspergillosis, pulmonary aspergilloma, and allergic bronchopulmonary aspergillosis (ABPA) (Stevens et al. 2000). Pulmonary aspergilloma, a saprophytic form of aspergillosis, usually develops in a pre-existing cavity in the lung and results from the ingrowth of colonized *Aspergillus*, while allergic bronchopulmonary aspergillosis is an immunological pulmonary disorder caused by hypersensitivity to *Aspergillus fumigatus* (Agarwal et al. 2013). However, there are also reports showing that, under certain circumstances, the colonization of airways by *Aspergillus* species can transform into an invasive disease (Shahi et al. 2015; Gago et al. 2018). Invasive aspergillosis is a devastating infection and is highly lethal in immunocompromised individuals. The lungs are the most common sites of primary invasive disease, while the central nervous system (CNS) is the secondary site of this invasive disease (Anagnostou et al. 2014; Mohammadi et al. 2015). Invasive aspergillosis leads to a mortality rate of around 50% even if diagnosed and treated, but delayed diagnosis may be 100% fatal (Latge 1999).

9.2.1.1 Diagnosis

Diagnosing aspergillosis remains an obstacle and early diagnosis in immunocompromised individuals is also one of the major challenges. *Aspergillus* is common in the environment and can be found in the saliva and sputum of healthy people. The symptoms of aspergillosis are similar to those of tuberculosis. Therefore, the isolation of *Aspergillus* spp. in sputum samples is highly determined by the immune status of the host (Garnacho-Montero et al. 2005). For instance, invasive aspergillosis cannot be ruled out by negative sputum samples; on the other hand, invasive disease can be easily established in immunocompromised patients. Apart from this, as high as 70% of negative sputum samples could still have invasive aspergillosis disease (Tang and Cohen 1992; Yu et al. 1986). Histopathological examination followed by bronchoalveolar lavage, transthoracic needle biopsy (TNB), or video-assisted thoracoscopic surgery are standard procedures for the diagnosis of invasive aspergillosis (Walsh et al. 2008). Chest X-ray and computed tomography (CT) scan are good tools to diagnose aspergilloma as well as the characteristic signs of invasive and allergic bronchopulmonary aspergillosis, but they have some limitations (Hope et al. 2013). Chest X-ray is of less significance in the early stages of invasive disease, while the CT scan in combination with high-resolution images (HRCT) is more useful (Caillot et al. 1997, 2001). A recent advancement in the diagnosis of

invasive aspergillosis is the detection of *Aspergillus* antigens, such as galactomannan and (1 → 3)-beta-D-glucan in the body fluids of patients. A double-sandwich enzyme-linked immunosorbent assay (ELISA) kit approved by the U.S. Food and Drug Administration (US FDA) for the detection of galactomannan that is released by *Aspergillus* during its growth in the patient's serum provides a non-culture-based diagnosis of invasive aspergillosis (Boutboul et al. 2002; Marr et al. 2004a). In this test, the patient's sputum is stained with a dye, which identifies the presence of *Aspergillus*.

9.2.1.2 Treatment

Oral corticosteroids are one of the best ways to treat bronchopulmonary aspergillosis (Rosenberg et al. 1978; Wang et al. 1979; Capewell et al. 1989). Antifungal medications are not very helpful in allergic bronchopulmonary aspergillosis, but combination with corticosteroids can improve lung condition and its functioning. In certain situations of aspergilloma, surgical resection is required to remove the fungal mass, but the surgical route should be avoided, as it is risky (Uflacker et al. 1985; Soltanzadeh et al. 1977; Massard et al. 1992).

The management of invasive pulmonary aspergillosis is difficult. Voriconazole is considered the first-line therapy for invasive aspergillosis infections caused by these pathogens (Johnson and Kauffman 2003; Sambatakou et al. 2006; Ghannoum and Kuhn 2002; Herbrecht et al. 2002; Limper et al. 2011; Walsh et al. 2008). Voriconazole, a broad-spectrum triazole, is available in both intravenous and oral formulations. Although voriconazole has a milder side-effect profile, it can potentially interact with many significant drugs, such as cyclosporine, warfarin, terfenadine, carbamazepine, quinidine, rifampin, statins, and sulfonylureas (Johnson and Kauffman 2003; Sambatakou et al. 2006; Ghannoum and Kuhn 2002; Herbrecht et al. 2002; Limper et al. 2011).

Isavuconazole, a newer triazole, has been approved for the treatment of invasive aspergillosis and is more tolerable than voriconazole (Spitzer et al. 2017). Amphotericin B products can be used as second-line agents in patients who cannot tolerate or are failing voriconazole therapy (Cornely et al. 2007). To reduce the side effects associated with amphotericin, which include nephrotoxicity, electrolyte disturbances, and hypersensitivity, a new lipid-based preparation of amphotericin B, such as liposomal amphotericin B and lipid complex amphotericin B, has been introduced and used for treatment (Cornely et al. 2007). Echinocandin derivatives such as caspofungin, micafungin and anidulafungin are also effective agents in the treatment of invasive aspergillosis and can be used as salvage therapy. Another broad-spectrum triazole, posaconazole, is effective and safe as salvage therapy in patients with invasive pulmonary aspergillosis refractory to standard antifungal therapy (Pitisuttithum et al. 2005; Spanakis et al. 2006; Cohen-Wolkowiez et al. 2006). The combined therapy of caspofungin and liposomal amphotericin B is more successful as a primary rather than a salvage therapy (Koulenti et al. 2014). In case

of failure of this therapy, the combination of caspofungin and voriconazole is also recommended (Marr et al. 2004b). Combinations of antifungal therapies have also been used as salvage therapy in many high-risk patients (Elizabeth et al. 2015).

9.2.2 Candidiasis

Candida species are the most common causative agents of fungal infections, which can range from a non-life-threatening state such as mucocutaneous illnesses to invasive infection that may affect many organs. There are more than 17 different species of *Candida* that have been identified to cause invasive candidiasis and, of these species, only five species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*) are responsible for more than 90% of invasive candidiasis (Pfaller and Diekema 2007). Among these *Candida* species, *C. albicans* is the most common infectious agent, while *C. glabrata* is the second most common species causing invasive candidiasis (Achkar and Fries 2010; Pfaller and Diekema 2007). *Candida* species form a highly structured biofilm under different environmental conditions. Surface-associated *Candida* can grow embedded in extracellular matrix or biofilm, which is composed of carbohydrates and proteins (Achkar and Fries 2010). *Candida albicans* is distinguished from many other fungal species by its ability to form both yeast cells and hyphae. Hyphae are an important structural component of *C. albicans* biofilm formation (Nobile and Johnson 2015). This is the most common member of the human microbiota, appearing in the oral cavity, gastrointestinal tract, female genital tract, or on the skin (Achkar and Fries 2010; Ganguly and Mitchell 2011; Nobile and Johnson 2015). In the healthy human population, *C. albicans* is harmless, while under the alteration of immune defenses, it overgrows and causes serious systemic disease and organ failure (Prieto and Pla 2015). *Candida albicans* also forms biofilm along with *Staphylococcus aureus* on the surface of implantable medical devices or organs (Peters et al. 2010). Such multispecies infection leads to higher mortality rates than single-species infection (Zago et al. 2015). In addition, infections caused by *C. albicans* can be acquired at hospitals and have become a cause of major health concerns. *Candida albicans* is identified as the fourth most common blood isolate in US hospitals, accounting for around 10% of hospital-acquired bloodstream infections (Horn et al. 2012).

9.2.2.1 Diagnosis

The diagnosis of invasive candidiasis is difficult and complicated because there are no specific clinical manifestations of the disease. Researchers have developed polymerase chain reaction (PCR)-based methods for the detection of *Candida* species in blood samples (Mirhendi and Makimura 2003), DNA-based fluorescence in-situ hybridization (FISH) (Bisha et al. 2011), as well as ELISA for the diagnosis of candidiasis infections (Lain et al. 2007).

9.2.2.2 Treatment

The treatment of *Candida* infections varies substantially and is determined by the anatomic location of the infection and the immunity status of the infected individual. Polyenes, triazoles (fluconazole, itraconazole, voriconazole, and posaconazole), echinocandins (caspofungin, anidulafungin, and micafungin), and flucytosine are the most common antifungal agents for the effective treatment of candidiasis (Pappas et al. 2007, 2009; Pfaller and Diekema 2007). Fluconazole is considered a first-line agent in non-neutropenic patients with invasive candidiasis. Amphotericin B is used for non-albicans species (e.g., *C. krusei*).

9.2.3 Cryptococcosis

Cryptococcosis is the one of the invasive fungal infections that cause significant morbidity and mortality in both immunocompromised and immunocompetent individuals worldwide. Cryptococcosis is caused by basidiomycetous fungi, *Cryptococcus* species. The two most common species that cause cryptococcosis are *C. neoformans*, which mostly affects immunocompromised individuals, and *C. gattii*, which can infect both immunocompromised as well as immunocompetent individuals (Oladele et al. 2017). These species are further divided into different genotypes (VNI, VII, VNB, VNIV, and VGI-IV) (Perfect and Bicanic 2015). The capsule of *C. neoformans* is made up of two polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), which act as a virulent factor (Zaragoza and Casadevall 2004; Bose et al. 2003; Zaragoza et al. 2009). Infection is caused by inhalation of these fungal spores, so the primary localization site is the lung, from where it spreads through the bloodstream to the CNS. *Cryptococcus* species have the propensity to locate in the CNS and cause fungal meningitis. Pulmonary cryptococcosis may be asymptomatic but meningitis is a common feature of infection, especially in HIV patients and solid-organ transplant recipients (Enoch et al. 2006). For instance, the HIV-infected patients are at higher risk of contracting cryptococcal infection, while the non-HIV patients also get infected by *Cryptococcus* spp., more often if have a history of transplantation or immunosuppressive therapies.

9.2.3.1 Diagnosis

Histopathology varies according to the immunological status of the host. In immunocompetent individuals, typical granulomas with multinucleated giant cells are formed at the site of cryptococcal infection, while in immunosuppressed hosts, cryptococcosis infects the CNS, skin, and the oral mucous membrane. The definitive diagnosis of cryptococcosis is established with periodic acid Schiff (PAS),

mucicarmine stain, and Fontana-Masson stain. The fungal wall stains with the PAS stain, mucicarmine stains the fungal capsule, while Fontana-Masson stains the fungal growth itself because it contains melanin, which is characteristic of cryptococci (Guarner and Brandt 2011). Diagnosis can also be done by the detection of fungal growth in cerebrospinal fluid (CSF) with India ink examination, isolation in tissue culture, and detection of cryptococcal polysaccharide capsular antigen (CrAg) in the serum and in the CSF through latex agglutination or ELISA (Oladele et al. 2017; Amaral et al. 2016).

9.2.3.2 Treatment

For many decades, amphotericin B has been used for treatment in transplant recipients and HIV patients (Sloan and Parris 2014). At present, the combination of liposomal amphotericin B and flucytosine (5-FC) treatment is considered as standard treatment due to its better performance in treating cryptococcal meningitis as compared to amphotericin B treatment alone (Larsen et al. 1990; de Gans et al. 1992; Brouwer et al. 2004; Dromer et al. 2008; Day et al. 2013; Sloan and Parris 2014). This combination is also a good treatment of choice for HIV-infected patients. This combination therapy improved early fungicidal activity (EFA) as well as lowered toxicity. However, although the high doses of amphotericin B and 5-FC have improved fungicidal activity, an increase in serious side effects has been reported (Bicanic et al. 2008). Fluconazole is also recommended as an alternative to flucytosine (Bellmann and Smuszkiewicz 2017; Perfect et al. 2010; Elizabeth et al. 2015). During combination of amphotericin B and fluconazole, higher fluconazole doses gave better outcomes compared to lower fluconazole doses (Yao et al. 2014; Elizabeth et al. 2015). Apart from this, other azoles, such as itraconazole, voriconazole, isavuconazole, and posaconazole, have also been tested for the treatment of cryptococcosis, but they seem unreliable (Chotmongkol and Jitpimolmard 1992; Pitisuttithum et al. 2005; Thompson 3rd et al. 2016; de Gans et al. 1992). Out of these, voriconazole has better absorption and penetration to the CNS than itraconazole and posaconazole and it has been used for cryptococcosis treatment in normal individuals (Yao et al. 2015). The survivors of cryptococcal meningitis HIV-infected patients have notably higher concentrations of cytokine stimulating factor, such as IFN-γ, TNF-α, IL-6 and -8, than the non-survivors of cryptococcal meningitis HIV-infected patients (Siddiqui et al. 2005). Researchers also found that the combination of liposomal amphotericin B, voriconazole, and IFN-γ is successful in the treatment of cryptococcal meningitis in HIV patients (Gamaletsou et al. 2012). In addition to all the antifungal treatments, a new technology is in progress for cryptococcal meningitis treatment. This technology, in combination with the antifungal therapies, would prove to be better for cryptococcal meningitis management (Cutshaw et al. 2016).

9.2.4 Mucormycosis

Mucormycosis is an infection caused by members of the order Mucorales (Ibrahim et al. 2012). Mucorales are distributed into six families and all members of the six families cause cutaneous and deep infections. *Rhizopus oryzae*, *R. microsporus*, and *R. pusillus* are the most common species isolated from patients with mucormycosis and are responsible for around 70% of all cases of mucormycosis. This causative agent is ubiquitous in nature and mostly found in soil and decaying organic matter. Because of the abundance of this fungus, the mouth and the nose inhale the spores very often. In a normal person, the macrophage cells phagocytize the spores, while in immunocompromised individuals, the spores germinate into the hyphae and cause infections in the sinuses and lungs.

9.2.4.1 Different Forms of Mucormycosis

Mucormycosis invades the blood vessels, producing thrombosis and tissue infarction. It causes five major forms of infections (Prabhu and Patel 2004): (i) rhino-orbitocerebral, (ii) pulmonary, (iii) disseminated, (iv) cutaneous, and (v) gastrointestinal. Rhino-orbitocerebral is the most common form of mucormycosis, while pulmonary mucormycosis is a rare pulmonary fungal infection and difficult to diagnose. After aspergillosis and candidiasis, mucormycosis is the third most common invasive fungal infection (Hammond et al. 2011).

9.2.4.1.1 Rhino-orbitocerebral Mucormycosis

This is the most common form of mucormycosis and is mostly found in diabetic patients. The disease starts from the nose and progresses into the sinuses, orbit, and then intracranially. The initial symptoms are sinusitis or periorbital cellulitis, facial pain, blurry vision, and soft tissue swelling. If untreated, infection spreads from the ethmoid sinus to the orbit, resulting in loss of extraocular muscle function (Spellberg et al. 2005). This is also divided into two major forms: (i) highly fatal rhino-orbitocerebral and (ii) less fatal rhino-orbitocerebral. The former involves ophthalmic and internal carotid arteries, while the latter involves the sphenopalatine and greater palatine arteries, resulting in thrombosis of turbinates and necrosis of the palate (Yanagisawa et al. 1977).

9.2.4.1.2 Pulmonary Mucormycosis

Pulmonary mucormycosis is the most common form found in neutropenic and stem cell-transplant patients. It may develop as a result of inhalation or by hematogenous or lymphatic spread. Its symptoms include dyspnea, cough, and chest pain. Patients

with untreated pulmonary mucormycosis usually die from disseminated disease before respiratory failure occurs (Tedder et al. 1994). The overall mortality rate of pulmonary mucormycosis is approximately 50–70%, but is >95% if the pulmonary mucormycosis is part of a disseminated process (Spellberg et al. 2005).

9.2.4.1.3 Disseminated Mucormycosis

Disseminated mucormycosis may originate from any primary site of infection. Pulmonary mucormycosis has the highest incidence of dissemination. The most common site of dissemination is the brain, but metastatic lesions may also be found in the spleen, heart, skin, and other organs. The mortality associated with dissemination to the brain is about 100%. Even without CNS involvement, disseminated mucormycosis has a mortality of >90%. The diagnosis of disseminated disease is difficult because patients are usually severely ill from multiple diseases. When disseminated mucormycosis is suspected, a careful search should be made for cutaneous lesions that can be biopsied for diagnostic purposes (Spellberg et al. 2005).

9.2.4.1.4 Cutaneous Mucormycosis

The mucormycosis agent cannot penetrate the skin but individuals with a disrupted protective cutaneous barrier are at high risk of cutaneous mucormycosis. Cutaneous mucormycosis can be very invasive and penetrate from the cutaneous and subcutaneous tissues into the adjacent fat, muscle, and even bone (Spellberg et al. 2005).

9.2.4.1.5 Gastrointestinal Mucormycosis

Gastrointestinal mucormycosis is a rare form of mucormycosis. It mainly occurs in extremely malnourished individuals, especially infants or children. The stomach, colon, and ileum are the most commonly involved sites during gastrointestinal mucormycosis. Non-specific abdominal pain and distention associated with nausea and vomiting are the most common symptoms (Spellberg et al. 2005).

9.2.4.2 Diagnosis

The diagnosis of mucormycosis is challenging and treatment should start as early as possible in order to decrease mortality. Although the diagnosis is difficult, some tests such as CT scan or magnetic resonance imaging (MRI) may be helpful to define the infections or tissue destruction. Histopathology is the gold standard for this diagnosis. The detection of fungal DNA in tissue samples by PCR is a non-culture-based method that may allow improved diagnosis of mucormycosis (Hammond et al. 2011).

9.2.4.3 Treatment

There are some important factors for the eradication of mucormycosis : rapidity of diagnosis, appropriate surgical removal of infected tissue, and appropriate antifungal therapy. For patients with mucormycosis, surgical treatment plus antifungal therapy is better than the use of antifungal therapy alone (Spellberg et al. 2005). Polyene-based antifungal therapy, such as amphotericin B, is the primary therapy for mucormycosis treatment. Amphotericin B deoxycholate (d-AmB) is the only antifungal agent for the treatment of mucormycosis that has been approved by the US FDA. d-AmB has been replaced by the various lipid formulations of amphotericin. Posaconazole is better than itraconazole and isavuconazole. Fluconazole and voriconazole have no meaningful activity against agents of mucormycosis. No other azoles except for posaconazole are recommended in the treatment of mucormycosis. Flucytosine lacks activity against agents of mucormycosis (Skiada et al. 2013). Liposomal amphotericin B is frequently used and is the most effective drug against mucormycosis. Lipid amphotericin B is also combined with echinocandins to treat mucormycosis (Spellberg and Ibrahim 2010).

9.2.5 Pneumocystis Pneumonia

Pneumocystis pneumonia is an opportunistic infection that occurs in immunosuppressed individuals, primarily individuals with HIV infections, caused by the invasion of the unicellular fungus, *Pneumocystis jirovecii*. Initially, it was misclassified as protozoan, on the basis of its morphology, but, later, it was considered as fungi (Thomas Jr. and Limper 2004). *Pneumocystis* isolated from one species cannot infect another species, meaning that the microbe is host-specific. There are five major species-specific *Pneumocystis* species: *P. carinii* and *P. wakefieldiae* in rats, *P. murina* in mice, *P. oryctolagi* in rabbits, and *P. jirovecii* in humans (Aliouat-Denis et al. 2008; Sokulska et al. 2015). Transmission occurs via aerosols from patients with pneumonia or from early-life contact with family or community members who carry the organism in their lungs (Brown et al. 2012). Its presence in lungs is asymptomatic. Pneumocystis pneumonia has been one of the main causes of morbidity and mortality among HIV-infected people (Sokulska et al. 2015), but the death rate in non-HIV individuals (30–60%) is significant higher than that in HIV-infected individuals (Sokulska et al. 2015; Schmiedel and Zimmerli 2016).

9.2.5.1 Diagnosis

Pneumocystis pneumonia is difficult to diagnose because of the non-specific symptoms and signs of infection. The diagnosis of pneumocystis pneumonia requires microscopic detection of *P. jirovecii* in respiratory tract specimens, followed by staining with dyes or antibodies. Cysts or trophozoites are morphologically

identified by methenamine silver nitrate or Giemsa stains (Sowden and Carmichael 2004). Pneumocystis from sputum, bronchoalveolar fluid, or lung tissue can also be identified by reverse transcription (RT)-PCR. Microscopic methods have limited sensitivity, while RT-PCR has high sensitivity and can be implemented as a rapid routine diagnostic test. (1 → 3)-beta-D-glucan in association with lactate dehydrogenase has been used as a biomarker in the case of HIV-infected patients (Esteves et al. 2014; Held et al. 2011). Up to now, attempts to culture *P. jirovecii* have been failures, except for recently, when a three-dimensional air–liquid interface culture system made up of differentiated pseudostratified airway epithelial cell line named as CuFi-8 cells has been demonstrated for culturing *P. jirovecii* (Schildgen et al. 2014).

9.2.5.2 Treatment

Typical antifungal drugs have been found to be not effective for *Pneumocystis* infection. Folic acid is needed for the synthesis of purines, glycine, and thymidylate, which are necessary for the proper functioning of an organism. The lifecycle on *P. jirovecii* also depends on folic acid synthesis but disruption of the folic acid synthesis pathway results in failed acquisition of the folic acid from the environment. Thus, the folic acid synthesis pathway has been used as a target for therapeutic agents for pneumocystis pneumonia treatment. Dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are two important enzymes involved in the folic acid synthesis pathway (Volpe et al. 1993). Trimethoprim (TMP) and a sulfa drug named sulfamethoxazole (SMZ) are inhibitors of the DHFR and DHPS enzymes, respectively, and have been used as agents to treat pneumocystis pneumonia (Lobo et al. 2013). The most effective treatment for pneumocystis pneumonia is the combination of these two drugs, as has been shown in HIV-negative individuals (Thomas Jr. and Limper 2004). This drug can be taken either orally or by intravenous infusion. However, in HIV-positive individuals, sulfamethoxazole causes allergic reactions, so sulfamethoxazole should be replaced by dapsone. Apart from this drug, pentamidine, prednisone, a combination of clindamycin and primaquine, and a combination of trimetrexate and leucovorin are also used. The inhibition effect of TMP-SMZ can be further enhanced by the addition of low doses of caspofungin, which is an inhibitor of the (1 → 3)-beta-D-glucan synthase, an enzyme required for the synthesis of cell walls of many fungi. Caspofungin and clindamycin have been reported to be a salvage therapy to treat immunocompromised patients having allergies or adverse reactions to TMP-SMZ (Li et al. 2016).

9.3 Conclusion and Future Implications

Fungi are opportunistic infectious agents and most of them are usually not pathogenic to normal healthy individuals. However, when they infect individuals at high risk of invasive fungal infection, they cause a wide range of diseases, ranging from superficial infections to disseminated infections of the vital internal organs. Invasive fungal infections continue to be a major problem due to improved medical care among individuals at high risk of invasive fungal infections. Despite the recent efforts, early diagnosis and intervention still need to be addressed due to their high associated mortality and morbidity rates. There have been significant advances in therapeutic options for invasive infections caused by *Aspergillus* and *Candida* species. Successful treatment of these opportunistic infections requires prompt diagnosis and aggressive therapy with antifungal agents. Inhalation of spores of these microorganisms is the most common mode of infection in a susceptible host (Deepa et al. 2014). To better understand the problem, we need to define accurate epidemiological data, the socioeconomic impact of the disease, and also stimulate scientific interest in this field. To tackle these challenges, we need to focus on: (i) development of better and rapid diagnostics tools which have an immediate impact on the mortality rate and (ii) development of more effective and less toxic antifungal drugs/vaccines, especially for the treatment of immunocompromised patients. By the use of combination drug therapy, we can increase the effectiveness of the drug and minimize the risk of development of resistance and decrease toxicity (Elizabeth et al. 2015). During vaccination development against invasive fungal infections, efforts should be focused on identifying epitopes with immunogenic T cell and adjuvants. Attention directed towards these goals would have a significant effect in reducing the global burden and negative impact of invasive fungal infections.

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Chapter 10
Animal Infections: The Role of Fungal Biofilms

Mukesh Kumar Yadav and Yogesh Malvi

10.1 Introduction

Microorganisms, throughout their evolution, modify their physical and metabolic habitats to adapt to the environmental conditions. Bacteria were initially believed to be unicellular; however, this was not accurate, as the pure planktonic mode of growth is uncommon and the bacteria frequently exist in complex communities (Marsh 1999). One of the biggest paradigms among microbiologists is the concept that bacteria are an asocial organism, which divide and reproduce identical copy progeny. However, it has been known for years that bacteria may demonstrate group behaviour, in which bacteria cross-talk with each other and act as a community (Aguilar-Romero et al. 2011). Now, with the use of advanced techniques, direct observation indicates that most microbes remain adherent to surfaces inside a structured ecosystem, called biofilms (Costerton et al. 1987). Microbial biofilm is defined as adherent microbial communities surrounded by self-produced extracellular polymeric substances (EPS) matrix. The microbial biofilm could be formed on biotic or abiotic surfaces, and could consist of a single microbial species or polymicrobial species, such as biofilms of bacteria and fungi. Biofilm formation is an important aspect of many bacteria diseases; however, many pathogenic filamentous fungi and yeasts, such as *Candida* spp., form biofilms. The composition and architecture of the fungal biofilms attribute tolerance to antifungal agents and require up to 10–1000 times greater concentrations of antifungal agents than planktonic cells to eradicate biofilms. In mature biofilms, the cell metabolism is slow and demonstrates differential gene expressions compared to the counterpart planktonic cells; therefore, the

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common antifungal agents are ineffective on active growing cells. In addition, the biofilm microbes are resistant to phagocytosis and, thus, are resistant to host responses (Amorena et al. 1999; Jefferson 2004). The biofilm increases the frequency of spread of resistant genes and gives rise to persister cells. As a result, biofilm infections are difficult to treat and increase the cost of treatment and recovery time.

Although filamentous fungi and yeast are also known to form biofilms, most of the fungi biofilms have been studied using a yeast like *Candida* spp. According to some researchers, the biofilm formation stages in yeast and filamentous fungi are not similar. The biofilm formation by yeast is similar to bacteria. However, the filamentous fungi form biofilms with some additional stages (Harding et al. 2009). The filamentous fungi secrete small proteins called hydrophobins, which facilitate the adhesion of hyphae to hydrophobic surfaces during biofilm formation (Harding et al. 2009; Wosten 2001). In animals, various fungal species, including *Candida* spp., *Cryptococcus* spp. (Ajesh and Sreejith 2012; Martinez and Casadevall 2006; Pettit et al. 2010; Martinez et al. 2010), *Malassezia* spp. (Bumroongthai et al. 2016), *Trichosporon* spp. (Iturrieta-Gonzalez et al. 2014), *Fusarium* spp. (Imamura et al. 2008; Mukherjee et al. 2012; Peiqian et al. 2013), *Scedosporium* spp., *Lomentospora prolificans* (Mello et al. 2016) and *Coccidioides* spp. (Davis et al. 2002), cause various biofilm-related infections, such as otomycosis, dermatitis, stomatitis, onychomycosis, vulvovaginitis, urinary tract infection and respiratory tract infection. In addition, the multi-species biofilm of fungi and bacteria is common in animal infections. In this review chapter, we present an overview of fungal biofilm-related infections in animals.

10.2 Fungal Biofilms and Infectious Disease

Although biofilm formation by *C. albicans* has been known for many years, recently, many studies have reported that various species of filamentous fungi possess the ability to form biofilms. The biofilm mode of growth plays an important role in infectious disease in both humans and animals. Paracoccidioidomycosis is a systemic, endemic mycosis caused by a dimorphic fungus *Paracoccidioides brasiliensis*. In the yeast phase, *P. brasiliensis* forms biofilm and increases the gene expressions of GP43, enolase, GAPDH and aspartyl proteinase, and decreases the expression of phospholipase, which are required for adhesion and biofilm formation (Sardi Jde et al. 2015). Histoplasmosis is a respiratory systemic mycosis caused by *Histoplasma capsulatum*, a dimorphic fungus that exists as biofilm in the yeast phase (Pitangui et al. 2012). In humans and animals, dermatophytes fungi invade keratinised tissues, producing dermatophytosis (Weitzman and Summerbell 1995; Costa-Orlandi et al. 2012). It has been reported that the fungi *Trichophyton rubrum* and *T. mentagrophytes* form biofilms that result in dermatophytosis and onychomycosis, which often relapse and show non-response to ward treatment (Burkhart et al. 2002; Costa-Orlandi et al. 2012). Moreover, the biofilms of *Histoplasma*,

Paracoccidioides and *Trichophyton* are resistant to antibiotics. The saprophytic fungi *Aspergillus* causes severe superficial and systemic infections (Gonzalez-Ramirez et al. 2016; Müller et al. 2011; Kaur and Singh 2014) in humans and animals. Aspergillosis caused by *Aspergillus* is the second major cause of nosocomial infection, which has a higher mortality rate (Kaur and Singh 2014; Ramage et al. 2011). *Aspergillus* is an opportunistic fungi and, in immune-suppressed conditions, causes aspergilloma, invasive pulmonary aspergillosis, allergic bronchopulmonary aspergillosis and even systemic dissemination (Kaur and Singh 2014; Ramage et al. 2011; Williams et al. 2016). The aspergilloma caused by *Aspergillus* is a fungal mass with biofilm characteristics (Ramage et al. 2011). The biofilm formations by *Aspergillus* take place within 24 h, and the mature biofilms demonstrate increased biofilm biomass, with channels developed between hyphae through which nutrients and fluid transportation occurs (Ramage et al. 2011; Villena et al. 2010). The extracellular matrix (ECM) composition of the biofilms has been detected and is composed of hydrophobins, α-1,3-glucans, galactomannan, polyols, melanin, monosaccharides and antigens (Ramage et al. 2011; Beauvais et al. 2007).

Cryptococcosis is an important fungal infection of animals and humans. It is one of the common infections of various domestic animals, including cats, dogs, ferrets, horses, camelids, goats, sheep, cattle, dolphins, birds, koalas and other marsupials (Malik et al. 2002; Sykes et al. 2010). It is caused by fungi belonging to *Cryptococcus* spp., *C. laurentii* and *C. albidus*, which, together, cause almost 80% of infections in humans and animals (Khawcharoenporn et al. 2007). *Cryptococcus laurentii* is an encapsulated saprophytic yeast and causes superficial infections like keratitis and deep-seated infections such as fungaemia and meningitis (Cheng et al. 2001; Shankar et al. 2006; Khawcharoenporn et al. 2006). *Cryptococcus neoformans* forms biofilms, has a worldwide distribution and infects immune-suppressed patients (Walsh et al. 1986; Ingram et al. 1993).

10.3 *Candida* spp. Biofilms

In animals, *C. albicans* causes otomycosis, dermatitis, stomatitis, onychomycosis, vulvovaginitis, subclinical mastitis, stomatitis, dermatitis and otitis, urinary tract infection and respiratory tract infection (Vijay and Pal 2013). The biofilm formation by *Candida* spp. is the most studied fungal biofilm and has been known since 1990. Many in vitro (Marcos-Zambrano et al. 2016) and in vivo studies revealed that *Candida* biofilms are heterogeneous and composed of hyphal, pseudohyphal, yeast blastospores and ECM (Henriques et al. 2006; Pires et al. 2011). In *Candida* biofilms, the yeast and hyphae are important structural components (Finkel and Mitchell 2011). The filamentation of fungi increases the suppression of biofilms and increases resistance to adverse conditions, such as sonication and vortexing. *Candida* biofilms express many genes involved in fungal adhesion, quorum sensing, ECM production and morphogenesis (Blankenship and Mitchell 2006; Bonhomme

and d'Enfert 2013; Finkel and Mitchell 2011). Within the genus *Candida*, the species *C. albicans*, *C.glabrata*, *C. parapsilosis* and *C. tropicalis* form biofilms; however, they vary in the carbohydrates and protein compositions in the biofilms' ECM and in their morphology. The biofilms of *C. albicans* and *C. parapsilosis* contain both yeast hyphae and pseudohyphae, while the biofilms of *C. glabrata* and *C. tropicalis* contain only yeast cells (Silva et al. 2009).

10.4 *Candida albicans* Multi-species Biofilms with *Streptococcus mutans*

Candida albicans is an opportunistic fungus found on various body sites in humans and on animals. However, it causes localised and systemic infection in immune-suppressed conditions (Tsui et al. 2016). *Candida* can form biofilms on biotic and abiotic surfaces, and frequently form multi-species biofilms with bacteria, which increases the virulence (Kojic and Darouiche 2004; Ramage et al. 2005). *Candida albicans* causes oral mucosal infections in both animals and humans, where the fungal interacts with the commensal viridans streptococci (Xu et al. 2014; Thein et al. 2009). Various researchers reported that *C. albicans* is usually absent in dental plaque biofilms of healthy individuals and it neither colonizes alone on the teeth of rodents nor co-colonizes with *S. mutans* in the absence of sucrose (Gregoire et al. 2011; Xiao et al. 2016). However, a *C albicans* and *S. mutans* interaction has been detected in plaque biofilms, with a high number of *S. mutans* causing aggressive tooth decay and rampant carious lesions (Falsetta et al. 2014; De Carvalho et al. 2006; Xiao et al. 2016). In this interaction, a secretary bacteria exo-enzyme called glucosyltransferase (Gtfs) plays an important role. The *S. mutans* can utilise host dietary sucrose through Gtfs and produce biofilm extracellular polymeric matrix, which mainly consists of α-glucans. Furthermore, the Gtfs can bind on the *C. albicans* cell surface and could produce EPS on the fungal surface, which results in enhanced bacterial binding and multi-species biofilm formation (Falsetta et al. 2014; Pereira-Cenci et al. 2008).

Candida albicans forms polymicrobial biofilms with *Staphylococcus aureus*. The fungus and bacteria has been detected in nosocomial and blood infections (Klotz et al. 2007). Many studies suggested that the polymicrobial interaction of *C. albicans* and *S. aureus* give rise to synergism and results in increased mortality in mice (Carlson 1982; Adam et al. 2002). Furthermore, the treatment and eradication of biofilms require a higher concentration of antibiotics. Indeed, *C. albicans* serves as a scaffold for the biofilm formation of *S. aureus*, and the bacterial cells were coated with matrix produced by *C. albicans* (Melphine and Mairi 2009).

10.5 Mode of Biofilm Formation

Both filamentous fungi and yeasts can form biofilms on biotic and abiotic surfaces; however, many reports are available for yeast biofilms but very few for filamentous fungi (Harding et al. 2009; Blankenship and Mitchell 2006). Many authors suggest that the definition of bacterial biofilms does not fit for filamentous fungi; therefore, fungal biofilms need to be revisited. Harding et al. proposed a model for filamentous fungi biofilms and suggested that the basic steps of biofilms formation are similar in filamentous fungi and bacteria, despite their distinct morphology (Harding et al. 2009). In addition, the filamentous fungi secrete small proteins called hydrophobins, which facilities the adhesion of hyphae to a hydrophobic surface during biofilm formation (Harding et al. 2009; Wosten 2001).

As per Harding et al. (2009), the biofilm formations of filamentous fungi are divided into various stages, as described in Fig. 10.1:

A. *Propagule adsorption*: involving the contact of spores, hyphal fragments or sporangia with a surface;
B. *Active adhesion*: in which adhesins are secreted by spores during germination and other reproductive structures;
C. *First microcolony formation*: which involves elongation and hyphal branching, forming a monolayer with the production of ECM;
D. *Second microcolony formation or initial maturation*: in which compact hyphae networks form in three dimensions, are covered by an ECM and the formation of water channels occurs;
E. *Final maturation*: in which fruiting bodies and other survivor structures are formed, depending on the fungi;
F. *And, finally, the dispersion or planktonic phase*: in which conidia and/or hyphae fragments are released, beginning a new cycle.

In yeasts, the *C. albicans* biofilms are the most often studied and the biofilm development stages are shorter than those of filamentous fungi and bear a close resemblance to those of bacterial biofilms (Chandra et al. 2001; Costa-Orlandi et al.

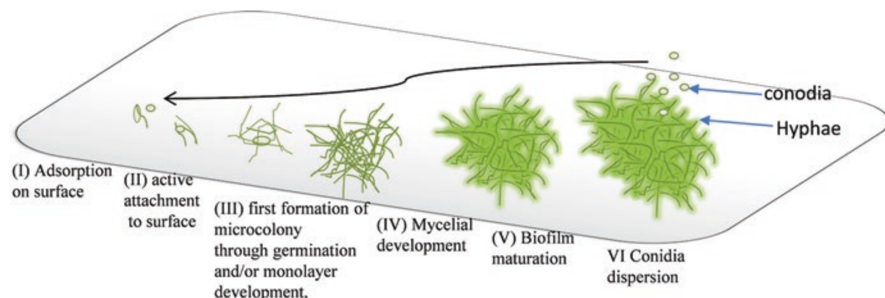

Fig. 10.1 Biofilm formation stages in filamentous fungi. (Figure 10.1 was reproduced from the original article published by Harding et al. (2009))

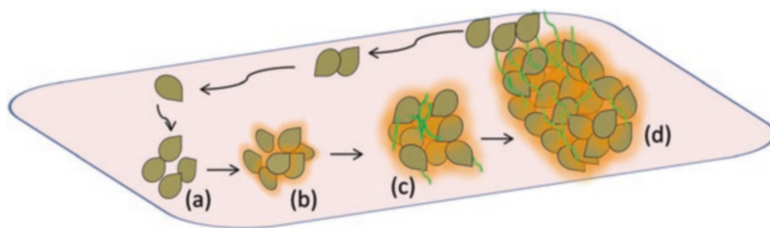

Fig. 10.2 Biofilm formation stages in yeast fungi. (Figure 10.2 was reproduced from the original article published by Harding et al. (2009))

2017). Harding et al. (2009) reported that the *C. albicans* biofilm development involves fewer stages compared to the filamentous fungi, as shown in Fig. 10.2:

(a) The adsorption of yeast cells to a surface
(b) Followed by initial adhesion
(c) Formation of basal layers of yeast with early development of hyphae and ECM
(d) Biofilm maturation containing a significant number of yeasts, hyphae, pseudo-hyphae, ECM and water channels that allow the movement of nutrients
(e) And cell dispersion.

10.6 Mechanism of Resistance in Fungal Biofilms

The biofilms of filamentous fungi and yeasts are difficult to eradicate, as they demonstrate increased resistance to antifungal agents (Scorzoni et al. 2017). The fungal biofilms demonstrate up to 1000-fold more resistance to antifungal agents than the planktonic form (Di Bonaventura et al. 2006; Tre-Hardy et al. 2008). The adaptive resistance acquired by fungal biofilms imparts resistance to antifungal agents (Ramage et al. 2012). The biofilms facilitate the adaptation of fungi to environmental conditions, the structure of biofilms protects cells and obstructs antifungal agent diffusion, the biofilm cells demonstrate altered metabolism and gene expression results in persister cells (Niimi et al. 2010; Rajendran et al. 2013). The adaptive factors which play a vital role in fungal biofilm resistance are shown in Fig. 10.3.

10.6.1 Biofilm Resistance Due to Extracellular Matrix

The bacterial biofilm ECM has been studied extensively. However, there is limited information on fungal biofilms' ECM. The main characteristic of all mature fungi biofilms, irrespective of the genus, is the presence of extracellular polymer matrix, which confers resistance to antifungal agents (Ramage et al. 2009). ECM production is a highly regulated process and the resistance to antimicrobial agents depends

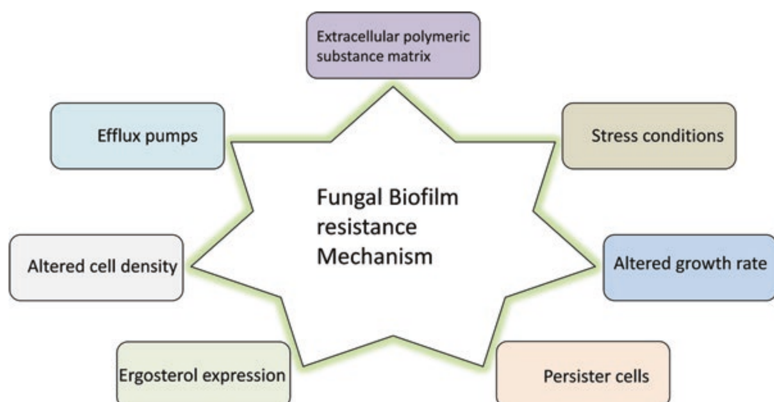

Fig. 10.3 Factors contributing resistance to fungal biofilms

on the diffusion and chemical compositions of the ECM. The ECM of *Candida* spp., including *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *C. albicans* has been characterised (Silva et al. 2009, 2011). The ECM of *C. albicans* and *C. tropicalis* biofilms is composed of protein, uronic acid, phosphorous, hexosamine and carbohydrates (Al-Fattani and Douglas 2006). It was detected that the biofilms were detached when treated with glucanase, indicating that β-1-3 glucans are the main carbohydrates components. β-Glucans also prevent neutrophil activation and release reactive oxygen species (ROS), which decreases the host defence response (Xie et al. 2012). In *C. albicans*, the ECM production is regulated by zinc regulator ZAP1 (Nobile et al. 2009). The other fungi which form biofilms and produce a significant amount of ECM are *C. neoformans*, *Apophysomyces elegans*, *Rhizopus oryzae*, *Aspergillus* species, *Blastoschizomyces capitatus*, *Saccharomyces cerevisiae*, *Lichtheimia corymbifera*, *Malassezia pachydermatis*, *Pneumocystis* species and *Rhizomucor pusillus* (Beauvais et al. 2009; Cannizzo et al. 2007; Cushion et al. 2009; D'Antonio et al. 2004; Martinez and Casadevall 2006; Singh et al. 2011).

10.6.2 Biofilm Resistance Due to Efflux Pump

The resistance of *C. albicans* against high-dose azoles is due to the increased efflux of drug mediated by the ATP-binding cassette and the major facilitator's superfamily transporters (Albertson et al. 1996; Lopez-Ribot et al. 1999; Sanglard et al. 1997). The primary function of efflux pumps is to maintain homeostasis during harsh environmental conditions; however, the exposure to high doses of antifungal agents increase efflux pump expression, resulting in resistance to antifungal agents (Piddock 2006; Bueid et al. 2010). Elevated levels of efflux pump expression have been detected in implanted catheters in animal models and in the *Candida* biofilm model (Andes et al. 2004; Nett et al. 2009; Bizerra et al. 2008).

10.6.3 Biofilm Resistance Due to Stress Responses

The phenotypic modulation of fungi in response to stress due to micro-environmental conditions and the adaptation of heterogeneity within fungi play vital roles in increasing resistance to antifungal agents (Shapiro et al. 2011). Thermal stress, oxidative stress, ionic stress and osmolality are micro-environmental stresses involved in fungal biofilms. Thermal, ionic and oxidative stressors, in addition to osmolality, are likely to be involved within the micro-environment of a biofilm, particularly in vivo. The adaptation of fungi to stress and biofilm development is controlled by the MAPK signal transduction network (Cannon et al. 2007). In *C. albicans*, biofilms development involves Mck1p and the mutation of this gene increased the sensitivity of biofilms towards azoles (Cannon et al. 2007; Kumamoto 2005). The calcineurin pathway is also involved in the stress tolerance response employed in fungi and plays an important role in biofilms and increases resistance (Steinbach et al. 2007).

10.6.4 Altered Synthesis of Ergosterol Increases Resistance

Azoles are fungicidal for moulds such as *Aspergillus* species and are fungi-static for yeasts, including *Candida* spp. However, gradually, *C. albicans* can acquire resistance to high levels of azoles due to the alteration of Erg11 (Anderson 2005). In the yeast, the azoles inhibit ergosterol biosynthesis, though blocking enzyme 14 α-demethylase encoded by ERG11 results in the accumulation of toxic sterol pathway intermediates (Akins 2005; Cannon et al. 2007). The main gene Erg11 could be overexpressed or develop mutation. A high level of ergosterol has been detected in early-phase biofilms, whereas it was lower in intermediate or mature biofilms (Mukherjee et al. 2003). Also, the ergosterol fluctuation in biofilms confers resistance against both azole- and polyene-derived antifungal agents (Khot et al. 2006).

10.6.5 Biofilm Resistance Due to Growth Rate

Most of the antimicrobial agents developed in the past are effective on the planktonic cells, which act on the active, rapidly growing cells. However, the cells in biofilms grow slowly and with altered metabolism in nutrient-limited, adverse conditions. For example, the azole inhibits actively growing cells; however, it is ineffective on slowly growing biofilm cells.

10.6.6 Biofilm Resistance Due to Cell Density

The fungal biofilms are characterised by high cell density, which confers resistance to biofilms. The fungal biofilms have a dynamic structure, which consists of high cell density arranged in a stack, micro-colonies and water channels for nutrients supply, aeration and waste transportation. The specialised architecture of biofilms prevents the diffusion of antifungal agents, which results in increasing resistance for antifungal agents (Chandra et al. 2001; De Beer et al. 1994; Lawrence et al. 1991). The high cell density facilitates the communication and coordination behaviours. It is well documented that fungi in biofilms communicate and coordinate their activities through a mechanism called quorum sensing, which is mediated by small molecules called auto-inducers (Miller and Bassler 2001). In *C. albicans*, farnesol is a quorum sensing molecule (Hornby et al. 2001).

10.6.7 Biofilms are a Source of Persister Cells

The biofilms' mode of growth prevents the antimicrobial agents from diffusing uniformly inside biofilms; therefore, cells deep inside the biofilm remain unaffected by antifungal agents. A subset of cell populations spontaneously enter in the dormant, non-dividing state, resulting in persister cells. The persister cells are a population of cells that are dormant and highly recalcitrant to antimicrobial challenge (Lewis 2010). Upon antimicrobial treatment, the persister cells survive and, on discontinuation of the antimicrobial therapy, the persistent cells can restore the biofilms. The persister cells with altered cell membrane and independence from efflux pumps have been detected in the *Candida* biofilms treated with amphotericin B (Khot et al. 2006; LaFleur et al. 2006; Al-Dhaheri and Douglas 2008).

10.7 Conclusions

Filamentous fungi and yeast both form biofilms on biotic or abiotic surfaces. Fungal biofilms are highly organised communities involved in various infections of both humans and animals. The filamentous fungi and yeasts form biofilms as single-species biofilms or in combination with bacteria, called multi-species biofilms. In multi-species biofilms, a cooperativity between bacteria and fungi has been detected, which enhances the survival opportunity of both species. Fungal biofilms are resistant to antifungal agents and the host defence. The ECM constituents and the architecture of the fungal biofilm contribute to the resistance of biofilms against antifungal agents. The biofilm matrix consists of polymer substances that hinder the diffusion of antifungal agents into the biofilms. As a result, the microbial cells deep inside the

biofilms are relatively unaffected by antimicrobial agents and give rise to persister cells. Upon antimicrobial treatment, the persister cells survive and, on discontinuation of the antimicrobial therapy, the persister cells can restore the biofilms. The persister cells, with altered cell membrane and independence from efflux pumps, have been detected in the *Candida* biofilms treated with amphotericin B. The biofilm mode of growth plays an important role in infectious disease in animals and a source of persister cells gives rise to difficult-to-treat infections, resulting in increased treatment cost and recovery time.

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