Drug resistance of *Aspergillus fumigatus* strains isolated from flocks of domestic geese in Poland

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ABSTRACT The aim of the study was to determine the in vitro susceptibility of 85 *Aspergillus fumigatus* strains isolated from domestic geese and from their environment to amphotericin B, clotrimazole, voriconazole, itraconazole, enilconazole, miconazole, ketoconazole, and tioconazole. Samples were collected from clinically healthy birds (oral cavity) and from birds with aspergillosis (lungs and air sacs). The study was carried out using the disk diffusion method according to the Clinical Laboratory and Standards Institute (CLSI) M44–A2 procedure in parallel with the microdilution broth method according to CLSI M38–A2. The disk diffusion method showed that the all of the strains, irrespective of source, were resistant to miconazole. Resistance to the remaining azoles and amphotericin B ranged from 90.6 to 70.6%. Complete susceptibility was noted for voriconazole and enilconazole. Determination of the minimum inhibitory concentration (MIC) confirmed the high resistance of the strains tested to clotrimazole (MIC_{90} = 16 $\mu g \cdot mL^{-1}$), amphotericin B (MIC_{90} = 16 $\mu g \cdot mL^{-1}$), varied susceptibility to itraconazole (MIC 0.5–8 $\mu g \cdot mL^{-1}$), and 100% susceptibility to enilconazole and voriconazole. A correlation was noted between the susceptibility of the strains and their source. The highest percentage of resistant strains was noted in isolates from the lungs (100% for amphotericin B and clotrimazole and 35.7% for itraconazole). To the best of our knowledge, this is the first monitoring conducted in Poland in this area of research.

Key words: aspergillosis, *Aspergillus fumigatus*, domestic geese, resistance

2014 Poultry Science 93:1106–1112
http://dx.doi.org/10.3382/ps.2013-03702

INTRODUCTION

Aspergillus fumigatus is currently considered to be the most important fungal respiratory pathogen in birds (Beernaert et al., 2010). It is a ubiquitous organism isolated mainly from soil, air, vegetation, and dead organic matter present in the environment. High concentrations of spores, a warm, moist environment, and poor ventilation are the main factors enabling infection. The disease affects all species of domesticated birds, aquatic birds, wild birds, and ornamental birds. Young individuals are particularly susceptible; infections are generally acute and the mortality rate is high (up to 90% of the flock). Milder cases are observed in older individuals, in which infections are mainly chronic, with poorly expressed, often atypical symptoms (dyspnea, lethargy, dehydration, and neurological symptoms such as ataxia, convulsions, paresis, and lameness). Prognoses for aspergillosis in birds are poor, mainly due to difficulties with diagnosis and the relatively low effectiveness of treatment (Beernaert et al., 2010).

Due to the limited knowledge of the pharmacodynamics and pharmacokinetics of antifungal preparations in individual bird species, the lack of clinically determined breakpoints for medications, and the growing phenomenon of acquired resistance among *A. fumigatus* strains, it is not possible to develop an optimal program of specific treatment, prophylaxis, or both for either breeding flocks or individuals (Beernaert et al., 2010).

Although data on the antifungal resistance of *A. fumigatus* strains isolated from humans or from the hospital environment are relatively extensive and straightforward (Espinel-Ingroff et al., 2010; Tashiro et al., 2012), there is little information concerning birds. A study by Beernaert et al. (2009) using the reference broth microdilution method [Clinical Laboratory and Standards Institute (**CLSI**, 2008) CLSI-M38-A2] showed that of 59 *A. fumigatus* strains isolated from domesticated and free-living birds from Belgium and the Netherlands, 4 were resistant to voriconazole (minimum inhibitory concentration, **MIC**, 4–8 $\mu g/mL$) and itraconazole (MIC > 16 $\mu g \cdot mL^{-1}$). Moreover, the results obtained

Received October 21, 2013.
Accepted January 1, 2014.
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by other authors (Redig and Duke, 1985; Silvanose et al., 2006) for thiabendazole, 5-fluorocytosine, fluconazole, ketoconazole, caspofungin, amphotericin B, itraconazole, and voriconazole for some *A. fumigates* are difficult to analyze and interpret in a broader context because the MIC values were determined nonstandard methods, and the test conditions (e.g., type of medium, temperature of incubation) significantly affect the evaluation and interpretation of results.

The current international standardization of antifungal susceptibility testing enables optimal in vitro selection of potentially effective preparations for targeted antifungal therapy, and also makes it possible to determine drug-resistance patterns for particular species of fungi (Pfaller et al., 2002; Espinel-Ingroff et al., 2007). Extensive epidemiological studies and monitoring of the susceptibility of *A. fumigatus* strains isolated from different environments and geographical regions using methods recommended by CLSI, EUCAST (The European Committee on Antimicrobial Susceptibility Testing), or both are essential for the development of optimal empirical strategies for treating aspergillosis and anticipating the directions of development of drug resistance (Pfaller, 2012).

Geese are a special group of breeding birds in Poland. The importance of maintaining high health of these birds in Poland stems from the fact that almost 100% of production (mainly carcasses) is destined for export. Goose meat is a product classified as a safe food; therefore, maintaining a high quality of health of birds is a priority. The aim of the present study was to determine the drug-susceptibility profiles of *A. fumigatus* strains isolated from goose farms in southeastern Poland and to attempt to determine the distribution of MIC values for therapeutic agents.

MATERIALS AND METHODS

The material for the study consisted of 85 *A. fumigatus* isolates obtained from 8 flocks of geese raised in Poland. Samples were collected from the oral cavity of live, clinically healthy birds, using swabs (n = 63) and from the lungs and air sacs of dead birds with symptoms of aspergillosis (n = 14), as well as from neighboring water bodies (n = 8). Strains were isolated and identified using classical mycological diagnostic methods: microscopic examination and microbiological cultures (37 and 48°C), including microcultures, using the filamentous fungi identification key provided by de Hoog et al. (2000) for confirmation. Species differentiation was performed according to Balajee et al. (2006).

Determination of the Susceptibility of the Strains

The drug susceptibility of the strains was determined according to CLSI methodology. Each assay was performed in triplicate and on 3 different days.

Inoculum

The isolated *A. fumigatus* strains were plated on Sabouraud medium and incubated at 37°C for 7 d. The material was washed from the medium with saline containing Tween 20. The density of the suspension was $2 \approx 3 \times 10^4$ cfu/mL. The suspensions were prepared directly before conducting the tests.

Disk Diffusion Test

The procedure was based on that described in CLSI (2009) document M44-A2, with our own modifications for *Aspergillus* spp., and on experiments by other authors (Espinel-Ingroff et al., 2007). Inocula of each strain were plated on Mueller-Hinton medium (Difco) with 2% glucose. The plates with disks were incubated at 35°C for 48 h, and the results were interpreted according to the producer's instructions (Dom Handlowy Nauki sp. z o.o. PAN in Krakow, Poland).

Commercial disks saturated with the antifungal agents were used for the assays, in the amount of 10 μg/disk for amphotericin B, enilconazole, itraconazole, clotrimazole, ketoconazole, miconazole, and tioconazole. Disks for voriconazole (1 μg/disk) were prepared according to the procedure described in CLSI M44–A2.

Microdilution Broth Method According to CLSI M38–A2 (2008)

The following antifungal preparations were used in the assays in their pure form: amphotericin B (Sigma Aldrich, Seelze, Germany), clotrimazole (Gedeon Richter, Grodzisk Mazowiecki, Poland), enilconazole (Vet-Agro, Lublin, Poland), itraconazole (Sigma-Aldrich), and voriconazole (Pfizer, New York, NY). Dilutions of each drug were carried out according to CLSI procedure M38–A2. Working solutions were prepared in dimethyl sulfoxide (Poch SA, Poland), with 0.2% glucose and 0.165 *M* MOPS buffer (morpholinepropanesulfonic acid). The final concentration of each drug ranged from 0.03125 to 32 μg/mL. Successive dilutions in the amount of 100 μL were poured into a 96-well microdilution plate and then 100 μL of the fungal inoculum with a density of $2 \approx 3 \times 10^4$ cfu/mL was introduced into each well. The plates were incubated at 35°C for 48 h. The MIC endpoint for the azoles and amphotericin B was defined as the lowest concentration that produced complete inhibition of growth. Quality control was ensured using the reference strain *A. fumigatus* ATCC 204305.

Interpretation of Results

Breakpoints (MIC μg/mL) for *A. fumigatus* were as follows for amphotericin B and azoles: MIC ≤1 μg/mL, susceptible; MIC = 2 μg/mL, intermediately suscep-

Table 1. In vitro activity of azole and amphotericin B against *Aspergillus fumigatus* strains

		MIC[2]				Disk diffusion method				
								%[4]		
A. fumigatus	Antifungal agent[1]	Range (μg/mL)	GM[3]	MIC_{50}	MIC_{90}	Range (mm)	GM ± SD	S	I	R
Throat	A	4–16	12.8	16	16	8–16	9.9 ± 2.2	7.5	7.5	85
(n = 63)	Cl	2–16	6.9	8	16	10–16	12.6 ± 1.3	—	16.9	83.1
	E	0.25–1	0.5	0.5	1	32–58	44.9 ± 6.5	100	—	—
	I	0.5–8	1.5	2	8	13–20	15.9 ± 1.6	13.2	73.6	13.2
	V	0.125–1	0.3	0.25	1	35–55	44.5 ± 5.1	100	—	—
	Mi		nt[5]			8–12	9.9 ± 1.3	—	—	100
	K		nt			10–14	11.9 ± 1.6	—	20.8	79.2
	Ti		nt			10–15	12.3 ± 1.7	—	43.4	56.6
Lungs	A	8–16	12.5	16	16	8–10	8.6 ± 0.8	—	—	100
(n = 14)	Cl	8–16	12.5	16	16	9–13	10.7 ± 1.1	—	—	100
	E	0.25–1	0.5	0.25	1	43–58	51.4 ± 4.4	100	—	—
	I	2–4	2.6	2	4	12–16	14.2 ± 1.3	—	64.3	35.7
	V	0.25–1	0.4	0.5	0.5	34–43	38.6 ± 3.1	100	—	—
	Mi		nt			8–11	8.5 ± 0.9	—	—	100
	K		nt			9–14	10.5 ± 1.3	—	7.2	92.8
	Ti		nt			8–16	10.8 ± 2.0	—	7.2	92.8
Water	A	8–16	12.3	16	16	8–11	8.8 ± 1.1	—	—	100
(n = 8)	Cl	4–16	8.7	8	16	11–16	13.1 ± 1.6	—	50	50
	E	0.25–2	0.4	0.25	0.5	55–64	59.8 ± 2.9	100	—	—
	I	1–2	1.5	2	2	14–20	16.2 ± 1.7	12.5	87.5	—
	V	0.25–1	0.4	0.5	0.5	40–57	47.9 ± 6.6	100	—	—
	Mi		nt			9–10	9.4 ± 0.4	—	—	100
	K		nt			11–15	12.0 ± 1.5	—	12.5	87.5
	Ti		nt			10–14	11.8 ± 1.3	—	12.5	87.5
Total	A	4–16	12.7	16	16	8–16	9.6 ± 2.1	4.7	4.7	90.6
(n = 85)	Cl	2–16	7.7	8	16	9–16	12.3 ± 1.5	—	15.3	84.7
	E	0.25–2	0.5	0.5	1	32–64	47.1 ± 7.5	100	—	—
	I	0.5–8	1.7	2	4	12–20	15.6 ± 1.7	9.4	76.5	14.1
	V	0.125–1	0.4	0.5	1	34–57	43.8 ± 5.7	100	—	—
	Mi		nt			8–12	9.6 ± 1.3	—	—	100
	K		nt			9–15	11.7 ± 1.7	—	15.3	84.7
	Ti		nt			8–16	12.0 ± 1.8	—	29.4	70.6

[1]A = amphotericin B; Cl = clotrimazole; E = enilconazole; I = itraconazole; V = voriconazole; Mi = miconazole; K = ketoconazole; Ti = tioconazole.

[2]MIC = minimum inhibitory concentration. MIC_{50} and MIC_{90} = MIC at which respectively 50 and 90% of the isolates tested were inhibited.

[3]GM = geometric mean.

[4]S = suscepible, I = intermediate, R = resistant.

[5]nt = not tested.

tible; MIC >2 μg/mL, resistant (Espinel-Ingroff et al., 2007; Verweij et al., 2009).

Statistical analysis was carried out using Statistica 9.1 software (StatSoft Polska, Kraków, Poland). The Kruskal-Wallis test was used to compare the degree of resistance to the antifungal agents depending on the source of the strains.

RESULTS

The *A. fumigatus* strains isolated from domestic geese had a high degree of resistance to the commonly used antifungal agents. The disk diffusion method (Table 1) showed that 100% of the strains, irrespective of source (throat, lungs, or water) were resistant to miconazole, and then in decreasing order to amphotericin B (90.6%), clotrimazole (84.7%), ketoconazole (84.7%), and tioconazole (70.6%). Complete susceptibility was noted for voriconazole (100%) and enilconazole (100%). In the case of itraconazole, most strains were intermediately susceptible (76.5%), whereas total resistance was observed in only 14.1% of the population. Determination of the MIC for the antifungal agents (Table 1) and their distribution (Figure 1) confirmed the high resistance of the strains tested.

The MIC ranged from 4 to 16 $\mu g \cdot mL^{-1}$ (MIC_{90} 16 $\mu g \cdot mL^{-1}$) for amphotericin B and from 2 to 16 $\mu g \cdot mL^{-1}$ (MIC_{90} 16 $\mu g \cdot mL^{-1}$) for clotrimazole. The MIC values for voriconazole and enilconazole were significantly lower (MIC_{90} 1 $\mu g \cdot mL^{-1}$). Susceptibility to itraconazole was varied; MIC ranged from 0.5 to 8 $\mu g \cdot mL^{-1}$ with MIC_{90} = 4 $\mu g \cdot mL^{-1}$. A correlation was noted between the susceptibility of the strains and their source (Table 1), though statistically significant differences were observed only in the case of amphotericin B, clotrimazole, and itraconazole (Figure 1). The highest percentage of resistant strains was noted in isolates from the lungs (100% for amphotericin B and clotrimazole and 35.7% for itraconazole; Figure 1). Adopting the interpretation of results proposed by Espinel-Ingroff et al. (2007) and

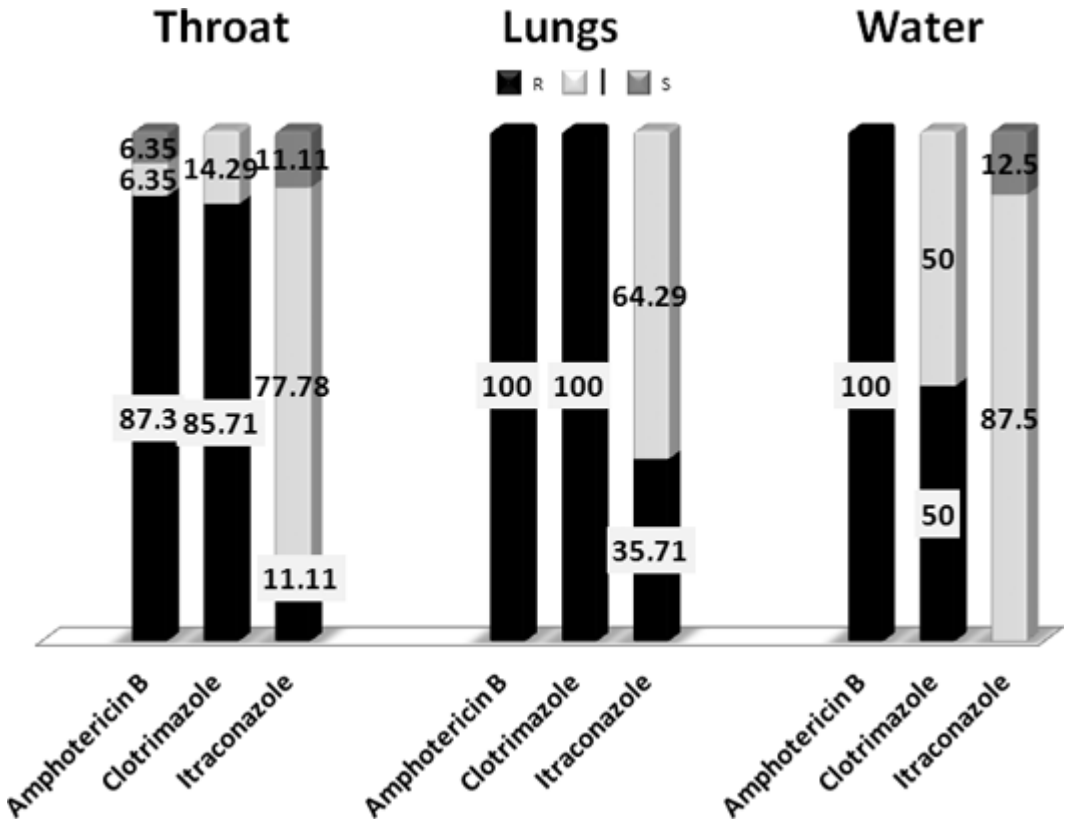

Figure 1. Distribution of minimum inhibitory concentration values in the population of isolated *Aspergillus fumigatus* strains (n = 85). S = susceptible; I = intermediate; R = resistant.

Verweij et al. (2009) (i.e., MIC $\leq$1 $\mu g \cdot mL^{-1}$ for susceptible strains, MIC = 2 $\mu g \cdot mL^{-1}$ for intermediate susceptibility, and MIC >2 $\mu g \cdot mL^{-1}$ for resistant strains), the level of agreement between the results of the disk diffusion test and the broth microdilution method (MIC) for each antifungal agent was determined as well (Table 2). Total agreement was noted for voriconazole and enilconazole (100%), whereas for amphotericin B, clotrimazole, and itraconazole agreement was 90.6, 77.6, and 43%, respectively. According to multidrug resistance classification criteria (Magiorakos et al., 2012), 100% of the strains were multidrug resistant (**MDR**). Two main resistance profiles were observed: I) strains resistant to amphotericin B and clotrimazole accounted for 35.3% (n = 30); II) strains resistant to amphotericin B, clotrimazole, and itraconazole accounted for 62.3% (n = 53). Only 2 strains had different patterns.

DISCUSSION

The present study on the resistance of *A. fumigatus* strains isolated from domestic geese to commonly available antifungal agents, the first such research conducted in Poland, found a high degree of resistance in the strains. The broad panel of antifungal agents included in the preliminary tests (disk diffusion) was narrowed in the next stage (determination of MIC according to CLSI M38–A) to representatives of particular groups of drugs: amphotericin B, until recently considered the gold standard in treatment of fungal infections, enilconazole and clotrimazole (imidazole), and itraconazole and voriconazole—new generation triazoles whose clinical effectiveness is currently high (Tashiro et al., 2012). Drugs such as flucytosine, nystatin, natamycin, and fluconazole, due to their complete lack of in vitro effect on

Table 2. Categorical agreement between the disk diffusion method and the broth microdilution method (CLSI, 2008, M38–A2)

	Number of errors			
Antifungal agent	Minor	Major	Very major	% Agreement
Amphotericin B	6	—	2	90.6
Clotrimazole	19	—	—	77.6
Enilconazole	—	—	—	100
Itraconazole	44	4	—	43
Voriconazole	—	—	—	100

the isolated *A. fumigatus* strains (no zone of inhibition in the disk diffusion method), were not included in the publication (unpublished data).

The high degree of resistance of the strains to amphotericin B was very surprising. Neither the MIC values of 4 to 16 $\mu g \cdot mL^{-1}$ nor their distribution is supported by available data from other research centers. Most research in this area has concerned *A. fumigatus* strains isolated from cases of aspergillosis in humans, and resistance to amphotericin B has not exceeded a few percent. A study by Guinea et al. (2005) analyzing the susceptibility of 596 *A. fumigatus* strains isolated in Spain from hospitalized patients, the hospital environment, and the external environment found only 1.5% of strains to be resistant to amphotericin B (MIC > 2 $\mu g \cdot mL^{-1}$), most of which were clinical isolates (6/9).

In a study by Espinel-Ingroff et al. (2008), among 292 clinical *A. fumigatus* isolates from the United States and Canada, for 31 strains the MIC for amphotericin B was ≥ 2 $\mu g \cdot mL^{-1}$, with a range of 0.25 to 4 $\mu g \cdot mL^{-1}$. Research conducted at other research centers has demonstrated relatively high in vitro activity of amphotericin B for clinical isolates of *A. fumigatus*; MIC has ranged from 0.5 to 8 $\mu g \cdot mL^{-1}$ with $MIC_{90} = 1$ $\mu g \cdot mL^{-1}$ (Pfaller et al., 2002; Arikan et al., 2008; Howard et al., 2009). Despite the widespread use of amphotericin B to treat aspergillosis in birds, few studies have dealt with the susceptibility of isolated strains to this drug. Silvanose et al. (2006) tested 16 *A. fumigatus* strains isolated from the respiratory system of falcons undergoing treatment at Dubai Falcon Hospital and found that they had relatively low in vitro susceptibility to amphotericin B; the MIC ranged from 0.25 to 12 $\mu g \cdot mL^{-1}$, with 31% resistant strains (MIC ≥ 2 $\mu g \cdot mL^{-1}$).

In a study by Beernaert et al. (2009), *A. fumigatus* isolates from ornamental and wild birds exhibited substantially higher susceptibility. The MIC values for amphotericin B ranged from 0.5 to 2 $\mu g \cdot mL^{-1}$, with MIC_{50} and $MIC_{90} = 2$ $\mu g \cdot mL^{-1}$ (Beernaert et al., 2009). The reasons for these discrepancies may be complex. Most importantly, due to the low toxicity of amphotericin B for birds (Kirchgessner, 2008), both local and general application (administered intravenously or via the air sacs or trachea) have been common, as has nebulization (Orosz, 2000). Nebulization has been used mainly in larger breeding establishments, not only for therapeutic purposes but also prophylactically (Orosz, 2000; Rochette et al., 2003). Direct contact of amphotericin B with the living environment of birds may have exerted selective pressure on the fungal species commonly occurring in it, including *A. fumigatus*, generating potentially resistant strains. This may offer an explanation for the high degree of resistance to amphotericin B in the *A. fumigatus* isolates examined in the present study.

Alongside resistance to amphotericin B, the strains tested had varied susceptibility to the antifungal agents from the azole group. Older generation drugs such as clotrimazole, miconazole, and tioconazole exhibited low in vitro activity, whereas the effectiveness of voriconazole, enilconazole, and in part, itraconazole was very high. The available data from recent years also indicate a marked progressive increase in the number of *A. fumigatus* strains resistant to azoles (Howard et al., 2009; Verweij et al., 2009; Espinel-Ingroff et al., 2010; Tashiro et al., 2012). These studies mainly focus on resistance of clinical isolates of *A. fumigatus* to itraconazole and voriconazole, which are currently recommended for treating aspergillosis in humans. The activity of the remaining azoles has rarely been monitored because their use in treating people is limited to local applications. The few studies conducted in this area of research have dealt mainly with aspergillosis treatment in birds.

The high in vitro activity of enilconazole demonstrated in the present study has been confirmed in vivo by numerous clinical studies. Its use in the form of smoke and spray on infected poultry farms has yielded satisfactory results as a therapeutic agent, as a biocide disinfectant and in protection against airborne infections (Braem, 1986; Redmann and Schildger, 1989). The high percentage of *A. fumigatus* strains isolated from domestic geese with intermediate sensitivity to itraconazole (MIC $= 2$ $\mu g \cdot mL^{-1}$ for over 40% of strains), together with over 14% resistant strains (MIC >2 $\mu g \cdot mL^{-1}$), indicates a marked growth trend for this phenomenon. Other studies have found a substantially lower percentage of *A. fumigatus* isolates from wild and domestic birds for which the MIC values for itraconazole were >1 $\mu g \cdot mL^{-1}$ (Silvanose et al., 2006; Beernaert et al., 2009). However, it should be taken into account that these are data from 4 yr ago (2009), and according to monitoring conducted by a major medical center in the Netherlands, resistance to itraconazole has shown an increasing trend from 0% in the years 1994 to 1999 to 6% in 2009 (Snelders et al., 2008). Moreover, in Denmark relatively high itraconazole resistance (MIC >4 $\mu g \cdot mL^{-1}$) was observed in environmental strains of *A. fumigatus* (Mortensen et al., 2010). It can be presumed that just as long-term antifungal therapy in humans has stimulated acquisition of azole resistance (Tashiro et al., 2012), prophylactic and therapeutic antifungal programs carried out on poultry breeding farms and in the environment for plant protection (Hof, 2001) have a similar effect.

Molecular research conducted in recent years has confirmed at the genome level the phenotypic phenomenon of resistance and cross resistance to drugs from the azole group (Mosquera and Denning, 2002; Howard and Arendrup, 2011). Cross-resistance patterns are varied and linked to the position of the mutation in the *cyp51A* gene (Howard et al., 2009), as well as to the molecular structure of the azoles (Xiao et al., 2004). In consequence, a similar susceptibility pattern is noted in *A. fumigatus* strains [e.g., susceptibility to itraconazole and posaconazole, but not to voriconazole and itraconazole (Mosquera and Denning, 2002; Verweij et al., 2002)].

In the present study, the high, 100% susceptibility of the *A. fumigatus* isolates to voriconazole (MIC < 1 $\mu g \cdot mL^{-1}$), along with the reduced antifungal activity of itraconazole, also suggests heterogeneous mechanisms of resistance to azoles in this group of fungi.

In regard to the source of the *A. fumigatus* strains as a determinant of their resistance patterns, a markedly lower percentage of strains resistant to clotrimazole and itraconazole was noted in strains isolated from the aquatic environment than in clinical strains. However, the pool of isolates studied was too small to allow for a reliable interpretation of the results. The prevailing view in the available literature is that the source of strains does not have a pronounced influence on their drug sensitivity (Guinea et al., 2005).

Because of the high economic losses generated by aspergillosis in poultry and the risk of environmental contamination by *Aspergillus* spores and of transmission of pathogenic strains to humans (Van Waeyenberghe et al., 2011), as well as the progression of drug resistance, there is a need for regular monitoring of the occurrence and drug susceptibility of *Aspergillus* strains in the environment (people, animals, water, air, and so on). Furthermore, in view of the lack of clinical breakpoints for *Aspergillus* spp., the MIC distribution among wild-type strains necessary to establish epidemiological cut-off values would be highly useful.

The results of the present study dealing with the environment of poultry breeding farms (a high-risk area) in Poland may be a contribution to such research. The anticipated continuation of research on a larger pool of isolates, derived from the immediate living environment of birds, expanded to determine their resistance at the genomic level, may in the future facilitate the development of effective treatment and prophylaxis programs for birds.

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