Chapter 10
Antifungal Resistance in Animal Medicine: Current State and Future Challenges

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10.1 Introduction

Antibiotic resistance is a dominant research area due to the high prevalence of nosocomial infections caused by multidrug-resistant bacterial strains, and the dramatic impact of such infections on the healthcare system and the global economy (Prestinaci et al. 2015). In contrast, resistance to antifungal drugs has received much less attention, even when the occurrence of fungal diseases is far from negligible (Delarze and Sanglard 2015). Actually, surveillance systems to monitor the incidence of fungal diseases and antifungal resistance are still suboptimal and often rely on not-for-profit initiatives, such as the "Global Action Fund for Fungal Infections" (GAFFI, http://www.gaffi.org/). Information about the burden of fungal infections and antifungal resistance is even scarcer in veterinary medicine, as fungal diseases of animals have been more neglected than human mycoses (Rochette et al. 2003; Kwon-Chung 2018).

The current limited antifungal armamentarium and the slow pace at which new drugs become available represent major challenges for clinicians (Beardsley et al. 2018). Furthermore, the antifungal drugs currently available have important limitations, including their high cost, remarkable toxicity to animal cells, poor bioavailability, and/or relative inefficacy (Beardsley et al. 2018; Elad 2018). In this context, the emergence and escalation of resistance to antifungal drugs are causing great concern in the scientific community, as exemplified by the inclusion of the

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multidrug-resistant species *Candida auris*, other drug-resistant species of genus *Candida*, and azole-resistant *Aspergillus fumigatus* in the updated list of "Antibiotic Resistance Threats in the United States" published by the Centers for Disease Control and Prevention, under the categories of "urgent threats," "serious threats," and "watch list," respectively (CDC 2019). Similarly, reports from different countries suggest that antifungal resistance is also prevalent among fungal isolates of animal origin (e.g., Cafarchia et al. 2012b, c, 2015; Cordeiro et al. 2015; Talbot et al. 2015; Álvarez-Pérez et al. 2016c; Brilhante et al. 2016). However, the actual impact of antifungal resistance on animal health and the farming system is mostly unknown, as animal mycoses have traditionally received much less attention than those affecting humans, and antifungal susceptibility testing of animal isolates is still uncommon (Rochette et al. 2003; Álvarez-Pérez et al. 2016c).

In this chapter, we present an overview of the current knowledge on antifungal resistance of animal pathogenic fungi. However, a detailed account of the information available for the different species of yeasts and filamentous fungi of veterinary importance and different animal groups (e.g., pets, farm animals, and wildlife) is beyond the scope of this chapter. Instead, we focus on some general aspects that may be of greater interest for the non-expert reader. In addition, we discuss some issues that, in our view, should be addressed in the near future to optimize antifungal therapies in the veterinary setting and minimize the impact caused by resistant strains.

10.2 Antifungal Therapy in Animal Medicine

Antifungal therapy is a central component of human and animal protection against fungal infections (Seyedmousavi et al. 2018). However, despite recent advances in antifungal pharmacology, therapeutic options are still limited. In particular, most antifungal drugs currently available belong to a few compound classes, namely the polyenes, the azoles, the echinocandins, the allylamines, and the nucleoside analogs (Table 10.1). Overall, the azoles are the antifungal class most widely used for the treatment and prophylaxis of human and animal mycoses, and these compounds also represent a mainstay for crop protection against fungal infections and material preservation (Fisher et al. 2018; Seyedmousavi et al. 2018). Chemically, the azoles are heterocyclic organic molecules that contain a core azole ring with two or three nitrogen atoms, and this characteristic is used to differentiate two subclasses: the imidazoles and the triazoles, respectively (Table 10.1). Other compounds with antifungal activity, such as griseofulvin, chlorhexidine, ciclopirox, salicylic acid, and tolnaftate, are often used to treat dermatophytosis and other superficial mycoses (Dias et al. 2013; Moriello et al. 2017; Bond et al. 2020). Additionally, several new antifungals that may be more advantageous than the current ones, both in terms of overcoming antifungal resistance and avoiding adverse effects and drug–drug interactions, are currently under preclinical and clinical evaluation (Wiederhold 2017;

Table 10.1 Overview of the main antifungal classes used in human and animal medicine[a]

Compound class	Mode of action	Representative compounds
Allylamines	Non-competitive inhibition of the squalene epoxidase, an enzyme that participates in the fungal ergosterol biosynthesis pathway	Terbinafine
Azoles	Inhibition of the synthesis of ergosterol from lanosterol in the fungal cell membrane by binding of the free nitrogen atom of the azole ring to the iron atom of the heme group of the fungal enzyme cytochrome P450 lanosterol 14-α-demethylase (CYP51 or Erg11p). Such inhibition depletes ergosterol, and methylated sterols accumulate in the cell membrane, which inhibits fungal growth or induces cell death	Imidazoles (2 N atoms in the azole ring): clotrimazole, enilconazole (imazalil), ketoconazole, and miconazole Triazoles (3 N atoms in the azole ring): fluconazole, isavuconazole, itraconazole, posaconazole, and voriconazole
Echinocandins	Inhibition of the β-1,3-D-glucan synthase, which catalyzes the biosynthesis of β-1,3-D-glucan, a key component of the fungal cell wall	Anidulafungin, caspofungin, and micafungin
Nucleoside analogs	Incorporation into RNA instead of uracil (after conversion into 5-fluorouracil [5-FU] and subsequent phosphorylation), which leads to miscoding and disruption of protein synthesis by fungal cells. Additionally, phosphorylated 5-FU is converted to its deoxynucleoside and can block DNA synthesis by inhibiting the thymidylate synthase, thus leading to the disruption of DNA replication.	Flucytosine (5-fluorocytosine).
Polyenes	Binding to the ergosterol in the fungal cell membrane, which results in the formation of transmembrane pores that disrupt cell membrane integrity and lead to cellular damage and, eventually, to cell death	Amphotericin B, nystatin

[a]Source of data: Foy and Trepanier (2010), Mazu et al. (2016), Fisher et al. (2018), Seyedmousavi et al. (2018), and Gintjee et al. (2020)

Gintjee et al. 2020), but the spectrum of action of such compounds and their applicability to the treatment of different animal species remain to be established.

One of the main limitations of antifungal therapy in human and animal medicine is the toxicity and other serious adverse effects of most available compounds, which prevent their prolonged use or dosage escalation (Wiederhold 2017; Antonissen and Martel 2018; Elad 2018). Drug–drug interactions and reduced water solubility are other drawbacks of most currently available antifungals (Gubbins and Amsden 2005; Wiederhold 2017; Antonissen and Martel 2018). In this regard, combination antifungal therapy is gaining popularity as a potential strategy to enhance the efficacy of treatments while reducing some of their side effects (Johnson and Perfect 2010; Belanger et al. 2015).

On the other hand, the difficulties in diagnosing some human and animal mycoses often lead to an advanced stage of the infection when the treatment is prescribed (Rochette et al. 2003; Ostrosky-Zeichner 2012). Early diagnosis of systemic fungal diseases remains challenging because the clinical signs are unspecific and, in most cases, there are no reliable non-invasive diagnostic tests available (Ostrosky-Zeichner 2012; Antonissen and Martel 2018). Consequently, antifungal therapy in human and animal patients is often administered empirically, before a definite diagnosis of fungal infection is made (Klastersky 2004; Antonissen and Martel 2018).

Apart from the aforementioned issues, antifungal therapy in the veterinary setting has some specific limitations. For example, there is still scarce information about the pharmacokinetics and optimal dosage of currently available antifungal drugs in most animal species, and such parameters can display large interspecies and even interindividual variability, which significantly determines drug safety and efficacy (Rochette et al. 2003; Antonissen and Martel 2018). Moreover, veterinary experience with some antifungals is yet too limited to allow a detailed analysis of their possible side effects in most animal species (Elad 2018). Additionally, only a few antifungals are licensed for use in animals, and, consequently, off-label use of drugs approved for human therapy is quite common (Rochette et al. 2003; Antonissen and Martel 2018; Seyedmousavi et al. 2018). Nevertheless, many of the newer drugs used in human medicine are cost-prohibitive in veterinary settings, thus limiting their use in the routine practice (Foy and Trepanier 2010; Elad 2018). Even when some antifungals that have come off patent are currently more accessible, they are still not an option for prolonged therapy. This issue is non-trivial and often results in discontinuation of the antifungal therapy before complete clinical recovery (Nakasu et al. 2020). The stress and/or other difficulties generated by repeated drug administration to some animal species, in particular to wild animals, should also be taken into account (Elad 2018).

Finally, environmental considerations have great importance in the management of animal mycoses, especially of those affecting farm animals. In general, fungal infections of livestock, poultry, and other farm animals should be treated as herd conditions rather than as individual infections. Although the infection source of animal mycoses may vary, this is in most cases the environment (Asfaw and Dawit 2016; Elad 2018; Elad and Segal 2018). Therefore, prevention measures mostly focus on reducing the environmental fungal load in the farm facilities and avoiding the unhygienic management of animals. For example, strategic treatment of conditions such as dermatophytosis should always include measures for environmental decontamination, so as to prevent re-infections and/or the spread of the infection to other animals or human hosts once the antifungal therapy is discontinued (Rochette et al. 2003). Environmental decontamination is also important to prevent outbreaks of avian aspergillosis (Nawrot et al. 2019). Some antifungals such as enilconazole (also known as imazalil), thiabendazole, or natamycin are available for environmental decontamination as emulsifiable concentrates and/or smoke generator formulations (Rochette et al. 2003). Environmental factors may also be important in the prevention and management of the fungal infections of pets, but individualized

antifungal prophylaxis and/or treatment based on the animal's clinical history are often crucial for a successful outcome (Moriello et al. 2017; Barrs and Talbot 2020).

10.3 Antifungal Resistance: General Concepts and Study Methods

The rapid worldwide emergence of resistance to antifungal drugs represents a major threat to human and animal health and food security (Fisher et al. 2018). Antifungal resistance can arise in the clinical setting under prolonged therapy or, alternatively, through resistance selection upon long-term exposure of the microorganism to sub-lethal concentrations of the compounds in the environment due to the widespread use of fungicides in diverse applications (e.g., agriculture, preservation of materials, disinfection of farm facilities, etc.) (Azevedo et al. 2015; Perlin et al. 2017; Beardsley et al. 2018; Seyedmousavi et al. 2018). A detailed analysis of the environmental origin of antifungal resistance is out of the scope of this chapter, but the reader is referred to the magnificent studies and review articles on this issue published in recent years (e.g., Berger et al. 2017; Schoustra et al. 2019). Besides, some fungi display intrinsic resistance to certain antifungals (Delarze and Sanglard 2015; Perlin et al. 2017). Regardless its origin, antifungal resistance can worsen the clinical outcome and even result in clinical failure (Beardsley et al. 2018).

At the molecular level, antifungal resistance occurs through various non-exclusive mechanisms, including the following: (a) non-synonymous point mutations within the gene encoding the target enzyme; (b) increased expression of the target enzyme; (c) decreased concentrations of the drug within fungal cells due to drug efflux; and (d) reduced production of the target of the antifungal drugs due to changes in the biosynthetic pathway (Perlin et al. 2017; Beardsley et al. 2018; Fisher et al. 2018; Seyedmousavi et al. 2018). Biofilm formation, which reduces the drug concentration by trapping it into polysaccharide-rich matrices, is another important resistance mechanism in some fungal species (Perlin et al. 2017). These mechanisms of antifungal resistance can occur either alone or concomitantly in a single isolate, and can produce additive effects or lead to cross-resistance among different drugs (e.g., different azoles) (Perlin et al. 2017).

In vitro susceptibility testing is key for comparing the susceptibility of different fungal species and strains against the different antifungal drugs and determining resistance rates. The most popular methods for in vitro antifungal susceptibility testing of filamentous fungi and yeasts are those based on the guidelines developed by the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2008b, c) and the European Committee on Antifungal Susceptibility Testing (EUCAST) (Arendrup et al. 2020a, b). Despite some technical differences, both the CLSI and EUCAST procedures are broth microdilution methods, where the growth of isolates is evaluated in a series of increasing concentrations of an antifungal agent, prepared by serial dilution with growth medium (Beardsley et al. 2018; Elad and Segal 2018;

Table 10.2 Basic concepts in antifungal susceptibility testing

Concept[a]	Definition
Minimum inhibitory concentration (MIC)	Lowest concentration of an antifungal agent that prevents visible growth of a fungal strain in a susceptibility test. The MIC refers to some defined test conditions (e.g., incubation time and temperature) and end point (e.g., 80% or 50% reduction in growth respective to the growth control).
Minimum effective concentration (MEC)	Lowest concentration of an antifungal drug resulting in morphological changes (growth of small, rounded, compact hyphal balls) compared with the filamentous hyphal growth seen in control wells. MECs are mostly defined for filamentous fungi and fungistatic drugs (e.g., echinocandins).
Minimal fungicidal concentration (MFC)	Lowest concentration of an antifungal drug required to achieve fungicidal killing, generally defined as a 99.9% reduction in the initial inoculum (colony-forming unit (CFU) count).
Clinical break point (CBP)	MIC threshold used to classify fungal isolates as "susceptible" or "resistant" to a given antifungal. CBPs for isolates that cannot be included in the aforementioned categories (e.g., "susceptible-dose dependent" and "intermediate" isolates) have also been defined for some species–antifungal combinations.
Epidemiological cutoff value (ECV/ECOFF[b])	MIC threshold used to classify fungal isolates as "wild type" (i.e., without any phenotypically expressed resistance mechanism) or "non-wild type" (i.e., showing phenotypically expressed resistance mechanism). For a given antifungal, the ECV/ECOFF is the upper limit of the wild-type population and usually includes 90–95% of the strains.

[a]MICs, MECs, MFCs, CBPs, and ECVs are usually expressed in terms of mg/l or μg/ml (but note that CBPs and ECVs can also refer to inhibition zone diameters)
[b]Abbreviations used by the CLSI and EUCAST, respectively

Sanguinetti and Posteraro 2018). The results of these tests are expressed as minimum inhibitory concentration (MIC), minimum effective concentration (MEC), or minimum fungicidal concentration (MFC) values (Table 10.2). There are also diverse commercial systems for antifungal susceptibility testing, including the agar-based Etest and the broth microdilution method Sensititre (Beardsley et al. 2018; Elad and Segal 2018; Sanguinetti and Posteraro 2018) (Fig. 10.1). Additionally, homemade or commercial four-well azole-supplemented screening plates containing itraconazole (4 mg/l), posaconazole (0.5 mg/l), voriconazole (2 mg/l), and no antifungal (growth control) in each of the wells have emerged as an inexpensive, rapid screening method for azole resistance in *A. fumigatus* and other aspergilli (Arendrup et al. 2017; Guinea et al. 2019).

Due to the difficulty of establishing reliable clinical breakpoints (CBPs) to classify fungal isolates as susceptible or resistant to a given drug, the CLSI and EUCAST have proposed the definition of epidemiological cutoff values (ECVs or ECOFFs) for different species–antifungal combinations (CLSI 2020; https://mic.eucast.org/Eucast2/). Such ECVs/ECOFFs split the fungal populations into wild-type strains and non-wild-type strains, where the latter are those strains that may present any phenotypically expressed resistance mechanism and are less likely to respond to a given antifungal agent (Beardsley et al. 2018; Sanguinetti and Posteraro 2018)

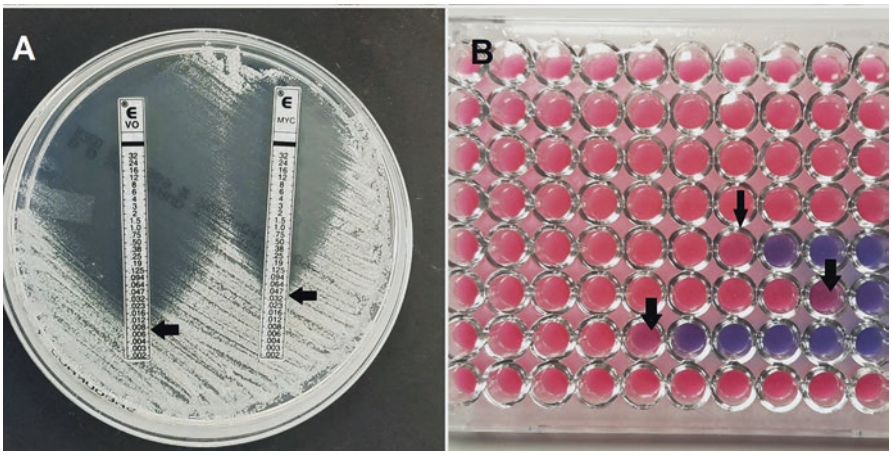

Fig. 10.1 Some examples of commercial methods for in vitro antifungal susceptibility testing. (**a**) Agar-based Etest, testing the susceptibility of a yeast strain against voriconazole (VO) and micafungin (MYC). Note the growth inhibition ellipses and the black arrows indicating the minimum inhibitory concentration (MIC; i.e., point where the inhibition halo intersects the Etest strip). (**b**) Sensititre YeastOne plate, testing (by a broth microdilution method) the susceptibility of the same yeast strain as in panel A against nine different antifungals (one per row of the microplate, plus an additional one in the last column). The MIC end points were defined as the lowest concentration of antifungal drug preventing the development of a pink color (i.e., first blue or purple well), and are indicated by black arrows

(Table 10.2). However, classification of a fungal isolate as resistant or non-wild type does not necessarily mean clinical failure, as there are many different factors that contribute to the clinical outcome, including host factors (e.g., animal species, immune state, type of infection, and comorbidities), therapeutic factors (e.g., pharmacokinetics/pharmacodynamics, dosage regime, administration route, toxicity, and compliance with the treatment), and ancillary factors (e.g., environmental decontamination) (Ostrosky-Zeichner and Andes 2017; Beardsley et al. 2018). In any case, the value of in vitro detection and characterization of antifungal resistance should not be overlooked, as these can assist clinicians to select the best drug regimen (Beardsley et al. 2018).

10.4 Antifungal Resistance in the Veterinary Setting

The results of recent studies dealing with the in vitro susceptibility testing of fungal isolates of animal origin suggest that antifungal resistance is relatively common among isolates from diverse host species, and that even healthy individuals can serve as a reservoir of resistant strains (Cordeiro et al. 2015; Cafarchia et al. 2012a, b, c, 2015; Talbot et al. 2015; Álvarez-Pérez et al. 2016c; Brilhante et al. 2016; Rocha et al. 2017). Nevertheless, resistance figures vary widely depending on the

animal species, the geographical location, and the methods used for in vitro susceptibility testing. In general, a major limitation of such studies is their small sample size, especially when compared with similar studies that focus on human isolates. Additionally, until recently, antifungal susceptibility testing of veterinary isolates was in most cases performed by non-standardized methods using different test conditions (antifungal panels, incubation time and temperature, MIC end points, etc.), which further hinders the direct comparison of results across studies. In particular, as not all fungi grow well in the synthetic medium (RPMI 1640) used in the CLSI and EUCAST protocols, some methodological adjustments have to be introduced for testing fungal species with special growth requirements. Such is the case, for example, of the yeast *Malassezia pachydermatis*, for which Sabouraud dextrose broth supplemented with 1% (v/v) of Tween 80 is recommended as test medium for the CLSI-based method (Cafarchia et al. 2012a, b, c, 2015; Álvarez-Pérez et al. 2014a). Additionally, susceptibility testing of slow-growing, scantly sporulating filamentous fungi such as *Microsporum canis*, *Trichophyton mentagrophytes*, and other dermatophyte species remains particularly challenging, as the MIC results depend largely on the type of inoculum used for the assays (conidia, hyphae + conidia, or arthroconidia) and other test conditions (Favre et al. 2003; Aneke et al. 2020).

Another limitation of most in vitro susceptibility studies testing animal isolates is that when antifungal-resistant isolates are detected, these are generally not studied for presence of gene mutations or other characteristics (potentially) responsible of the resistant phenotype. Luckily, this aspect is changing in recent years, and the number of publications reporting mechanisms of antifungal resistance in isolates of animal origin is increasing, as illustrated by the examples below.

One of the most detailed reports of antifungal resistance among fungal isolates of animal origin is that of Rocha et al. (2017), who studied potential mechanisms of azole resistance in *Candida albicans* strains recovered from different mammal and avian species. The authors concluded that azole resistance in this yeast species is a multifactorial process that involves increased efflux pump activity and the overexpression of different genes, including *ERG11*, which encodes the azole target 14α-sterol demethylase, the multidrug-resistant 1 (*MDR1*) gene of the major facilitator superfamily (MFS), which encodes proton-dependent efflux pumps, and the efflux pump genes for *Candida* drug resistance 1 (*CDR1*) and 2 (*CDR2*) (Rocha et al. 2017). In contrast, the ergosterol content of fungal cell walls showed no significant differences between resistant and susceptible strains (Rocha et al. 2017). The results of Castelo-Branco et al. (2020) further confirmed that efflux-mediated mechanisms are involved in the azole resistance of *Candida* spp. isolates from animals.

Another study dealing with potential mechanisms of antifungal resistance in *Candida* isolates of animal origin is our report of multi-azole resistance acquisition by *Candida tropicalis* in a dog with urinary candidiasis (Álvarez-Pérez et al. 2016b). Multi-azole resistance appeared after prolonged fluconazole therapy followed by a five-day course of bladder irrigation with amphotericin B, both of which were unsuccessful in controlling the yeast infection. Notably, pre- and post-azole

treatment isolates were clonally related and had identical silent mutations in the *ERG11* gene, but post-treatment isolates displayed increased azole MICs. Furthermore, a novel frameshift mutation in the *ERG3* gene, which encodes for sterol $\Delta^{5,6}$-desaturase, was found in some isolates recovered after resistance development, so it is unlikely that this mutation was responsible for the multi-azole-resistant phenotype (Álvarez-Pérez et al. 2016b).

Mechanisms of antifungal resistance have also been studied in non-*Candida* yeasts of veterinary origin, including *M. pachydermatis* and *Cryptococcus gattii*. For example, Kim et al. (2018) compared the whole genome sequences of a ketoconazole-resistant isolate of *M. pachydermatis* retrieved from the ear canal of a dog with otitis externa and the type strain of the same yeast species and found that a ~ 84-kb region in the chromosome 4 of the clinical isolate was tandemly quadruplicated. Notably, such quadruplicated region contained 52 protein-encoding genes, including homologs of *ERG11* and *ERG4* (which encodes sterol C-24 reductase). Moreover, transcriptome analysis indicated an overexpression of both *ERG11* and *ERG4* (3.68- and 2.81-fold, respectively) in the ketoconazole-resistant isolate (Kim et al. 2018). Soon thereafter, Kano et al. (2019) reported the isolation of a strain of *M. pachydermatis* from a case of canine dermatitis that displayed elevated MICs to itraconazole and ketoconazole. The combination of itraconazole and the calcineurin inhibitor FK506, which can reverse multidrug resistance in different types of eukaryotic cells by blocking ATP-dependent efflux pumps, exerted an additive effect against the azole-resistant strain (Kano et al. 2019). Furthermore, the studied strain had two missense mutations (A412G and C905T) in the sequence of the *ERG11* open reading frame, but the relationship between those mutations and azole tolerance was not further investigated (Kano et al. 2019). More recently, the same research group studied the in vitro susceptibility to ravuconazole of 13 isolates of *M. pachydermatis* retrieved from clinical cases of canine dermatitis and detected one isolate with an MIC >32 mg/l (Kano et al. 2020). The ravuconazole-resistant isolate was also resistant to clotrimazole, miconazole, and voriconazole, and had a G1382A substitution in the *ERG11* gene (Kano et al. 2020). In contrast, Sykes et al. (2017) did not find any mutation in the sequences of *ERG11* and the efflux pump gene *PDR11* of isogenic fluconazole-susceptible and fluconazole-resistant isolates of *C. gattii* retrieved from a case of invasive cryptococcosis in a domestic longhair cat. However, an increase in the number of copies and overexpression of *ERG11* and *PDR11* were detected in the post-treatment-resistant isolate compared to the fluconazole-susceptible isolate collected prior to initiation of antifungal therapy (Sykes et al. 2017). Moreover, reversion to wild-type susceptibility was observed when the resistant isolate was maintained in antifungal-free media, thus confirming the in vivo development of fluconazole resistance (Sykes et al. 2017).

Regarding the filamentous fungi, potential mechanisms of antifungal resistance among animal isolates have been mainly investigated in *A. fumigatus* (but see, for example, Talbot et al. (2019) for a study focusing on the members of the *Aspergillus viridinutans* species complex). For instance, Wang et al. (2014) examined *A. fumigatus* collected in avian farms from France (n = 57) and southern China (n = 51) where azole chemoprophylaxis was and was not performed, respectively. Although

all tested isolates were susceptible to itraconazole, posaconazole, and voriconazole, sequencing of the *cyp51A* gene, which encodes the cytochrome P450 14-α sterol demethylase in *A. fumigatus*, for a selection of 61 isolates revealed 11 isolates with a total of 20 point mutations (Wang et al. 2014). Eleven of such point mutations were silent, but the other nine yielded amino acid substitutions (Wang et al. 2014). Similarly, Talbot et al. (2015) analyzed the azole resistance in canine and feline isolates (n = 46 and 4, respectively) of *A. fumigatus* collected between 1988 and 2014, and identified an isolate from 1992 showing multi-azole resistance and a F46Y point mutation in the *cyp51A* gene that seems to be associated with azole resistance. Finally, Bunskoek et al. (2017) reported a case of azole-resistant invasive aspergillosis in a female captive bottlenose dolphin (*Tursiops truncatus*). The *A. fumigatus* strain recovered from this case showed in vitro resistance to itraconazole, posaconazole, and voriconazole, and harbored the TR_{46}/Y121F/T289A mutation in the *cyp51A* gene (Bunskoek et al. 2017), which is associated with environmental resistance selection (van der Linden et al. 2013). Fortunately, the animal was successfully treated with high-dose posaconazole that reached plasma levels >3 mg/l (Bunskoek et al. 2017).

The main conclusion extracted from the aforementioned examples is that susceptibility testing and the study of gene mutations and other mechanisms involved in antifungal resistance can provide very useful information for veterinary professionals, including, for instance, data about the epidemiology of antifungal resistance in the studied animal population(s) and about the treatment options to fight infections that are refractory to standard treatments.

10.5 Future Challenges

Despite recent advances in the study of the prevalence and mechanisms of antifungal resistance among fungal pathogens of animals, there are still some issues that, in our view, should be further addressed in order to optimize antifungal therapies in animal medicine and minimize the impact caused by resistant species and strains including, for example: (a) species-level identification of animal pathogenic fungi; (b) establishment of meaningful breakpoints for antifungal resistance of veterinary isolates; and (c) reduction of the environmental impact of antifungal use. These aspects are briefly described below.

10.5.1 Species-Level Identification of Animal Pathogenic Fungi

Many fungal species cannot be reliably identified based on phenotypic features alone, and a polyphasic approach combining morphological, metabolic, ecological, and (phylo)genetic data is often required for better taxonomic resolution (Crous et al. 2015). This is the case, for example, of many members of the genera *Aspergillus* and *Candida* (Howard 2014; Criseo et al. 2015; Paulussen et al. 2017; Barrs and Talbot 2020), including relevant animal pathogens. Furthermore, the term "cryptic species" has been coined to describe recognized morphospecies that represent a suite of (almost) indistinguishable taxa according to macro- and microscopic criteria, but are clearly different based on phylogenetic inference (Crous et al. 2015). Notably, some of these cryptic species also have different ecology (including host range and pathogenicity), geographic distribution, and/or antifungal susceptibility patterns than their sibling species (Cendejas-Bueno et al. 2012; Howard 2014; Crous et al. 2015; Barrs and Talbot 2020). For example, *Aspergillus felis* is an emerging agent of invasive aspergillosis in cats, dogs, and humans which phenotypically resembles its close relatives *A. viridinutans* and *A. fumigatus* but can be differentiated from these by molecular-based methods and often displays itraconazole and voriconazole cross-resistance (Barrs et al. 2013; Álvarez-Pérez et al. 2014b; Barrs and Talbot 2020). Therefore, accurate identification of clinical isolates to the species level may be helpful for effective antifungal treatment. However, some cryptic species do not have predictable susceptibility patterns, so in vitro susceptibility remains as an invaluable tool to aid directed antifungal therapy (Howard 2014). Unfortunately, to date, polyphasic identification and susceptibility testing of fungal isolates recovered from clinical cases are not included in the routine of most veterinary diagnostic laboratories, but are mostly executed during the course of research projects.

10.5.2 Establishment of Meaningful Breakpoints for Antifungal Resistance of Veterinary Isolates

Current efforts for defining CBPs and ECVs for antimicrobial drugs used in veterinary medicine, such as those headed by CLSI's Subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST) (CLSI 2008a, 2013) and EUCAST's subcommittee for Veterinary Antimicrobial Susceptibility Testing (VetCAST) (Toutain et al. 2017), mostly focus on antibacterial compounds. Furthermore, antifungal susceptibility testing has been standardized mostly using human isolates, and its predictive value for animal isolates remains to be determined (Elad 2018).

In absence of breakpoints for antifungal resistance of fungal isolates of animal origin, published reports on this issue show MIC results without any interpretation of these in terms of susceptibility or resistance (or wild type/non-wild type) (e.g.,

Table 10.3 Comparison of the tentative epidemiological cutoff values (ECVs) for animal isolates of *Candida* spp. with those previously established for human isolates[a]

Candida species	Origin of isolates	ECVs (μg/ml)				
		AMB	CAS	FCZ	ITZ	VCZ
C. albicans	Human	2	0.125	0.5	0.125	0.0312
	Animal	1	0.25	≥64	≥16	1
C. parapsilosis 'sensu lato'	Human	2	1	2	0.5	0.125
	Animal	1	2	4	0.5	NA
C. tropicalis	Human	2	0.125	2	0.5	0.0625
	Animal	1	1	≥64	≥16	1

[a]Abbreviations: *AMB* amphotericin B, *CAS* caspofungin, *ECVs* epidemiological cutoff values, *FCZ* fluconazole, *ITZ* itraconazole, *NA* not available, *VCZ* voriconazole. Source of data: Cordeiro et al. (2017), Castelo-Branco et al. (2020), and references therein

Álvarez-Pérez et al. 2014a; Talbot et al. 2019; Aneke et al. 2020), establish their own in-house breakpoints (e.g., Cafarchia et al. 2012b, c), or use the CBPs/ECVs established for human isolates of the tested fungal species (e.g., Wang et al. 2014; Cordeiro et al. 2015; Talbot et al. 2015; Álvarez-Pérez et al. 2016c; Brilhante et al. 2016) or other different species (e.g., Cafarchia et al. 2012a). Tentative ECVs for animal isolates of some yeast species such as *Candida albicans*, *Candida parapsilosis* 'sensu lato' and *Candida tropicalis* (Cordeiro et al. 2017; Castelo-Branco et al. 2020), and *Malassezia pachydermatis* (Cafarchia et al. 2015) have also been proposed. Notably, the fluconazole, itraconazole, and voriconazole ECVs proposed by Castelo-Branco et al. (2020) for *C. albicans* and *C. tropicalis* are remarkably higher than those determined for human isolates (Table 10.3), thus emphasizing the importance of azole resistance among *Candida* isolates from animals. Nevertheless, these tentative ECVs should be further validated by testing larger collections of fungal isolates of animal origin and by determining if non-wild-type isolates actually display any mechanism of antifungal resistance. Furthermore, it would be desirable to establish specific ECVs for different animal groups (e.g., small animals, horses, and ruminants), as the epidemiology of fungal infections and antifungal pressures may be different in each group.

10.5.3 Reduction of the Environmental Impact of Antifungal Use

Residuals of the antifungal compounds used in veterinary medicine can eventually enter the environment, especially when the treatment is applied topically or the compounds are used for fungal decontamination of hatcheries and other farm facilities (Chen and Ying 2015; Bártíková et al. 2016). Such environmental contamination with antifungal residuals may affect non-target fungi and potentially alter key ecosystem functions (Dijksterhuis et al. 2011; Dimitrov et al. 2014; Chen and Ying 2015; Álvarez-Pérez et al. 2016a). Furthermore, the presence of sublethal

concentrations of antifungals in the environment can select for fungal species and strains that are less susceptible to these compounds and, eventually, result in the emergence of resistant phenotypes that may become a threat for human and animal hosts (Faria-Ramos et al. 2014; Buil et al. 2019; Schoustra et al. 2019). Therefore, there is an urgent need to minimize the amount of antifungals released to the environment and reduce their potential side effects.

10.6 Conclusion

Despite recent advances in the study of the prevalence and mechanisms of antifungal resistance among fungal isolates of animal origin, there is still little public awareness about the relevance of antifungal resistance in veterinary medicine, especially when compared with the current focus on the emergence of antibiotic-resistant bacteria. Therefore, in our modest view, veterinarians and other animal health professionals should take action to demand more resources for improving the monitoring of fungal infections and antifungal resistances in veterinary clinics and the farming system worldwide. Additionally, a closer collaboration and improved data sharing between researchers working on animal mycology would also be welcome.

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