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Azole resistance in canine and feline isolates of *Aspergillus fumigatus*

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ABSTRACT

Azole resistance is an emerging cause of treatment failure in humans with aspergillosis. The aim of this study was to determine if azole resistance is emerging in *Aspergillus fumigatus* isolates from canine and feline sino-nasal aspergillosis cases. Susceptibilities of isolates collected between 1988 and 2014 from 46 dogs and 4 cats to itraconazole, posaconazole, voriconazole, fluconazole and ketoconazole were assessed using Sensititre YeastOne microdilution trays; and to enilconazole and clotrimazole, following the CLSI M38-A2 standard. For the majority of isolates MICs were high for ketoconazole, low for enilconazole and clotrimazole, and less than established epidemiological cut-off values for itraconazole, posaconazole and voriconazole. One canine isolate from 1992 had multiazole resistance and on *Cyp51A* gene sequencing a mutation associated with azole resistance (F46Y) was detected. There is no evidence of emerging azole resistance among *A. fumigatus* isolates from dogs and cats and topical azole therapy should be effective against most isolates.

1. Introduction

Azole antifungal drugs are pillars of treatment for aspergillosis in humans and animals. Since 1997, there has been an increasing number of reports of azole resistance amongst isolates of *Aspergillus fumigatus* from human patients with both invasive and non-invasive forms of aspergillosis. Azole resistance is associated with treatment failures and increased mortality [1–3]. Resistance to itraconazole alone or multiazole or panazole resistance has been reported globally [4–7].

Fungal azole resistance can be intrinsic or acquired. Intrinsic resistance is reported for cryptic *Aspergillus* species, whereas acquired resistance is increasingly described for *A. fumigatus sensu stricto* isolates [8,9]. Resistant strains have been identified in the environment and in human patients naïve to and previously exposed to azole therapy [10]. Resistance is associated with mutations in the *cyp51A* gene which encodes lanosterol 14α-demethylase, a component of the ergosterol synthesis pathway targeted by triazole drugs [9,11]. Emergence of resistance in Europe has been linked to triazole fungicide use in agriculture causing altered gene expression [12].

Clinical antifungal resistance is defined as persistence of infection despite treatment with an antifungal with proven *in vitro* activity against the infecting fungus [13]. *In vitro* resistance is difficult to define as although standardised methods of antifungal susceptibility testing have been developed [14–16], clinical breakpoints based on minimum inhibitory concentrations (MICs), pharmacological parameters, animal data and clinical outcomes, have not yet been established for *Aspergillus* species. Instead, epidemiological cut-off values (ECVs) of wild-type *A. fumigatus* isolates are used to evaluate clinical isolate minimum inhibitory concentrations (MICs). Correlation between *in vitro* and clinical outcomes is reported from invasive and non-invasive human aspergillosis cases [1–3].

Canine and feline sino-nasal aspergillosis (SNA) is most commonly caused by members of *Aspergillus* section *Fumigati*. Canine SNA is usually caused by *A. fumigatus* [17] while feline SNA is most commonly caused by *A. fumigatus* and *Aspergillus niger* [18]. These infections present therapeutic challenges for veterinarians, and involve the use of endosurgical procedures and topical and/or systemic antifungal azole administration. Topical clotrimazole (CLO) or enilconazole (ENL) are the mainstays of therapy, although there are limited *in vitro* studies to support the use of these drugs [19,20].

Abbreviations: SNA, sino-nasal aspergillosis; SOA, sino-orbital aspergillosis; ITZ, itraconazole; POS, posaconazole; FLU, fluconazole; KET, ketoconazole; VOR, voriconazole; AMB, amphotericin B; CAS, caspofungin; 5-FC, 5-flucytosine; CLO, clotrimazole; ENL, enilconaozle; MIC, minimum inhibitory concentration; MEC, minimum effective concentration.

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Response rates to treatment vary [18,21,22]. Whether emerging azole resistance contributes to treatment failures is unknown. To date no studies have systematically investigated the prevalence of azole resistance amongst *A. fumigatus* isolates from dogs and cats. To determine whether azole resistance is emerging amongst *A. fumigatus* isolates from dogs and cats susceptibilities to a panel of antifungal drugs were assessed in a retrospective surveillance study.

2. Materials and methods

2.1. Isolates

Fifty archived isolates of *A. fumigatus* from dogs ($n = 46$) and cats ($n = 4$) with confirmed SNA were retrieved from the fungal culture biobank of the Small Animal Infectious Diseases Clinical Research Group at the University of Sydney [17,21,23]. Isolates originated from Australia ($n = 33$), United States of America ($n = 10$) and Belgium ($n = 7$). Collection dates ranged from 1988 to 2013 inclusive [1988 ($n = 1$); 1992 ($n = 1$); 1994 ($n = 1$); 2000 ($n = 1$); 2004 ($n = 1$); 2005 ($n = 1$); 2006 ($n = 1$); 2007 ($n = 5$); 2008 ($n = 7$); 2009 ($n = 5$); 2010 ($n = 6$); 2011 ($n = 3$); 2012 ($n = 14$); 2013 ($n = 3$)].

2.2. Antifungal susceptibility testing

Isolates were sub-cultured onto malt extract agar and grown in the dark at 25 °C until sporulation occurred (7–14 days), then inoculum was prepared according to the Clinical Laboratory Standards Institute (CLSI) M38-A2 standard for filamentous fungi [16].

Susceptibility ($n = 50$) to ENL and CLO (BOVA Compounding Chemist, St Marys, Australia) was assessed following the recommendations for drug preparation, dilution, inoculum preparation, incubation and endpoint reading stipulated by the CLSI M38-A2 standard [16]. Final concentrations tested were two fold dilutions within the range 0.03–16 μg/mL for both compounds. The concentrations selected were based on *in vitro* susceptibilities of *A. fumigatus* to other azole drugs [16,24,25] and are lower than concentrations of 1–2% (10–20 mg/mL) used in topical preparations of clotrimazole and enilconazole for treatment of canine and feline SNA [19–21,24,25]. Quality control strains used as a standard to assess repeatability and accuracy of testing procedures were reference strains of *Candida parapsilosis* (ATCC 22019) and *A. fumigatus* (ATCC MYA-3626), according to the CLSI M38-A2 standard.

Susceptibility to amphotericin B (AMB), itraconazole (ITZ), ketoconazole (KCZ), voriconazole (VCZ) (range 0.008–16 μg/mL for all four compounds), posaconazole (POS) (range 0.008–8 μg/mL), fluconazole (FLU) (0.12–256 μg/mL) and 5-flucytosine (5-FC) (0.03–64 μg/mL) was assessed using Sensititre YeastOne YO8 microdilution trays according to the manufacturer's instructions. Susceptibility to caspofungin (CSP) (range 0.008–16 μg/mL) was assessed using the CLSI M38-A2 standard [16]. For those drugs for which pharmacokinetic data are available in dogs and/or cats including itraconazole, fluconazole, ketoconazole and posaconazole, reported C_{MAX} in serum or plasma after therapeutic dosing were within the range of drug concentrations tested in this study [26–31]. *C. parapsilosis* (ATCC 22019) was used as the quality control strain (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia).

For all isolates and both tray types, 10 μL from the growth control well was spread onto a Sabouraud's agar plate immediately after plate inoculation as a purity and inoculum density check. All trays were incubated at 35 °C for 72 h ± 2 h in an ambient air incubator. Endpoints for all drugs were determined after 72 h incubation. For CLO and ENL the endpoint was the lowest two-fold dilution revealing 100% growth inhibition (MIC). For AMB, ITZ, POS, VCZ, FLU, KCZ and 5-FC, the endpoints were as per the manufacturer's instructions (Trek Diagnostic Systems, Thermo Fisher Scientific, Scoresby, Australia). For CSP endpoints were read as MECs at the lowest dilution of round compact hyphal forms as per CLSI M38A2 [16]. Geometric mean, MIC_{50} and MIC_{90} values were calculated using Windows Excel (Microsoft Office, 2007, Redmond, United States of America).

In vitro azole resistance was defined as the absence of endpoint antifungal activity in the range of drug concentrations tested, except for FLU and 5-FC, as most filamentous fungi have intrinsic resistance to these drugs [16]. Multiazole resistance was defined as resistance to more than one antifungal azole. Decreased susceptibility was defined as MICs greater than established ECVs for wild-type *A. fumigatus*, established using CLSI standards [16].

2.3. Cyp51A gene sequencing

DNA was extracted from those isolates with possible ITZ resistance (*i.e.* MICs > 1 μg/mL) using the Roche High Pure PCR Template Preparation Kit (Castle Hill, Australia), with an additional bead beating step [32]. The coding region of *cyp51A* was sequenced as described previously [11]. Sequences were aligned with *cyp51A A. fumigatus* reference strain, GenBank reference AF338659 [33] and sequence from a reference laboratory isolate with a L98H *cyp51A* resistance mutation (obtained from National Mycology Reference Centre, SA Pathology, Adelaide, South Australia, 2014).

3. Results

Antifungal susceptibility results for all 50 *A. fumigatus* isolates from clinical specimens from dogs and cats with sino-nasal aspergillosis are listed in Table 1 and compared to established

Table 1
Antifungal susceptibility results for 50 *A. fumigatus* isolates from clinical specimens from dogs and cats and comparison with epidemiological cut-off values.* MIC/MEC (μg/mL) values reflect the number of isolates within the specific cut-off value.

Drug	MIC/MEC (μg/mL) distribution among tested isolates												GM	MIC_{50}	MIC90	ECV (μg/mL)
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	>16				
AMB						1	8	34	7				1.92	2	4	2
ITZ		2	13	16	17	1						1	0.13	0.12	0.25	1
VCZ			2	3	24	16	3	1	1				0.34	0.25	0.5	1
POS	5	13	20	8	3	1							0.06	0.06	0.12	0.5
KCZ							1	2	28	15	3	1	5.09	4	8	–
CSP†	16	25	8	1									0.03	0.03	0.06	1
CLO					2	31	15	1	1				0.64	0.5	1	–
ENL			7	27	11	4		1					0.15	0.12	0.25	–

* ECV, epidemiological cut off value [46–48]; MIC, minimum inhibitory concentration; MEC, minimum effective concentration (†); AMB, amphotericin-B; ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; KCZ, ketoconazole; CSP, caspofungin; CLO, clotrimazole; ENL, enilconazole; GM, geometric mean; MIC_{50}, minimum inhibitory concentration at which ≥50% of isolates in the group were inhibited; MIC_{90}, minimum inhibitory concentration at which ≥90% of isolates in the group were inhibited.

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