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Original article

Susceptibility testing of *Aspergillus niger* strains isolated from poultry to antifungal drugs – a comparative study of the disk diffusion, broth microdilution (M 38-A) and Etest® methods

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Abstract

The aim of this study was to determine the sensitivity of *Aspergillus niger* strains isolated from birds to available antifungal drugs using different *in vitro* assays – classical disk diffusion, Etest® and broth microdilution NCCLS/CLSI M 38-A. The study material consisted of about 2.000 swabs and samples from different species of birds. *A. niger* (n=10) was accounted for 6.81% of the total pool of strains isolated. Determinations were made for 13 antifungal drugs using the disk diffusion method. The *A. niger* exhibited high susceptibility to enilconazole, terbinafine, voriconazole, tioconazole and ketoconazole, low susceptibility to clotrimazole, miconazole and nystatin, and resistance to amphotericin B, itraconazole, pimaricin, fluconazole and 5-fluorocytosine. Minimum inhibitory concentration (MIC) was determined for 9 antifungal drugs using the micromethod of duplicate serial dilutions in a liquid medium. *A. niger* strains were most susceptible to enilconazole and voriconazole. MIC ranged from 0.0625 to 0.5 μg/ml for enilconazole, with MIC_{90}-0.5 μg/ml and MIC_{50}-0.125 μg/ml. The corresponding values for voriconazole were 0.25-1 μg/ml, 1 μg/ml and 0.5 μg/ml. MIC for amphotericin B and terbinafine ranged from 0.5 to 4 μg/ml, while the values for the remaining drugs were highly varied. MIC was measured by the gradient diffusion method using Etest® for 5 antifungal drugs: amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole. By far the highest susceptibility was obtained in the case of voriconazole, with MIC ranging from 0.0625 to 1 μg/ml. MIC for amphotericin B ranged from 0.25 to 4 μg/ml, for itraconazole and ketoconazole ranging from 0.5 to 16 μg/ml. Methods available for this purpose are not always applicable in field conditions. The present results indicate that the Etest® technique, due to its high percentage of agreement with the M 38-A microdilution method, should find application in medical and veterinary practice.

Key words: *Aspergillus niger*, poultry, disk diffusion method, broth microdilution (M 38-A) method, Etest®

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Introduction

Aspergillosis is the most common fungal infection of the respiratory tract in birds. The illness affects all species of poultry, particularly geese and ducks, as well as ornamental birds and wild birds kept in aviaries. The highest sensitivities are found in embryos and chicks, in which the course of the disease is acute. Infection in adults occurs predominantly in its chronic form.

Aspergillosis is usually caused by *Aspergillus fumigatus* and less commonly by *A. flavus* or *A. niger* (Akan et al. 2002, Martin et al. 2007, Khosravi et al. 2008).

In nature, *A. niger* is found in soil and litter, in compost and on decaying plant material (Schuster et al. 2002). It is generally regarded as safe and non-pathogenic. Nevertheless, in recent years an increasing number of aspergillosis cases inducted by *A. niger* have been found, particularly in wild birds kept in captivity, ducks and geese raise on farms (Akan et al. 2002, Silvanose et al. 2006, Ziółkowska and Tokarzewski 2007). It is mainly associated with stress and lowered immunity, and in other cases with farms where very large numbers of birds, i.e. from several thousand to tens of thousands, are assembled in one place, and with the microclimate characteristic of these farms.

Prevention and treatment of aspergillosis in birds is difficult and depends on maintenance conditions. In poultry flocks, treatment mainly involves disinfection of hatcheries and farm buildings with an antifungal disinfecting agent such as enilconazole (Van Cutsem 1983, Ziółkowska and Tokarzewski 2006).

In pigeons, ornamental birds and wild birds (especially parrots and falcons) individual empirical therapy is conducted (Silvanose et al. 2006, Tokarzewski et al. 2007, Flammer et al. 2008). This requires not only the application of an effective drug for each case, but also the development of an appropriate dosing regimen, route and method of administration for the drug. Existing research determining susceptibility to available antifungal preparations *in vitro* and *in vivo* has focused on the main etiological agent of aspergillosis, i.e. *A. fumigatus* (Ziółkowska and Tokarzewski 2007), while information on other species of *Aspergillus*, such as *A. niger*, is limited.

There are no recommended methods for determining the sensitivity of filamentous fungi to drugs. The techniques currently in use are the classical disk diffusion method, the Etest® (bioMerieux, AB Biodisk, France) method and the broth microdilution assay, performed as described by the NCCLS/CLSI (National Committee for Clinical Laboratory Standards/Clinical and Laboratory Standards Institute, USA) (CLSI/NCCLS 2002, 2004).

These methods differ in sensitivity, reproducibility, labour-intensity and difficulty which significantly affects interpretation of results and their comparison between laboratories.

The aim of this study was to determine the sensitivity of *A. niger* strains isolated from birds to available antifungal drugs using different *in vitro* assays (two agar-based susceptibility testing methods – classical disk diffusion and Etest® and broth microdilution NCCLS/CLSI M 38-A).

Materials and Methods

The poultry flocks used in the study were from south-eastern Poland. The farms were provided with permanent veterinary inspection and prophylactic programmes.

The study material consisted of about 2.000 swabs from poultry.

Mycological examinations. *Aspergillus* species were isolated and identified in accordance with generally accepted methodologies and recommendations for mycological diagnostics. The samples were inoculated on Sabouraud dextrose agar with chloramphenicol (0.05 mg/ml) and incubated at 25°C and 35°C, in aerobic conditions for 14 days. The fungal cultures obtained were identified by microscopic examination and microbiological culture, including microcultures using the identification key for filamentous fungi by de Hoog et al. (2000). Susceptibility of strains was determined immediately following isolation and identification.

Inoculum suspension. Spore suspensions of *A. niger* were prepared in sterile 0.85% NaCl with Tween 20 from fresh colonies grown on Sabouraud dextrose agar at 35°C for 5 days. Cell concentration was adjusted to final concentrations of 0.4×10^4 to 5×10^4 CFU/mL. These suspensions were used directly for the inoculation.

Disk diffusion method. The procedure was based on that described in CLSI document M 44-A (CLSI/NCCLS 2004), with our own modifications for *Aspergillus spp.*, and on experiments by other authors (Espinel-Ingroff 2007). Standard disks were used for the disk diffusion test: amphotericin B, clotrimazole, fluconazole, itraconazole, ketoconazole, miconazole, pimaricin, tioconazole (concentration 10 µg/disk) and 5-fluorocytosine (0.5 µg/disk) (Dom Handlowy Nauki, Poland). Additionally, we applied voriconazole (1 µg/disc), terbinafine (10 µg/disk) and enilconazole (10 µg/disk) prepared in-house according to CLSI M 44-A guidelines, using concentrations based on data from the literature (Espinel-Ingroff 2007, Espinel-Igroff et al. 2007, Mendez et al. 2008).

Table 1. Disk diffusion method – zone of inhibition (mm) – DHN Kraków, Poland.

Antifungal agent	Zone of inhibition (mm)		
	S – susceptible	I – intermediate	R – resistant
Amphotericin B	≥ 16	12-16	< 12
5 – Fluorocytosine	≥ 20	16-20	< 16
Clotrimazole *Fluconazole* *Itraconazole* *Ketoconazole* *Miconazole* *Nystatin* *Pimaricin* *Tioconazole*	≥ 18	14-18	< 14

Table 2. Degree of sensitivity of *Aspergillus niger* to antifungal drugs – disk diffusion method.

Antifungal agent	Disc diffusion method (mm)										
	A. niger ATCC 16404	*A. niger* (n=10)									
		1	2	3	4	5	6	7	8	9	10
5 – Fluorocytosine	10	No growth inhibition									
Amphotericin B	12	12	9	12	10	11	13	13	10	9	11
Clotrimazole	13	15	12	14	18	17	19	15	16	14	16
Enilconazole	53	38	51	56	52	55	34	36	40	48	52
Fluconazole	14	No growth inhibition									
Itraconazole	12	8	15	11	10	12	11	10	10	12	12
Ketoconazole	17	10	14	27	23	28	31	17	20	20	22
Miconazole	10	18	11	17	18	20	21	14	14	13	18
Nystatin	15	17	12	14	15	14	14	21	16	15	17
Pimaricin	16	17	17	15	15	17	12	10	10	15	13
Terbinafine	26	29	25	26	42	33	37	18	25	31	32
Tioconazole	14	21	13	23	27	29	30	20	20	19	15
Voriconazole	45	56	45	52	55	52	53	53	55	47	40

A volume of 200 µl of the inoculum was plated on Sabouraud dextrose agar and then antifungal disks were applied, no more than four disks per plate (90 mm petri dishes full plate). Zone diameters (in mm) were determined after 48 h of incubation at 35°C and measured at the point at which total growth inhibition was noted. Each assay was performed in triplicate and on three different days, and the mean diameters were reported (Table 1, 2).

CLSI M 38-A broth microdilution method (CLSI/NCCLS 2002). The antifungal drugs used in this study, obtained as reagent-grade powders from their manufacturers, were amphotericin B (Sigma-Aldrich Chemie, Germany), clotrimazole (Gedeon Richter Polska, Poland), enilconazole (Vet-Agro, Poland), fluconazole (Polfarmex, Poland), itraconazole (Sigma-Aldrich Chemie, Germany), ketoconazole (Anpharm, Servier Group, Poland), miconazole (Gedeon Richter Polska, Poland), terbinafine (Hetero Drugs Limited, India) and voriconazole (Pfizer, USA). The broth microdilution method was performed according to CLSI guidelines. Stock solutions of the antifungals were prepared in dimethyl sulfoxide (Poch SA, Poland). All drugs were diluted 100 times to their final concentration, further diluted in RPMI 1640 medium buffered to pH 7.0 with morpholinopropane sulfonic acid, and dispensed into 96-well microdilution trays. Trays containing a 100 µl aliquot in each well of the appropriate drug solution (2 x final concentration) were subjected to quality control, then sealed and stored at -70°C until use. The final concentration of the drugs in the wells ranged from 0.03 to 16 µg/mL. The wells were then inoculated with 100 µl of the test strain and the inoculated microdilution trays

Table 3. Degree of sensitivity of *Aspergillus niger* to antifungal drugs – broth microdilution CLSI M 38-A method.

Antifungal agent	MIC (μg/ml)																	
	A. niger ATCC 16404	*A. niger* (n=10)										Range	AM	GM	SD	SE	50%	90%
		1	2	3	4	5	6	7	8	9	10							
Amphotericin B	2	1	1	1	4	4	0.5	4	4	2	1	0.5-4	2.25	1.74	1.55	0.49	1	4
Clotrimazole	2	0.5	4	1	1	16	4	8	4	1	4	0.5-16	4.35	2.64	4.69	1.48	4	8
Enilconazole	2	0.5	0.5	0.125	0.125	0.125	0.0625	0.5	0.5	0.25	0.125	0.0625-0.5	0.28	0.22	0.19	0.06	0.125	0.5
Fluconazole	16	8	16	4	4	16	8	4	4	4	8	4-16	7.6	6.5	4.79	1.51	4	16
Itraconazole	4	2	2	1	4	4	4	1	8	8	2	1-8	3.6	2.83	2.59	0.82	2	8
Ketoconazole	4	0.25	4	0.25	0.5	2	1	8	2	4	2	0.25-8	2.4	1.41	2.4	0.76	2	4
Miconazole	8	0.5	16	1	2	1	4	2	0.5	4	1	0.5-16	3.2	1.74	4.68	1.48	1	4
Terbinafine	2	2	2	0.5	2	2	2	4	1	0.5	2	0.5-4	1.8	1.52	1.005	0.32	2	2
Voriconazole	0.5	0.5	1	0.25	0.5	0.5	0.5	1	0.5	0.5	0.5	0.25-1	0.58	0.54	0.24	0.08	0.5	1

AM – arithmetic mean, GM – geometric mean, SD – standard deviation, SE – standard error

Table 4. Degree of sensitivity of *Aspergillus niger* to antifungal drugs – Etest®.

Antifungal agent (concentration gradient)	Etest® (μg/ml)																	
	A. niger ATCC 16404	*A. niger* (n=10)										Range	AM	GM	SD	SE	50%	90%
		1	2	3	4	5	6	7	8	9	10							
Amphotericin B (0.002-32 mg/mL)	0.5	0.5	0.5	1	0.5	0.25	1	0.5	4	2	0.5	0.25-4	1.08	0.76	1.14	0.36	0.5	2
Fluconazole (0.016-256 mg/mL)	16	16	16	16	16	16	32	8	32	8	32	8-32	19.2	17.15	9.39	2.97	16	32
Itraconazole (0.002-32 mg/mL)	8	4	1	1	2	4	8	0.5	8	0.5	2	0.5-8	3.1	2	2.88	0.91	2	8
Ketoconazole (0.002-32 mg/mL)	8	1	8	2	1	0.5	1	16	4	4	2	0.5-16	3.95	2.3	4.8	1.52	2	8
Voriconazole (0.002-32 mg/mL)	0.5	0.25	0.5	0.0625	0.5	0.25	0.25	0.5	1	0.5	0.0625	0.0625-1	0.39	0.29	0.28	0.09	0.25	0.5

AM – arithmetic mean, GM – geometric mean, SD – standard deviation, SE – standard error

Table 5. Summary of test results for all methods.

Antifungal agent	Disc diffusion method (DD)			**MIC**			Etest		
	Degree of sensitivity (% of strains)								
	R	I	S	**R**	**I**	**S**	R	I	S
Amphotericin B	60	–	40	**40**	**10**	**50**	10	10	80
Itraconazole	90	10	–	**50**	**30**	**20**	40	20	40
Ketoconazole	10	20	70	**30**	**30**	**40**	40	20	40
Voriconazole	–	–	100	**–**	**–**	**100**	–	–	100
Fluconazole	100	–	–	**100**	**–**	**–**	100	–	–
Clotrimazole	10	80	10	**60**	**–**	**40**	ND		
Enilconazole	–	–	100	**–**	**–**	**100**	ND		
Terbinafine	–	–	100	**10**	**60**	**30**	ND		
Miconazole	20	60	20	**30**	**20**	**50**	ND		
Tioconazole	10	10	80	**ND**			ND		
Nystatin	10	80	10	**ND**			ND		
Pimaricin	40	60	–	**ND**			ND		
5-Fluorocytosine	100	–	–	**ND**			ND		

ND – no determined, R – resistant, I – intermediate, S – susceptible

were incubated at 35°C and read at 48 h. The MIC endpoint was defined as the lowest concentration producing complete inhibition of growth.

The broth microdilution tests were done in triplicate and on three different days, and then the geometric mean values were reported.

Etest®

Susceptibility testing with Etest®. Etest strips containing a continuous concentration gradient of amphotericin B (0.002-32 µg/mL), fluconazole (0.016-256 µg/mL), itraconazole (0.002-32 µg/mL), ketoconazole (0.002-32 µg/mL) and voriconazole (0.002-32 µg/mL) were obtained from bioMerieux (France). All strips were stored at -20°C until use and left at room temperature for 20 min.

Etest® procedure for *A. niger.* The test strain in a volume of 200 µl was inoculated on Sabouraud dextrose agar. The plates were then dried at room temperature for 15 min before Etest strips were applied, and then incubated at 35°C for 24-48 h. The minimum inhibitory concentration (MIC) was defined as the drug concentration present at the point where the fungal ellipse intersected the MIC scale on the Etest strip. Each assay was performed three times on different days, and the geometric mean values were reported.

Quality control. Quality control was performed using *A. niger* ATCC 16404 (Mecconti, Italy).

Interpretation of results. For all tests, P values < 0.05 were considered statistically significant. Statistical analysis was carried out using STATISTICA 6.0 software (STATSOFT).

Due to the lack of understanding regarding breakpoints for fungi of the genus *Aspergillus* (Espinel-Igroff et al. 2007) the following interpretation of the results for amphotericin B and azoles has been considered:

MIC ≤ 1 µg/mL – susceptible strains

MIC = 2 µg/mL – intermediate

MIC ≥ 4 µg/mL – resistant

Discrepancies between MIC's of no more than 3 dilutions (0.5; 1.0; 2.0) were used to calculated the percent agreement (for example Etest, Broth Microdilution Method); percent agreement between tests for each drug was established by comparing the results of categorizing the various strains (susceptible, intermediate, resistant) in the methods (Espinel-Igroff et al. 2007).

Results

Overall 2.000 specimens were tested, from which 147 strains of *Aspergilllus* were isolated and then classified into seven species (*A. fumigatus*, *A. flavus*, *A. niger*, *A. glaucus*, *A. nidulans*, *A. clavatus*, *and A. ustus*).

A. niger (n=10) was isolated from 10 flocks of geese and chickens and accounted for 6.81% of the total pool of strains isolated.

Evaluation of the susceptibility of the *A. niger* strains was carried out in parallel using three methods. The results are presented in Tables 2, 3, 4 and 5.

Table 6. Percent agreement between tests.

% agreement between tests			
Antifungal agent	DD/MIC	DD/ET	MIC/ET
Amphotericin B	70	60	70
Fluconazole	100	100	90
Itraconazole	70	70	90
Ketoconazole	30	30	90
Voriconazole	100	100	90
Enilconazole	100	ND	
Miconazole	70	ND	
Clotrimazole	50	ND	
Terbinafine	30	ND	
5 – Fluorocytosine	ND		
Tioconazole	ND		
Nystatin	ND		
Pimaricin	ND		

ND – no determined

Determinations were made for 13 antifungal drugs using the disk diffusion method. The *A. niger* strains tested exhibited high susceptibility to enilconazole, terbinafine, voriconazole, tioconazole and ketoconazole, low susceptibility to clotrimazole, miconazole and nystatin, and resistance to amphotericin B, itraconazole, pimaricin, fluconazole and 5-fluorocytosine (Table 2, Fig. 1).

MIC was determined for 9 antifungal drugs using the micromethod of duplicate serial dilutions in a liquid medium (Table 3). Here also the *A. niger* strains were most susceptible to enilconazole and voriconazole. MIC ranged from 0.0625 to 0.5 µg/mL for enilconazole, with MIC_{90}-0.5 µg/mL and MIC_{50}-0.125 µg/mL. The corresponding values for voriconazole were 0.25-1 µg/mL, 1 µg/mL and 0.5 µg/mL respectively. MIC for amphotericin B and terbinafine ranged from 0.5 to 4 µg/mL, while the values for the remaining drugs were highly varied (Table 3).

MIC was measured by the gradient diffusion method using Etest for 5 antifungal drugs: amphotericin B, fluconazole, itraconazole, ketoconazole

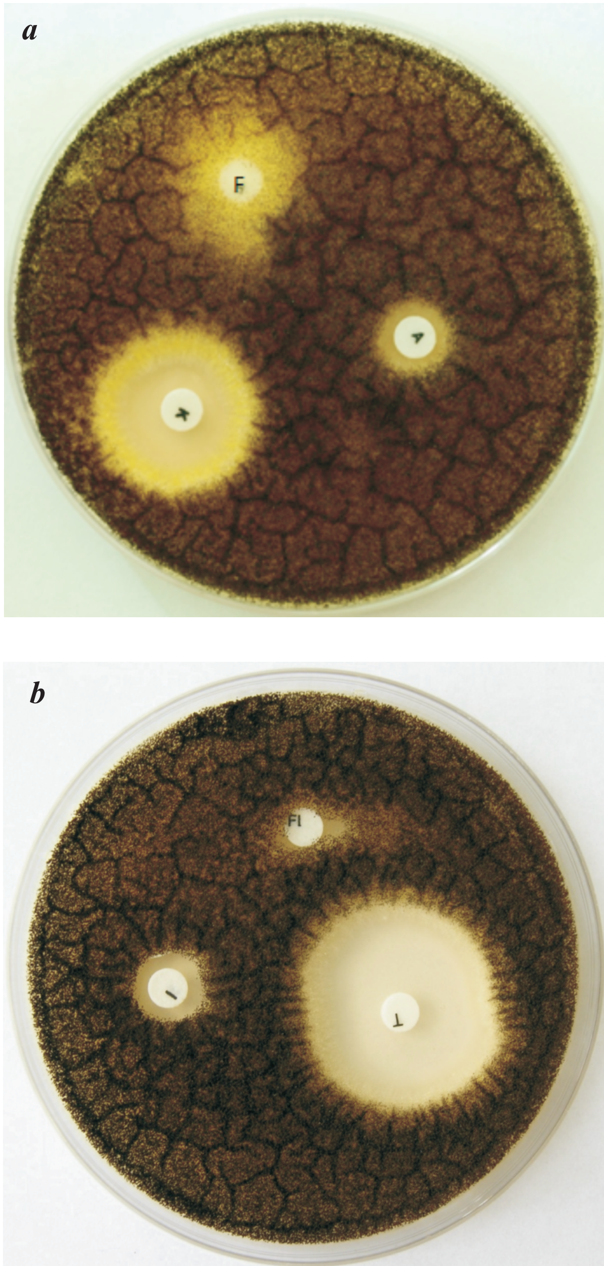

Fig. 1. Disk diffusion metod: *a* – A – amphotericin B, F – fluconazole, K – ketoconazole, *b* – Fl – 5-Fluorocytosine, I – itraconazole, T – tioconazole

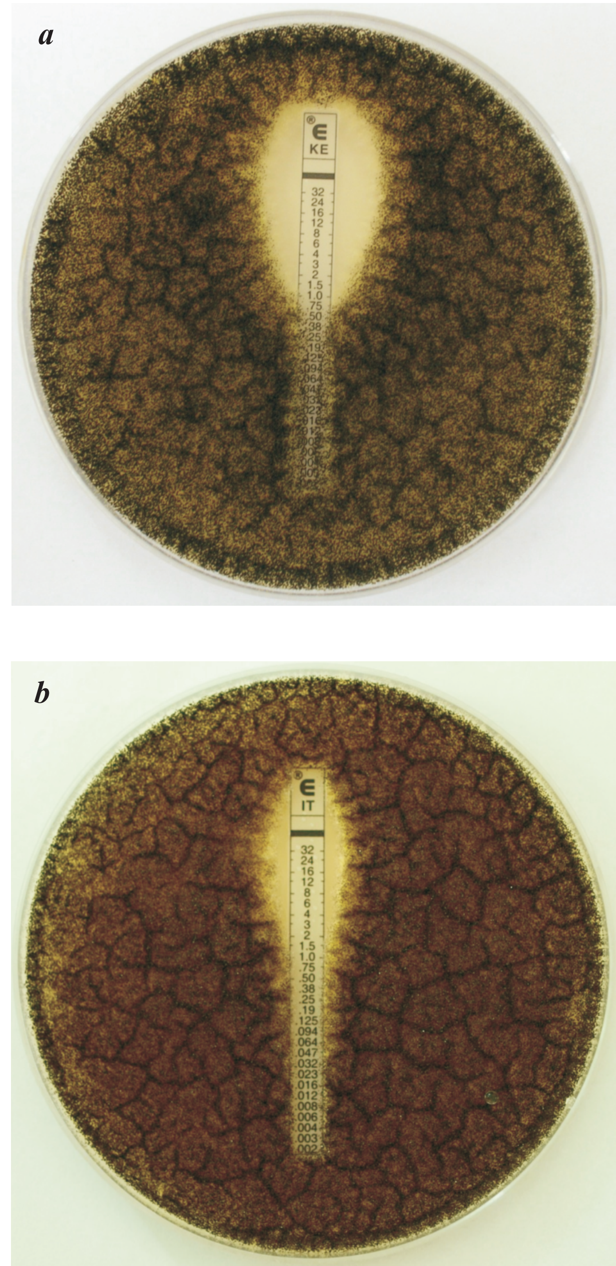

Fig. 2. Etest® metod: *a* – ketoconazole, *b* – itraconazole

and voriconazole (Table 4, Fig. 2). The zones of inhibition for the *A. niger* strains were relatively distinct and not difficult to read. By far the highest susceptibility was obtained in the case of voriconazole, with MIC ranging from 0.0625 to 1 µg/ml, MIC_{90}-0.5 µg/mL, and MIC_{50}-0.25 µg/mL. MIC for amphotericin B ranged from 0.25 to 4 µg/mL, with MIC_{90}-2 µg/mL and MIC_{50}-0.5 µg/mL. The results for itraconazole and ketoconazole were far less concentrated around the mean and more scattered, with MIC ranging from 0.5 to 16 µg/mL. The highest variation was observed in the case of fluconazole.

To determine the correlation between the methods applied, the percentage of agreement between tests was calculated (Pfaller et al. 2000, Espinel-Igroff and Rezusta 2002, Espinel-Igroff et al. 2007). The results obtained are presented in Table 6. Agreement of 100% was noted for the disk diffusion method and MIC determination by microdilution for fluconazole, voriconazole and enilconazole. A similar percentage of agreement was obtained in comparing the disk diffusion method and Etest for fluconazole and voriconazole. For the remaining antifungals, the percentage of agreement was 50-90%, with the exception of ketoconazole and terbinafine, for which it was only 30%.

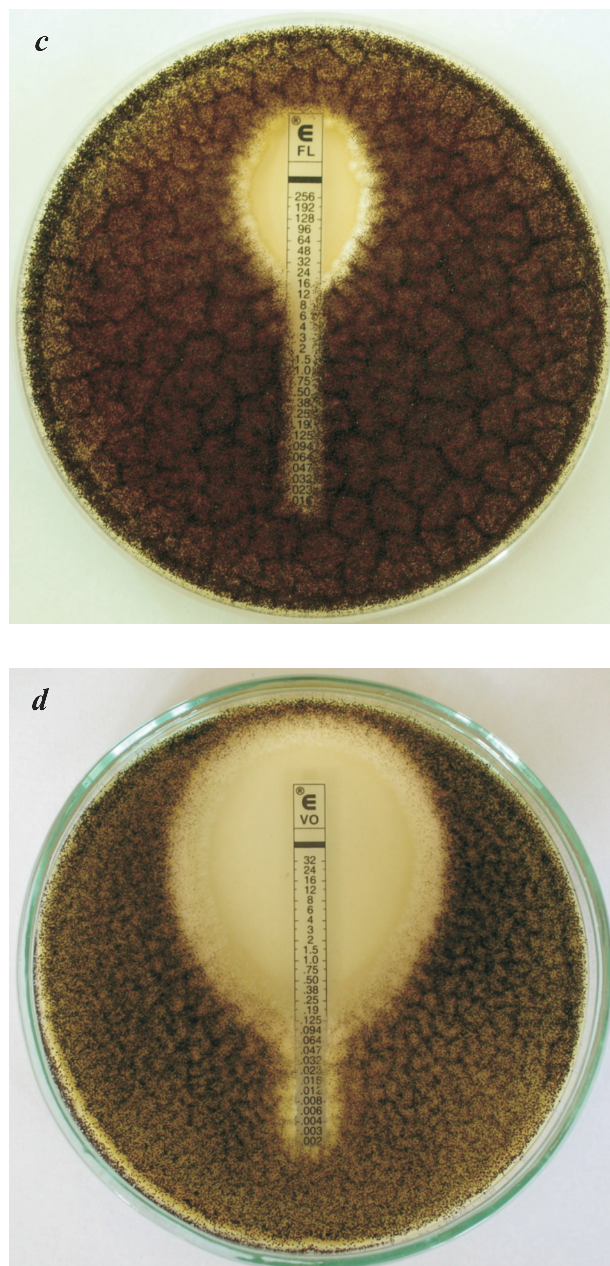

Fig. 2. Etest® metod: *c* – fluconazole, *d* – voriconazole

Discussion

The rise in recent years in the number of cases of *Aspergillus* fungi isolation, both on poultry farms and from ornamental or wild birds, calls for the development of effective prophylactic and treatment programmes. In view of both the changing profile of *Aspergillus* species that are pathogenic for birds and the growth of drug resistance, it also seems that regular monitoring should be conducted that takes into account both of these phenomena.

The present study demonstrated a relatively high percentage of *Aspergillus niger* – 6.81% in the total pool (n=147) of *Aspergillus spp.* strains isolated from poultry. Due to the lack of analogous data from other research centres, it is difficult to make a comparative evaluation of the epidemiological situation. The isolated strains displayed *in vitro* resistance to amphotericin B, itraconazole and fluconazole (in the disk diffusion method) with MIC_{90} values of 4 μg/mL, 8 μg/mL and 16 μg/mL, respectively. High antifungal activity was observed in the case of miconazole (100% of strains), enilconazole (100% of strains) and terbinafine (90% of strains), for which MIC_{90} attained values of 4 μg/mL, 0.5 μg/mL and 2 μg/mL, respectively.

The susceptibility of *A. niger* strains determined at other research centres has varied, and differs from the values obtained in the present study. Araujo et al. (2007) determined the susceptibility profiles for strains isolated from clinical cases of aspergillosis in humans and found 100% susceptibility of *A. niger* strains to amphotericin B (MIC 0.125-0.5 μg/mL) and voriconazole (MIC 0.06-1 μg/mL), and 70% susceptibility to itraconazole (MIC 0.06-2 μg/mL), assuming that the breakpoint MIC $\leq$ 1 μg/mL.

Espinel-Ingroff et al. (2008) analysed analogous data from various clinical laboratories and found somewhat different values: MIC_{90} ranged from 0.5 to 1 μg/mL for amphotericin B, from 0.25 to 8 μg/mL for itraconazole, and from 0.12 to 2 μg/mL for voriconazole.

Susceptibility similar to that of the strains isolated in the present study was observed in clinical *A. niger* isolates from falcons. MIC for amphotericin B, itraconazole and voriconazole was 0.064-16 μg/mL, 1-12 μg/mL and 0.094-0.75 μg/mL, respectively (Silvanose et al. 2006).

It can be assumed that as in the case of other *Aspergillus* species, the effectiveness of antifungal agents may depend not only on the type of fungus (Ziółkowska et al. 2010), but also on the site of isolation (environment, patient) (Araujo et al. 2007), and even the species of the host (human, animal, bird).

The lack of standard procedures for *in vitro* determination of the susceptibility of fungi to therapeutic agents and the lack of uniform standards, based on clinical research, for interpreting the results obtained, is a further difficulty in developing effective therapy.

Despite that, just as in the case of bacteria, the gold standard for determining the sensitivity of fungi to drugs is considered the MIC determination double dilution method, and even the developed methodology for the implementation of this procedure (M 38-A) (NCCLS/CLSI), is due to the difficulty in standardization, its application requires each adaptation and optimize the conditions for the implementation.

Nevertheless, because this method is costly, labour-intensive and time-consuming, it is used mainly in reference and research laboratories. In medical and veterinary practice, the most commonly used method is disk diffusion (qualitative evaluation), and more recently (to a limited extent, due to its high cost) MIC determination by the Etest method (quantitative evaluation). In order to standardization *in vitro* assessment of drug susceptibility in fungi, the degree of agreement between results obtained by different types of testing must be determined. Research on this question has been conducted for over 10 years but has not been fully satisfactory. Agreement between M 38-A and Etest is generally considered to be relatively high, ranging from 80 to 100% (Martin-Mazuelos et al. 2003, Pfaller et al. 2003a,b, Guinea et al. 2007,), depending both on the species of fungus (Szekely et al. 1999, Buchta et al. 2008) and on the drug tested (Colombo et al. 1995, Chen et al. 1996).

The present study has confirmed the relatively high degree of agreement between the above-mentioned methods – 90% for fluconazole, itraconazole, ketoconazole and voriconazole and 70% for amphotericin B.

These values are somewhat lower than those obtained by other authors, who found that in the case of the genus *Aspergillus* agreement between M 38-A and Etest was 98%, 100% and 100% for amphotericin B, itraconazole and voriconazole, respectively (Guinea et al. 2007) or, for voriconazole and itraconazole, it was either 96.3% and 97.8% (Pfaller et al. 2003b) or 96.3% and 82.2% (Espinel-Igroff and Rezusta 2002, Espinel-Igroff et al. 2007).

Agreement closer to that found in our study was attained by Martin-Mazuelos et al. (2003) – 88.5% for amphotericin B and 67.2% for itraconazole.

The discrepancies that occur may be the result of testing methodology that is not fully standardized in terms of enrichment media, inoculum density, incubation time and endpoint determination (Colombo et al. 1995).

An additional difficulty, particularly in the case of fungal pathogens, is the lack of breakpoints determined for particular antifungal agents (Araujo et al. 2007), including amphotericin B, which is considered the gold standard in treatment of systemic mycoses. Clinical interpretation of results, as well as comparison of MIC values with the results of a disk diffusion test, are generally based on data available from the literature.

Based on clinical assessment of the effectiveness of antifungal treatment and a parallel assessment of the drug susceptibility of particular fungus species *in vitro*, strains are considered susceptible if MIC values for amphotericin B and azole antifungals are less than 1 μg/mL (Espinel-Igroff et al. 2001).

Other research centres propose significantly higher breakpoint values. Strains are classified as resistant to amphotericin B if they have MIC values > 2 μg/mL (Lass-Florl et al. 1998) and resistant to azoles if MIC ≥ 8 μg/mL (Denning et al. 1997a,b).

In the present study, where *A. niger* strains with MIC > 1 μg/mL were considered to be resistant, the disk diffusion method was shown to have a relatively low percentage of agreement with M 38-A and Etest (Tables 6). Perhaps the values would have been higher if different criteria had been used to classify the strains.

In summing up, it should be noted that the relatively frequent isolation of *A. niger* in poultry breeding flocks can be a potential health hazard. Effective prevention and treatment of aspergillosis requires regular monitoring of the drug susceptibility profiles of currently isolated strains.

Methods available for this purpose are varied and not always applicable in field conditions. The results of the present study indicate that the Etest technique, due to its high percentage of agreement with the M 38-A microdilution method (the gold standard for this type of testing) should find application in medical and veterinary practice.

The assessment of the susceptibility of the isolated *A. niger* strains to commonly used antifungal agents should be confirmed on a wider range of material, in particular with respect to their high resistance to amphotericin B and itraconazole, which are commonly used in experimental treatment of aspergillosis in ornamental and wild birds.

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