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Examination of *cyp51A* and *cyp51B* expression level of the first Polish azole resistant clinical *Aspergillus fumigatus* isolate*

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***Aspergillus fumigatus* is one of the most prevalent airborne fungal pathogens causing infections worldwide. Most *A. fumigatus* strains are susceptible to azoles, which are administered as the first line therapeutics. However, during last decade the acquired resistance to triazoles by these species has been described. There is a number of publications concerning the examination of clinical *A. fumigatus* strains from different countries, however there has been no report from Poland. Here, we describe for the first time, an examination of *cyp51A* and *cyp51B* expression level of 11 clinical *A. fumigatus* strains isolated during 2007–2014 period from the collection of Medical University in Wrocław. Their susceptibility to itraconazole, voriconazole and posaconazole has been examined. The MIC values of triazoles for one of the examined isolates were respectively: >8 mg/L for itraconazole, 2 mg/L for voriconazole and 0.5 mg/L for posaconazole. The *cyp51A* gene with its promoter region of all isolates was sequenced. It was found that the resistant isolate harbors the TR_{34}/L98H mutation in the *cyp51A* gene and when cultured on media supplemented with voriconazole exhibits overexpression of both, *cyp51A* and *cyp51B* genes. The level of *cyp51A* gene expression was about 50 times higher than *cyp51B*.**

Key words: *Aspergillus fumigatus*, azole resistance, *cyp51A*, *cyp51B*

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INTRODUCTION

Aspergillus (A.) fumigatus is a saprophytic, cosmopolitan fungus present in the soil, rotting plant debris, damp walls or in-house dust (Soubani *et al.*, 2002). It plays an important role in the carbon and nitrogen cycle in the environment. However, it is also recorded as the etiological agent of aspergillosis. Modern diagnostics and application of antifungals improved the outcome of aspergillosis treatment, however, mortality of immunocompromised patients with invasive aspergillosis reaches 40–90% (Shapiro *et al.,* 2011). Among all potential antifungal agents for treatment of *A. fumigatus* infections, antibiotics from the group of azoles, polyenes and echinocandins, are being administered. Because of the broad spectrum, relatively low toxic azoles are the first-line therapeuticals. (Valiante *et al.*, 2015) The activity of azoles is based on inhibition of lanosterol 14-α-demethylase (Odds *et al.*, 2003). Azoles are a group of antifungal compounds which are applied in clinical medicine and in agriculture (Verweij *et al.*, 2009). In 1997, the first itraconazole resistant *A. fumigatus* strains were identified in the Netherlands (Denning *et al.*, 1997). The number of azole resistant strains still increases for e.g. in the Netherlands from 2% in 2000 to 8% in 2009. In Great Britain the highest increase was observed — 5% in 2004, 14% in 2008 and 20% in 2009. However, the published data are probably underestimated due to the lack of routine testing of susceptibility of *A. fumigatus* isolates (Perlin *et al.*, 2015). Most of the azole resistant strains harbor the TR_{34}/L98H mutation in the *cyp51A* gene, which is related to azole resistance (Mellado *et al.*, 2007). Another suggested mechanism of acquiring the azole resistance is connected to the expression level of the *cyp51* genes (Shapiro *et al.*, 2011; van Ingen *et al.*, 2015). In this paper we present the results of examination of *cyp51A* and *cyp51B* genes expression level of 11 clinical *A. fumigatus* isolates grown on Sabouraud agar and Sabouraud agar supplemented with voriconazole.

MATERIALS AND METHODS

Isolates. Eleven clinical *A. fumigatus* isolates from the collection of Wrocław Medical University were included in this study. Minimal Inhibitory Concentration (MIC) was assessed for itraconazole (ITR), voriconazole (VOR) and posaconazole (POS) using broth microdilution methods, according to European Committee for Antimicrobial Susceptibility Testing E.Def 9.1 (Rodriguez-Tudela *et al.*, 2008). One of the tested isolates (no. 55) has been characterized as VOR and POS intermediate susceptible (MIC for VOR: 2 mg/L and POS: 0.25 mg/L) and resistant to itraconazole (MIC for ITR: 32 mg/L).

PCR. *A. fumigatus* DNA was extracted by an earlier described procedure (Brillowska-Dąbrowska *et al.*, 2010). Briefly, DNA from pieces of mycelium of 3–5 mm diameter was extracted by a 10-min incubation of the sample in 100 µl of extraction buffer (60 mM sodium bicarbonate ($NaHCO_3$), 250 mM potassium chloride (KCl) and 50 mM Tris, pH 9.5) at 95°C and subsequent addition of 100 µl of 2% bovine serum albumin. After vortexing, this DNA-containing solution was used for amplification of ITS1, 5.8S RNA , ITS2 regions (White *et al.*, 1990), a fragment of β-tubulin gene (Alcazar-Fuoli *et al.*, 2008) and the *cyp51A* sequence (cyp51Afor 5' ATGGTGCCGATGCTATG-

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Abbreviations: PCR, polymerase chain reaction; TR, tandem repeat

New sequences: none

GCT 3' and cyp51Arev 5' ACCGCTTCTCCCAG-CCGA 3'). PCR products were purified (Clean-up, A&A Biotechnology) and sequenced (Macrogen). The analysis of the sequences was performed by VectorN-TI (Informax).

A PCR-based assay was applied to screen for the presence of the 34-bp tandem repeat in the promoter region of the *cyp51A* gene as it was described elsewhere (Bromley *et al.*, 2014). 2x Master Mix HighGC (A&A Biotechnology) was applied for all of the PCR assays performed.

Examination of *cyp51A* and *cyp51B* expression level. All isolates were grown on Sabouraud agar plates for 72–96 h at room temperature (with no antimycotics and supplemented with 1 mg/L of voriconazole, respectively). Mycelia from the whole surface of the plate (diameter 900 mm) were placed in 2 ml tubes with zirconia/silica beads (A&A Biotechnology) and 400 µl of de-ionized water was added. The tubes were placed in a bead beater and shaken for 2 min at 2300 rpm. Lysates were then transferred into 1.5 ml tubes. The next steps of RNA extraction were performed by means of Total RNA Mini kit (A&A Biotechnology). Thus obtained RNA was treated by a DNase and further purified with Clean up RNA Concentrator (A&A Biotechnology). The concentration was determined using the NanoDrop1000 (Thermo Scientific). cDNA synthesis was carried out by means of TranScriba kit (A&A Biotechnology) with an oligo(dT)18 solution, 1 µg RNA and the rest of components from the kit.

Quantitative analysis of expression of the *cyp51A*, *cyp51B* (Buied *et al.*, 2013) and reference β-tubulin (Glass N.L., 1995) genes was performed by real-time PCR with a LightCycler Nano PCR Real-Time System (Roche). Real-time PCR was performed in 20 µl reaction volume containing the following reagents: 10 µl RealTime 2 X PCR Master Mix SYBR (A&A Biotechnology), 1 µl each primer solution (10 mM), 1 µl of total cDNA sample and distilled water. All reactions were performed in triplicates. The experiments were also repeated in triplicate for each specimen. Quantitative analysis of the level of expression of the investigated genes was carried out using the $R = 2^{-\Delta\Delta Cq}$ formula, also known as the Livak's method (Livak *et al.*, 2001). The azole resistant *A. fumigatus* strain from Centraalbureau voor Schimmelcultures (accession number CBS 133436) was applied as a calibrator.

RESULTS

Species identification and analysis of *cyp51A* sequence

The results of rRNA fragment and β-tubulin gene sequencing confirmed classical identification of the examined isolates as *A. fumigatus*. Amplification of the promoter region of *cyp51A* gene revealed presence of a 34 bp tandem repeat. Sequencing of *cyp51A* gene confirmed point mutation in the azole resistant isolate (no. 55) resulting in L98H substitution.

Examination of *cyp51A* and *cyp51B* expression level

The expression level of the *cyp51A* gene of 9/11 *A. fumigatus* isolates grown on Sabouraud agar with voriconazole was higher when compared to isolates cultured without any azoles. Two isolates cultured in the presence of voriconazole exhibited a lower expression level of the *cyp51A* gene. However, the dramatic increase from R = 0.354 to R = 2036 was detected in the azole resistant isolate no. 55 harboring the TR_{34}/L98H mutation (in comparison, the highest increase of azole susceptible isolate reached R = 1.777 from R = 0.008). Similar results were obtained for examination of the expression level of the *cyp51B* gene. We observed a change of R = 0.044 for the resistant isolate grown without access to voriconazole, to R = 42.942 for the same isolate cultured in the presence of voriconazole. However, the increase in expression level of *cyp51B* was lower when compared to an increase in *cyp51A* gene expression (Table 1).

DISSCUSSION

The relation between the occurrence of the TR_{34}/L98H mutation and resistance to azoles is well documented (Diaz-Guerra *et al.*, 2003; Howard *et al.*, 2011; Mellado *et al.*, 2007; Snelders *et al.*, 2011). We report here a great difference in expression of *cyp51A* and *cyp51B* genes of the azole resistant *A. fumigatus* isolate

Table 1. Expression level of *A. fumigatus* genes

Isolate no.	Expression level of the *cyp51A* gene		Expression level of the *cyp51B* gene	
	Isolates grown on Sabouraud agar	Isolates grown on Sabouraud agar with 1 mg/L voriconazole	Isolates grown on Sabouraud agar	Isolates grown on Sabouraud agar with 1 mg/L voriconazole
2	0.127	0.03	0.369	0.36
6	0.008	0.401	0.01	0.305
22	0.012	0.415	0.012	0.616
31	0.103	0.12	0.031	0.183
34	0.105	0.937	0.043	0.521
39	0.125	0.098	0.243	0.122
47	0.079	0.123	0.034	0.231
49	0.012	0.21	0.018	0.324
55	0.354	2036.675	0.044	42.942
71	0.028	0.346	0.014	0.482
82	0.008	1.777	0.016	0.349

harboring the TR_{34}/L98H mutation when grown on Sabouraud agar and Sabouraud agar supplemented with 1 mg/L voriconazole respectively. Our findings indirectly confirm the hypothesis of azole resistance induction by the presence of azoles in the environment (Snelders *et al*., 2009, Snelders *et al*., 2012, Stensvold *et al*., 2012, Verweji *et al*., 2009), as the elevated expression level of *cyp51* genes can be the reason for azole resistance occurrence. However, further examination of other resistance mechanisms is necessary, not only due to a dramatic increase of expression level of *cyp51A* gene in isolate no. 55, but also as most likely the azole resistance is a result of combination of different factors. Beside tandem repeats in the promoter region of the *cyp51A* gene and a point mutation in this gene, some transcription factors can also be involved in upregulation of *cyp51A*. For example, the SrbA protein is a regulating factor from the SREBP family (sterol regulator element binding protein), which is involved in the sterol biosynthesis. It is possible that SrbA can interfere with the *cyp51A* gene and influence its activity (Blosser *et al*., 2011). Other mechanisms may also induce azole resistance – for e.g. a mutation in the transcription factor HapE which binds the CCAAT sequence (Wei *et al*., 2015), overexpression of MFS (major facilitator superfamily) transporters (Shapiro *et al*., 2011) or overexpression of ABC transporters (ATP binding cassette superfamily).

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