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Brief Report

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Low rate of azole resistance in cases of avian aspergillosis in Germany

Amelia E. Barber [1,*,†], Sandra Scheufen [1,†], Grit Walther [2], Oliver Kurzai [1,2] and Volker Schmidt [3]

[1]Institute for Hygiene and Microbiology, University of Wuerzburg, Wuerzburg, Germany, [2]National Reference Center for Invasive Fungal Infections (NRZMyk), Leibniz Institute for Natural Product Research and Infection Biology, Hans Knoell Institute, Jena, Germany and [3]Clinic for Birds and Reptiles, Faculty of Veterinary Medicine, University of Leipzig, Germany

*To whom correspondence should be addressed. Amelia Barber, PhD, Institute for Hygiene and Microbiology, University of Wuerzburg, Germany, 97080 Würzburg. E-mail: amelia.barber@leibniz-hki.de

†Denotes equal contribution.

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Abstract

Aspergillosis is the most common fungal disease of the avian respiratory tract. Due to delayed diagnosis and treatment failure, the outcome of these infections is often poor. We investigate 159 cases of avian aspergillosis among captive birds in Germany to define clinical features as well as the frequency of *in vitro* triazole resistance. Adult birds were more likely to present with clinical signs compared to juvenile birds, and dyspnoea was the most common clinical sign, present in 53% of birds. Molecular species identification indicated that all infections were caused by *Aspergillus fumigatus*. Only one of 159 independent isolates was azole resistant.

Key words: avian aspergillosis, azole resistance, *Aspergillus fumigatus*, veterinary infectious disease.

Aspergillosis is an important cause of respiratory disease in birds, most commonly caused by the saprophytic mould *Aspergillus fumigatus*.[1] These infections are frequently detected late, and a gold standard for diagnosis is lacking.[2] Clinical signs can be related to compromised respiratory function (rhinitis, change in vocalization, dyspnoea) but are often nonspecific (lethargy, loss of appetite, and anorexia). A definitive diagnosis of aspergillosis requires successful isolation of the fungal pathogen, and confirmation by histopathological or cytological findings.[1,2] Given these challenges in diagnosing aspergillosis in birds, one goal of this study was to define the incidence and prevalence of clinical features among infections caused by *A. fumigatus* in captive birds in Germany.

Voriconazole is currently the most effective compound for treating aspergillosis in avian cases.[3] However, resistance to voriconazole and other azoles in *A. fumigatus* strains from domestic and wild birds has been reported.[3–5] Acquired azole resistance in *A. fumigatus* also presents an increasing problem in human medicine.[6] As current models predict that the majority of azole resistance in *A. fumigatus* emerges in the environment, we aimed to define the incidence of azole resistance among cases of avian aspergillosis in captive birds in Germany.

Between 12/2015 and 11/2018, 159 cases of avian aspergillosis caused by *Aspergillus* section *Fumigati* were identified by the University of Leipzig Clinic for Birds and Reptiles. Cases were included in this study based on the fungal morphology following 5 days of growth on Sabouraud-Chloramphenicol-Gentamicin Agar (Oxoid, Germany) at a selective temperature of 41°C. Differential diagnosis was made through a combination of clinical presentation, radiology, endoscopy, bloodwork, and clinical microbiological and histopathological findings. Necropsies were also performed on 83 birds. Psittacines (*Psittaciformes*)

Table 1. Number of birds with avian aspergillosis listed according to their taxonomic order, natural origin, and husbandry.

	Natural origin			Accommodation			Husbandry condition		
Taxonomic order	Tropical	Temperate/subtropical	Subpolar	Indoor	Outdoor*	Zoo	Single	Pair	Group
Psittaciformes ($n = 93$)	86	7	0	66	26	1	39	47	7
Falconiformes ($n = 15$)	0	2	13	0	15	0	5	0	10
Passeriformes ($n = 10$)	5	5	0	1	5	4	0	1	9
Sphenisciformes ($n = 7$)	0	0	7	0	0	7	0	0	7
Accipitriformes ($n = 5$)	0	5	0	0	3	2	1	0	4
Columbiformes ($n = 5$)	1	4	0	1	3	1	0	1	4
Galliformes ($n = 4$)	3	1	0	0	0	4	0	0	4
Gruiformes ($n = 4$)	2	2	0	0	0	4	0	2	2
Anseriformes ($n = 3$)	0	2	1	0	2	1	0	0	3
Strigiformes ($n = 3$)	0	1	2	0	1	2	0	1	2
Charadriiformes ($n = 2$)	0	2	0	0	0	2	0	0	2
Ciconiiformes ($n = 2$)	1	1	0	0	0	2	0	1	1
Pelecaniformes ($n = 2$)	2	0	0	0	0	2	0	0	2
Trogoniformes ($n = 2$)	2	0	0	0	0	2	0	0	2
Bucerotiformes ($n = 1$)	1	0	0	0	0	1	0	1	0
Otidiformes ($n = 1$)	0	1	0	0	0	1	0	0	1
Total: $n = 159$	103 (65%)	33 (21%)	23 (14%)	68 (43%)	55 (35%)	36 (23%)	45 (28%)	54 (34%)	60 (38%)

*Including four wild birds.

were the most common taxonomic order affected ($n = 93$; 58%), followed by birds of prey (*Falconiformes*, *Accipitriformes*) ($n = 20$; 13%), passerines (*Passeriformes*) ($n = 10$; 6%), and penguins *Sphenisciformes*) ($n = 7$; 4%). Classification of birds according to their natural origin revealed 103 (65%) tropical birds, including the majority of the psittacines and the most common affected species, the African grey parrot (*Psittacus erithacus*) ($n = 39$; 25%). In sum, 23 (14%) birds of subpolar natural origin were present, including 13 (8%) gyrfalcons (*Falco rusticolus*) and their hybrids, as well as seven (4%) Humboldt penguins (*Spheniscus humboldti*) (Table 1, Suppl Table 1). Birds were most frequently kept by the owners in the household ($n = 68$; 43%) or outdoor aviaries ($n = 51$; 32%) or were sent from zoos ($n = 36$; 23%) and included both sexes (79 males; 70 females; 10 unknown) and various ages (43 juveniles; 116 adult) (Table 1). Additionally, one adult and three juvenile birds were free-living (wild). Of note, 35% of the examined psittacines had a previous history of antifungal treatment, primarily with voriconazole. In contrast, only one bird in the other taxonomic orders had a previously received antifungal therapy (Suppl. Table1).

The most common clinical signs were dyspnoea without respiratory sound ($n = 57$; 36%) and with respiratory sound ($n = 27$; 17%) (Suppl. Table 1). Rhinoliths were present exclusively in adult psittacines ($n = 33$; 21%) and were one cause of stridor in affected birds (Pearson correlation coefficient $\phi = 0.471$; $P < .001$). The other cause of dyspnoea with respiratory sound was aspergillomas in the syrinx ($\phi = 0.315$; $P = .001$). Birds with dyspnoea without respiratory sound were more often associated with aspergillomas of the lower respiratory tract (44 of 57 birds with dyspnoea without respiratory sound; 77%; $\phi = 0.248$; $P = .002$), especially of the air sacs (36 of 57 birds with dyspnoea without respiratory sound; 63%; $\phi = 0.211$; $P = .08$). All other clinical signs, including apathy, anorexia, regurgitation, wasting, or central nervous signs were not significantly associated with diagnosis of avian aspergillosis (Suppl. Table 1). However, lack of clinical signs prior to death was also common in birds with aspergillosis (50 of 83 necropsied birds; 60%; $\phi = 0.532$; $P < .001$). Adult birds with avian aspergillosis presented more often with clinical signs compared to juvenile birds (73% vs. 42%; $\phi = 0.285$; $P < .001$). The main postmortem finding was aspergillosis of the lung (86%), followed by aspergillosis of the air sacs (46%) or both (43%). Chronic infection, as defined by the presence of fibrinous aspergillomas was observed in 61% of postmortem cases. Acute aspergillosis of a single site represented 20% of postmortem cases, while 11% were invasive infections involving multiple sites.

From the described avian aspergillosis cases, we obtained 159 *Aspergillus* section *Fumigati* isolates. Isolates came from nasal swabs, endoscopic sampling of lungs or air sacs, or necroscopies. Species-level identification was made through partial sequencing of the calmodulin gene as in Hong et al.,[7] and all isolates were found to be *A. fumigatus*, with no cryptic species identified (CaM sequences deposited into GenBank under the accession numbers listed in Suppl. Table 2).

Azole susceptibility was screened using the agar-based VIPcheck™ assay (Mediaproducts BV, NL) following the manufacturer's suggested protocol.[8] Only two isolates showed growth on voriconazole agar (2 mg/l), with one of these isolates also showing growth on itraconazole-supplemented agar (4 mg/l). No isolates showed growth on the posaconazole

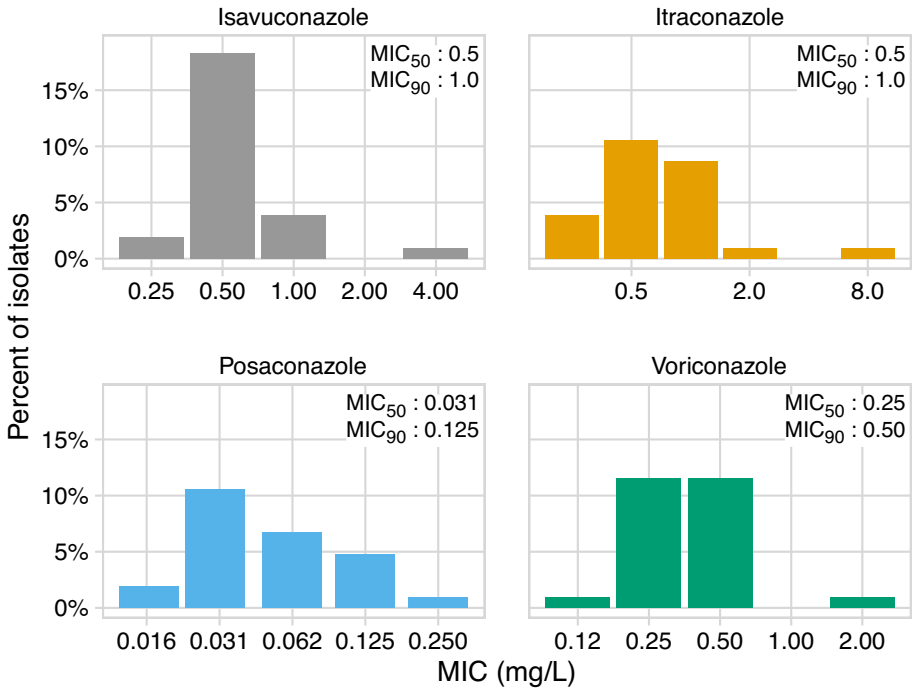

Figure 1. Azole susceptibility in avian *Aspergillus fumigatus* isolates. MIC distribution, MIC_{50}, and MIC_{90} values for isavuconazole, itraconazole, posaconazole, and voriconazole among 26 avian *A. fumigatus* isolates. MICs were determined using broth microdilution following EUCAST protocol E.DEF 9.3.1.

test wells (0.5 mg/l). These two isolates, plus an additional 24 randomly selected isolates were subjected to broth microdilution using EUCAST methodology to determine minimum inhibitory concentrations (MICs) to isavuconazole, itraconazole, posaconazole, and voriconazole. All four antifungals showed unimodal distributions, with no evidence of acquired resistance in the population (Fig. 1). No breakpoints exist for predicting the clinical response to therapy in birds, so MIC_{50} and MIC_{90} values were calculated and are indicated in Figure 1. Only the isolate that demonstrated growth on both itraconazole and voriconazole agar in the VIPcheck assay showed atypical azole MICs and would be considered resistant using the EUCAST breakpoints for human medicine. Sanger sequencing of the azole target gene, *cyp51a*, in this isolate as in Weber et al.[9] did not show any base pair substitutions definitively associated with azole resistance (sequence deposited in GenBank, acc. no. MT309495). However, this strain carries the previously reported polymorphisms at amino acid positions 46, 172, 248, 255, and 427 that are present in both azole-resistant and susceptible strains.[10] This isolate came from an indoor-housed grey parrot with no prior history of antifungal treatment. As azole-resistant, *cyp51a*-wildtype isolates are known to circulate in the environment, albeit at low frequency,[11–13] the most likely explanation for this occurrence, is that the bird was exposed to and became infected with one such strain via the normal environmental exposure route.

This study highlights the challenges of diagnosing aspergillosis in avian patients, as 60% of birds displayed no clinical signs prior to death. Dyspnoea with or without respiratory sound was the most common clinical sign and present in only 53% of birds. In line with previous studies, *A. fumigatus* was the primary etiologic agent of avian aspergillosis.[14,15]

The overall rate of triazole resistance in our study was low—less than 1% (1/159), similar to what has been observed in studies of poultry and domestic geese,[4,5,16] but lower than in another study of avian aspergillosis.[3] The observed frequency is significantly lower than rates reported from clinical *A. fumigatus* in Germany.[17,18] Whereas culture will not be positive in the majority of human patients, isolation of the causative organism is possible for most infected birds due to massive fungal growth in avian airways. Thus, determining resistance rates in strains cultured from avian infections may be more representative than culture-based studies on human infections. On the other hand, birds acquire *A. fumigatus* from different sources than human patients—for example, mulch used in aviaries—and these sources may carry different populations of *A. fumigatus* than human patients.

Supplementary material

Supplementary material is available at *MMYCOL* online.

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Declaration of interest

OK is an advisor to and has received lecture honoraria from Astellas, Basilea, and Pfizer. The other authors have no conflicts of interest to declare.

References

1. Beernaert LA, Pasmans F, Van Waeyenberghe L, Haesebrouck F, Martel A. *Aspergillus* infections in birds: a review. *Avian Pathol*. 2010; 39: 325–331.
2. Savelieff MG, Pappalardo L, Azmanis P. The current status of avian aspergillosis diagnoses: veterinary practice to novel research avenues. *Vet Clin Pathol*. 2018; 47: 342–362.
3. Beernaert LA, Pasmans F, Van Waeyenberghe L et al. Avian *Aspergillus fumigatus* strains resistant to both itraconazole and voriconazole. *Antimicrob Agents Chemother*. 2009; 53: 2199–2201.
4. Ziolkowska G, Tokarzewski S, Nowakiewicz A. Drug resistance of *Aspergillus fumigatus* strains isolated from flocks of domestic geese in Poland. *Poult Sci*. 2014; 93: 1106–1112.
5. Wang DY, Gricourt M, Arne P et al. Mutations in the Cyp51A gene and susceptibility to itraconazole in *Aspergillus fumigatus* isolated from avian farms in France and China. *Poult Sci*. 2014; 93: 12–15.
6. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science*. 2018; 360: 739–742.
7. Hong SB, Cho HS, Shin HD, Frisvad JC, Samson RA. Novel *Neosartorya* species isolated from soil in Korea. *Int J Syst Evol Microbiol*. 2006; 56: 477–486.
8. Guinea J, Verweij PE, Meletiadis J et al. How to: EUCAST recommendations on the screening procedure E.Def 10.1 for the detection of azole resistance in *Aspergillus fumigatus* isolates using four-well azole-containing agar plates. *Clin Microbiol Infect*. 2019; 25: 681–687.
9. Weber M, Schaer J, Walther G et al. FunResDB — A web resource for genotypic susceptibility testing of *Aspergillus fumigatus*. *Med Mycol*. 2018; 56: 117–120.
10. Chowdhary A, Sharma C, Meis JF. Azole-Resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *J Infect Dis*. 2017; 216: S436–S444.
11. Bromley MJ, Van Muijlwijk G, Fraczek MG et al. Occurrence of azole-resistant species of *Aspergillus* in the UK environment. *J Glob Antimicrob Resist*. 2014; 2: 276–279.
12. Tsitsopoulou A, Posso R, Vale L, Bebb S, Johnson E, White PL. Determination of the prevalence of triazole resistance in environmental *Aspergillus fumigatus* strains isolated in South Wales, UK. *Front Microbiol*. 2018; 9: 1395.
13. Jeanvoine A, Rocchi S, Reboux G, Crini N, Crini G, Millon L. Azole-resistant *Aspergillus fumigatus* in sawmills of Eastern France. *J Appl Microbiol*. 2017; 123: 172–184.
14. Sabino R, Burco J, Valente J et al. Molecular identification of clinical and environmental avian *Aspergillus* isolates. *Arch Microbiol*. 2019; 201: 253–257.
15. Talbot JJ, Thompson P, Vogelnest L, Barrs VR. Identification of pathogenic *Aspergillus* isolates from captive birds in Australia. *Med Mycol*. 2018; 56: 1038–1041.
16. Nawrot U, Wieliczko A, Wlodarczyk K, Kurzyk E, Brillowska-Dabrowska A. Low frequency of itraconazole resistance found among *Aspergillus fumigatus* originating from poultry farms in Southwest Poland. *J Mycol Med*. 2019; 29: 24–27.
17. van der Linden JWM, Arendrup MC, Warris A et al. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg Infect Dis*. 2015; 21: 1041–1044.
18. Steinmann J, Hamprecht A, Vehreschild MJ et al. Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *J Antimicrob Chemother*. 2015; 70: 1522–1526.