

## Morphological changes and induction of antifungal resistance in *Aspergillus fumigatus* due to different CO<sub>2</sub> levels

Sima Darabian<sup>1</sup>, Sayed Jamal Hashemi<sup>2</sup>, Sadegh Khodavaisy<sup>2</sup>, Somayeh Sharifynia<sup>3</sup>, Mohammad Kord<sup>2</sup>, Maryam Akbari Dana<sup>2</sup>, Farzad Aala<sup>4</sup>, Sassan Rezaie<sup>2\*</sup>

<sup>1</sup> Department of Medical Mycology and Parasitology, School of Public Health, International Campus, Tehran University of Medical Sciences, Tehran, Iran

<sup>2</sup> Department of Medical Mycology and Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

<sup>3</sup> Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>4</sup> Department of Medical Mycology and Parasitology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran

### Article Info

#### Article type:

Original article

#### Article History:

Received: 04 December 2017

Revised: 31 December 2017

Accepted: 17 January 2018

#### \* Corresponding author:

Sassan Rezaie

Division of Molecular Biology,  
Department of Medical Mycology  
and Parasitology, School of Public  
Health, Tehran University of Medical  
Sciences, Tehran, Iran.

Email: srezaie@tums.ac.ir

### ABSTRACT

**Background and Purpose:** Aspergillosis is one of the most common opportunistic fungal infections in immunocompromised and neutropenic patients. *Aspergillus fumigatus* (*A. fumigatus*) is the most common causative agent of this infection. Due to variable CO<sub>2</sub> concentrations that pathogens are exposed to during the infection process and to understand the role of CO<sub>2</sub>, we examined the effects of various CO<sub>2</sub> concentrations as one of the environmental factors on morphological changes and induction of antifungal resistance in *A. fumigatus*.

**Materials and Methods:** *A. fumigatus* strains were cultured and incubated under 1%, 3%, 5%, and 12% CO<sub>2</sub> atmospheres, each time for one, two, and four weeks. The control culture was maintained for one week without CO<sub>2</sub> atmosphere. Morphological changes were investigated and antifungal susceptibility test was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document. The results of different CO<sub>2</sub> atmospheres were compared with that of the control sample.

**Results:** We found that 1%, 3%, 5%, and 12% CO<sub>2</sub> atmospheres were associated with morphological colony changes. Macroscopically, the colonies were shallow dark green, smooth, crisp to powdery with reduced growth; microscopic examination revealed the absence of conidiation. The induction of antifungal resistance in the susceptible strains to itraconazole, voriconazole, and amphotericin B increased after exposure to 12% CO<sub>2</sub> atmosphere and four weeks of incubation. The MIC values for itraconazole, voriconazole, and amphotericin B were 16 µg/ml, 1 µg/ml, and 16 µg/ml, respectively. These values for the control group were 0.125 µg/ml, 0.125 µg/ml, and 2 µg/ml, respectively.

**Conclusion:** Exposure to different CO<sub>2</sub> atmospheres induced morphological changes in *A. fumigatus*, it seems to increase the MIC values, as well. In parallel, resistance to both itraconazole and voriconazole was also observed.

**Keywords:** *Aspergillus fumigatus*, Carbon dioxide, Itraconazole, Voriconazole

#### ➤ How to cite this paper

Darabian S, Hashemi SJ, Khodavaisy S, Sharifynia S, Kord M, Akbari Dana M, Aala F, Rezaie S. Morphological changes and induction of antifungal resistance in *Aspergillus fumigatus* due to different CO<sub>2</sub> levels. *Curr Med Mycol.* 2017; 3(3): 21-26. DOI: 10.29252/cmm.3.3.21

## Introduction

**A** *aspergillus* species are widely distributed in the environment and have a wide range of manifestations from asymptomatic colonization or allergic reaction to invasive infection depending on host immunity [1, 2]. Invasive aspergillosis is the most severe manifestation with high mortality rate in immunocompromised patients [3, 4]. Amphotericin B (a member of the polyene antibiotics) and several azole-based medicines such as itraconazole, voriconazole, posaconazole, and isavuconazole are used for the treatment and prophylaxis of aspergillosis [5-7].

Recent reports from different European countries, the United States, South America, China, Japan, Iran, and India showed an increase in the frequency distribution of *A. fumigatus* isolates, indicating phenotypic resistance to itraconazole, voriconazole, and posaconazole [8-13].

Besides, specific point mutations in the *cyp51A* gene in combination with tandem repeats in the *cyp51A* promoter region are indicated as major environmentally derived mutations among azole-resistant *A. fumigatus* isolates. Additionally, *cyp51A*-

independent resistance mechanisms, such as the upregulation of drug efflux transporters, have been recognized [14, 15].

Acquired resistance in *A. fumigatus* may be caused by long-term treatment and exposure of the fungal cells to azole fungicides used in agriculture. Along with these factors, environmental changes including the nutrient sources, humidity, temperature, and air pollution could potentially lead to physiological changes in microorganisms [16]. Atmospheric CO<sub>2</sub> is considered an important agent for the biosphere homeostasis, which may cause changes in the physiological and morphological characteristics and the allergenic properties of saprophytic and pathogenic fungi. Such changes have been shown in *Cryptococcus neoformans* and *Candida albicans* during colonization and infection [17]. In addition, the effect of CO<sub>2</sub> pressure on growth and aflatoxin production in *Aspergillus flavus* was previously reported [18-21]. Other studies revealed that increasing CO<sub>2</sub> atmosphere in the culture medium could influence the pathogenicity of *A. fumigatus* [22, 23]. Due to the effectiveness of CO<sub>2</sub> pressure as one of the environmental factors, we examined the effects of different CO<sub>2</sub> concentrations on morphology and induction of antifungal resistance in *A. fumigatus*.

## Materials and Methods

### *A. fumigatus* strain

The wild-type strain of *A. fumigatus* (ATCC 1028) was cultured on Potato Dextrose Agar (PDA; Merck, Germany) and incubated at 35°C for 5-7 days. The suspension was prepared according to Clinical And Laboratory Standard Institute (CLSI M38-A2) protocol [24]. Briefly, fungal spores were harvested from 3 to 7 day-old cultures (in logarithmic growth phase) and mixed with distilled water containing 0.05% Tween 40; the mixture was adjusted at the final concentration of 1×10<sup>6</sup> CFU/ml by spectrophotometer (530 nm) [24]. Afterwards, 200 µl of the obtained suspension was added to PDA culture medium plates [25]. The plates were incubated at 35°C for 1, 2, and 4 weeks under 1%, 3%, 5%, and 12% CO<sub>2</sub> atmospheres separately.

CO<sub>2</sub> atmosphere was provided by using a 20-L cell culture CO<sub>2</sub> incubator (SLS, USA). The desired CO<sub>2</sub> injection was controlled by the calibrated automatic control panel in this incubator. The control culture was prepared under the same conditions without CO<sub>2</sub> pressure.

### Macroscopic and microscopic examinations

Macroscopic and microscopic criteria were investigated during culture under various CO<sub>2</sub> atmospheres. The color and texture features of the colony (i.e., flat, granular, downy to powdery, and radial grooves) were studied during the incubation time. By using Lactophenol Cotton Blue, the microscopic criteria were considered to assess the morphological changes [26, 27]. All the cultures

revealing morphological changes under CO<sub>2</sub> atmospheres were inoculated in the Tryptic Soy Broth (TSB) medium (Merck, Germany) and stored at -20°C. After three weeks, the stocked cultures in TSB medium were sub-cultured in PDA medium and incubated at 35°C without CO<sub>2</sub> atmosphere, and their morphological aspects were checked for reversibility evaluation.

### Antifungal susceptibility test

The minimum inhibitory concentrations (MICs) of three antifungal agents were evaluated according to CLSI (M38-A2) protocol [24, 28]. Itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer, NY, USA), and amphotericin B (Bristol-Myers-Squibb, Woerden, the Netherlands) were obtained as reagent-grade powders dissolved in dimethyl sulfoxide (DMSO) and were diluted in standard RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) buffered to pH 7.0 with 0.165 mol.L<sup>-1</sup> morpholinepropanesulfonic acid buffer with L-glutamine without bicarbonate (MOPS, Sigma-Aldrich, St. Louis, MO, USA). The final concentrations of itraconazole, voriconazole, and amphotericin B ranged from 0.016 to 16 µg/ml. The plates were stored at -20°C prior to use [29]. All the cultured fungal cells under CO<sub>2</sub> pressure were scraped after sporulation and the obtained spores were suspended in distilled water containing 0.05% Tween-40. The optical density (OD) of the supernatant was adjusted spectrophotometrically at 530 nm. The final concentrations of the stock inoculum suspensions were within the range of 0.5-4 × 10<sup>4</sup> CFU/ml. Microdilution plates were incubated at 35°C and examined visually for MIC determination within 48 h of incubation. The MICs for itraconazole, voriconazole, and amphotericin B were defined as the concentration that fully inhibits fungal growth. The strain *Candida parapsilosis* ATCC 22019 was used as the negative quality control. All the tests were performed in duplicate.

This study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (code: IR.TUMS.SPH.REC.1396.2289).

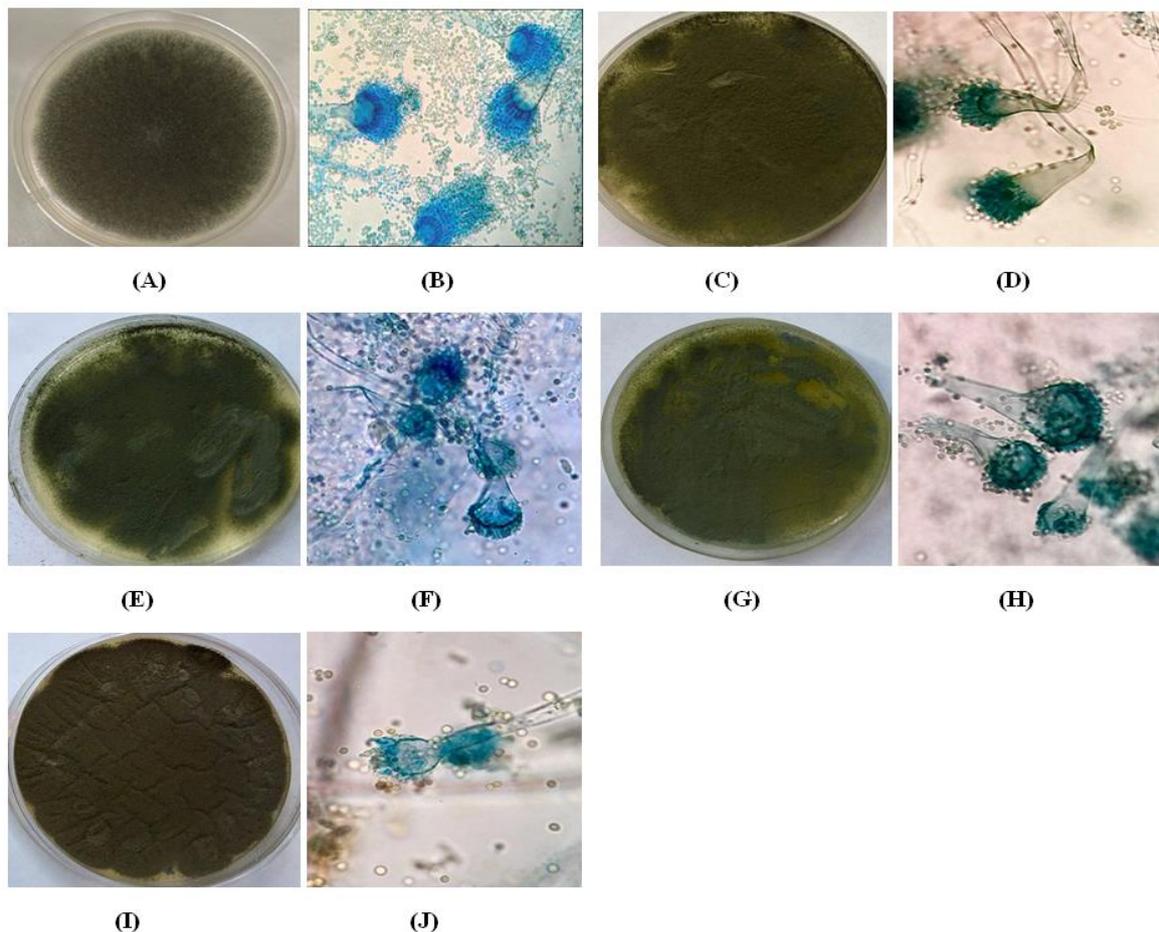
## Results

We observed significant changes in the macroscopic and microscopic characteristics of *A. fumigatus* wild-type in different situations (CO<sub>2</sub> atmosphere and incubation duration). The initial macro- and micro-colony changes occurred in the presence of low CO<sub>2</sub> pressure (1%). However, the minimum of such changes were observed under 3% and 5% CO<sub>2</sub> atmospheres after one, two, and four weeks, while maximum changes happened under 12% CO<sub>2</sub> atmosphere after four weeks. The major macroscopic changes appeared in the culture media that included shallow colonies with dark green color, smooth and crisp to powdery appearance, and reduced growth. However, the major microscopic changes appeared in exposed fungal cells with short

conidiophores, deformed vesicles, and declined conidia (Figure 1). These results were obtained by comparison with the control colonies, which were blue-green, fast-growing, fluffy to powdery colonies with normal conidiophores, arranged vesicles, phialides, metulae, and smooth hyaline conidia (Figure 1). The reversibility of morphological changes was reviewed through culture of the samples after four weeks in the absence of CO<sub>2</sub> atmosphere.

The obtained results indicated that *A. fumigatus* could maintain the described changes even in the absence of CO<sub>2</sub> atmosphere.

The MIC values for itraconazole, voriconazole, and amphotericin B under various CO<sub>2</sub> atmospheres and incubation times are listed in Table 1. The MIC values after 48 h of incubation increased with elevated CO<sub>2</sub> pressure and prolonged incubation time. Under 12% CO<sub>2</sub> atmosphere and after four weeks of



**Figure 1.** Photographs of macroscopic morphological changes of *A. fumigatus* following exposure to different levels of CO<sub>2</sub> after four weeks. (A) Macroscopic examination with the original colony; (B) microscopic examination with the normal features of conidiation; (C, D) inception of changes were observed under 1% CO<sub>2</sub> atmosphere, (E, F) 3% CO<sub>2</sub> atmosphere, (G, H) 5% CO<sub>2</sub> atmosphere, and (I, J) the most changes occurred under 12% CO<sub>2</sub> atmosphere.

**Table 1.** In vitro susceptibility of *A. fumigatus* to itraconazole, voriconazole, and amphotericin B under different CO<sub>2</sub> atmospheres

Strain	CO <sub>2</sub> level	Time (week)	Minimum inhibitory concentration (µg/ml)		
			ITR	VOR	AMB
<i>A. fumigatus</i> (ATCC 1028)	1% CO <sub>2</sub>	1	0.125	0.5	4
		2	0.5	0.125	4
		4	1	0.125	8
	3% CO <sub>2</sub>	1	1	0.5	8
		2	1	0.125	8
		4	1	0.5	8
	5% CO <sub>2</sub>	1	0.5	0.5	8
		2	2	0.5	8
		4	8	0.5	16
	12% CO <sub>2</sub>	1	1	0.5	8
		2	2	0.5	8
		4	16	1	16
Control cultures			0.125	0.125	1

Abbreviations: ITR: itraconazole; VOR: voriconazole; AMB: amphotericin B

incubation, the MIC values for itraconazole, voriconazole, and amphotericin B were 16 µg/ml, 1 µg/ml, and 16 µg/ml, respectively. However, these values were 0.125 µg/ml, 0.125 µg/ml, and 1 µg/ml, respectively, in the control group (Table 1).

## Discussion

*A. fumigatus* is one of the most important fungal pathogens and the main causative agent of invasive aspergillosis. This species is affected by environmental conditions such as CO<sub>2</sub> [23, 30]. Studies showed that CO<sub>2</sub> could significantly affect fungal physiology and morphology [31]. Previous studies on the effects of CO<sub>2</sub> atmosphere on pathogenesis and metabolism of *C. albicans* and *Cryptococcus neoformans* revealed the induction of invasion to blood in *C. albicans* cells under 5% CO<sub>2</sub> atmosphere. Besides, filamentation and pseudohyphal formation were reported to be induced in such cells [25, 32, 33]. Capsular polysaccharide synthesis of *C. neoformans* was stimulated after exposure to 5% CO<sub>2</sub> atmosphere [23, 34-36]. Another study indicated the occurrence of arthroconidium formation, a resistance factor in *T. rubrum*, after 10 days of incubation on Sabouraud Dextrose Agar medium at 37°C under 10% CO<sub>2</sub> atmosphere [37]. Some investigations have considered the effect of CO<sub>2</sub> on fungal growth and found a general inhibitory effect under CO<sub>2</sub> atmospheres [31]. Coelho et al. reported that high CO<sub>2</sub> pressure could have detrimental effects on growth and metabolism of yeasts, and therefore, could contribute to the inactivation of *Saccharomyces cerevisiae* cells [38, 39]. Due to its antimicrobial activity, CO<sub>2</sub> can be particularly used for storing food commodities through the inactivation of the microorganisms [40]. Changed CO<sub>2</sub> atmosphere, carbonic anhydrases (CAs), and C: N ratio have been reported to affect growth by influencing the production of proteins and the content of fungal spores [41, 42]. The effects of CO<sub>2</sub> on morphology, growth, and citric acid production in *A. niger* were also reported by McIntyre and McNeil [31].

Recent investigations indicated that *A. fumigatus* and *A. flavus* spores grown under varying CO<sub>2</sub> atmosphere showed higher allergenicity [22, 32]. In line with the previous investigations, we appraised the effect of CO<sub>2</sub> on morphological changes in *A. fumigatus*. The obtained results indicated that CO<sub>2</sub> atmosphere could enhance some morphological (macroscopic and microscopic) changes such as sporulation, vesicle deformation, and the development of *Chlamydia* spores in *A. fumigatus* strains. The majority of the mentioned changes were also indicated to occur under 12% CO<sub>2</sub> atmosphere and after four weeks of incubation. Previous reports revealed the capability of pathogenic *Aspergillus* species in growth under CO<sub>2</sub> atmosphere; they also showed that this capacity might affect the pathogenicity and antifungal activity of these species [20]. Our findings may help with deeper understanding of this process. Besides,

variation in CO<sub>2</sub> atmosphere in environments where *A. fumigatus* is able to grow reveals the importance of effective factor and CO<sub>2</sub> absorb regulator genes in these fungi.

Our results manifested that MICs of itraconazole, voriconazole, and amphotericin B against *A. fumigatus* increased near high CO<sub>2</sub> environments. However, the mechanism of resistance induction by CO<sub>2</sub> remains inconspicuous. Some medicines and environmental factors have been used for the induction of drug resistance in *A. fumigatus* [43]. In some studies, different types of resistance mechanisms, including changes in the target lanosterol-14-demethylase and induction of high-capacity drug efflux pumps, have been shown to promote drug resistance following UV-induced mutations [44-46]. In the study of Escribano et al., the MICs of itraconazole, voriconazole, and posaconazole against *A. fumigatus* cells were determined before and after exposure of the fungal cells to itraconazole. The obtained results indicated that the final MICs were substantially higher than the initial ones [47]. Faria-Ramos et al. investigated cross-resistance in *A. fumigatus* to clinical azoles after the exposure of fungal cells to prochloraz (an agronomical azole) and found that resistance to prochloraz increased after the initial exposure [48]. In addition, prochloraz exposure caused some morphological changes in fungal colony such as changing the appearance to white-colored colonies, losing the typical pigmentation, and absence of conidiation [48]. Our results also indicated the macroscopic and microscopic morphological changes in *A. fumigatus* due to CO<sub>2</sub> pressure. However, these morphological changes were not similar to the alterations described by Faria-Ramos et al.

The results of these studies indicate that *A. fumigatus* can be resistant to antifungal compounds due to antifungal agents in medicine, fungicides used in agriculture, and environmental factors. The results of this study also indicated that high concentrations of CO<sub>2</sub> could be considered as an environmental factor affecting the occurrence of drug resistance in fungi.

## Conclusion

Different levels of CO<sub>2</sub> exposure induced morphological changes in *A. fumigatus*, an evident increase in MIC values, and the development of cross-resistance to itraconazole and voriconazole. Our next step is assessing the underlying molecular resistance mechanisms in these induced resistant strains, as well as in isolates with naturally high MIC values in different levels of CO<sub>2</sub> and evaluating resistance to antifungal drugs without any prior in vitro induction.

## Acknowledgments

We wish to thank the Division of Molecular Biology, Tehran University of Medical Sciences, Tehran, Iran, for providing us laboratory facilities for performing tests. This study was financially supported by the School of Public Health, International Campus,

Tehran University of Medical Sciences (TUMS; grant No: 9223687002).

### Author's contribution

S. R., S.J. H., and S. K. designed and managed the project. S. D. performed the tests and wrote the first draft of the manuscript. S. R. edited the final manuscript. S. S., M. K., M. A.D., and F.A. were the project partner.

### Conflicts of interest

None declared.

### Financial disclosure

No pecuniary interest related to the material of this writing has been declared.

### References

- Rocchi S, Reboux G, Millon L. Azole resistance with environmental origin: what alternatives for the future? *J Mycol Med*. 2015; 25(4):249-56.
- Abdolrasouli A, Rhodes J, Beale MA, Hagen F, Rogers TR, Chowdhary A, et al. Genomic context of azole resistance mutations in *Aspergillus fumigatus* determined using whole-genome sequencing. *MBio*. 2015; 6(3):e00536.
- Denning DW, Pleuvry A, Cole DC. Global burden of allergic bronchopulmonary *aspergillosis* with asthma and its complication chronic pulmonary *aspergillosis* in adults. *Med Mycol*. 2013; 51(4):361-70.
- Meersseman W, Lagrou K, Maertens J, Van Wijngaerden E. Invasive *aspergillosis* in the intensive care unit. *Clin Infect Dis*. 2007; 45(2):205-16.
- Maertens JA, Blennow O, Duarte RF, Muñoz P. The current management landscape: *aspergillosis*. *J Antimicrob Chemother*. 2016; 71(2):ii23-9.
- Patterson TF, Thompson GR 3rd, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice guidelines for the diagnosis and management of *Aspergillosis*: 2016 update by the infectious diseases society of America. *Clin Infect Dis*. 2016; 63(4):e1-60.
- Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, et al. Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother*. 1997; 41(6):1364-8.
- Seyedmousavi S, Hashemi SJ, Zibafar E, Zoll J, Hedayati MT, Mouton JW, et al. Azole-resistant *Aspergillus fumigatus*, Iran. *Emerg Infect Dis*. 2013; 19(5):832-4.
- Nabili M, Shokohi T, Moazeni M, Khodavaisy S, Aliyali M, Badiie P, et al. High prevalence of clinical and environmental triazole-resistant *Aspergillus fumigatus* in Iran: is it a challenging issue? *J Med Microbiol*. 2016; 65(6):468-75.
- Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ, Verweij PE. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol*. 2009; 75(12):4053-7.
- Gonçalves SS, Souza AC, Chowdhary A, Meis JF, Colombo AL. Epidemiology and molecular mechanisms of antifungal resistance in *Candida* and *Aspergillus*. *Mycoses*. 2016; 59(4):198-219.
- Escribano P, Peláez T, Muñoz P, Bouza E, Guinea J. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? *Antimicrob Agents Chemother*. 2013; 57(6):2815-20.
- Chowdhary A, Kathuria S, Xu J, Meis JF. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog*. 2013; 9(10):e1003633.
- Shalhoub S, Luong ML, Howard SJ, Richardson S, Singer LG, Chaparro C, et al. Rate of cyp51A mutation in *Aspergillus fumigatus* among lung transplant recipients with targeted prophylaxis. *J Antimicrob Chemother*. 2015; 70(4):1064-7.
- Wang HC, Huang JC, Lin YH, Chen YH, Hsieh MI, Choi PC, et al. Prevalence, mechanisms, and genetic relatedness of the human pathogenic fungus *Aspergillus fumigatus* exhibiting resistance to medical azoles in the environment of Taiwan. *Environ Microbiol*. 2018; 20(1):270-80.
- da Silva Ferreira ME, Capellaro JL, dos Reis Marques E, Malavazi I, Perlin D, Park S, et al. In vitro evolution of itraconazole resistance in *Aspergillus fumigatus* involves multiple mechanisms of resistance. *Antimicrob Agents Chemother*. 2004; 48(11):4405-13.
- Sheth CC, Johnson E, Baker ME, Haynes K, Mühlshlegel FA. Phenotypic identification of *Candida albicans* by growth on chocolate agar. *Med Mycol*. 2005; 43(8):735-8.
- Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW. *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. *Microbiology*. 2007; 153(6):1677-92.
- Ellis W, Smith J, Simpson BK, Khanizadeh S, Oldham JH. Control of growth and aflatoxin production of *Aspergillus flavus* under modified atmosphere packaging (MAP) conditions. *Food Microbiol*. 1993; 10(1):9-21.
- Hall LA, Denning DW. Oxygen requirements of *Aspergillus* species. *J Med Microbiol*. 1994; 41(5):311-5.
- Gilbert MK, Mack BM, Payne GA, Bhatnagar D. Use of functional genomics to assess the climate change impact on *Aspergillus flavus* and aflatoxin production. *World Mycotoxin J*. 2016; 9(5):665-72.
- Lang-Yona N, Levin Y, Dannemiller KC, Yarden O, Peccia J, Rudich Y. Changes in atmospheric CO<sub>2</sub> influence the allergenicity of *Aspergillus fumigatus*. *Glob Change Biol*. 2013; 19(8):2381-8.
- Tobal JM, Balieiro ME. Role of carbonic anhydrases in pathogenic micro-organisms: a focus on *Aspergillus fumigatus*. *J Med Microbiol*. 2014; 63(1):15-27.
- Wayne P. Reference method for broth dilution antifungal susceptibility testing of *filamentous* fungi. Approved standard-second edition. *Clin Lab Stand Instit*. 2008; 28(16):6-12.
- Sasani E, Khodavaisy S, Agha Kuchak Afshari S, Darabian S, Aala F, Rezaie S. Pseudohyphae formation in *Candida glabrata* due to CO<sub>2</sub> exposure. *Curr Med Mycol*. 2016; 2(4):49-52.
- Latgé JP. *Aspergillus fumigatus* and *aspergillosis*. *Clin Microbiol Rev*. 1999; 12(2):310-50.
- McClenny N. Laboratory detection and identification of *Aspergillus* species by microscopic observation and culture: the traditional approach. *Med Mycol*. 2005; 43(Suppl 1):S125-8.
- Wayne PA. Clinical and Laboratory Standards Institute; 2011. CLSI Performance standards for antimicrobial susceptibility testing 20th Informational Supplement CLSI document M100-S21. *Clin Lab Stand Instit*. 2011; 31(1):1-162.
- Khodavaisy S, Badali H, Hashemi SJ, Aala F, Nazeri M,

- Nouripour-Sisakht S, et al. In vitro activities of five antifungal agents against 199 clinical and environmental isolates of *Aspergillus flavus*, an opportunistic fungal pathogen. *J Mycol Med*. 2016; 26(2):116-21.
30. Mousavi B, Hedayati MT, Hedayati N, Ilkit M, Syedmousavi S. *Aspergillus* species in indoor environments and their possible occupational and public health hazards. *Curr Med Mycol*. 2016; 2(1):36-42.
31. Papagianni M. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnol Adv*. 2004; 22(3):189-259.
32. Bahn YS, Cox GM, Perfect JR, Heitman J. Carbonic anhydrase and CO<sub>2</sub> sensing during *Cryptococcus neoformans* growth, differentiation, and virulence. *Curr Biol*. 2005; 15(22):2013-20.
33. Du H, Guan G, Xie J, Cottier F, Sun Y, Jia W, et al. The transcription factor Flo8 mediates CO<sub>2</sub> sensing in the human fungal pathogen *Candida albicans*. *Mol Biol Cell*. 2012; 23(14):2692-701.
34. Mogensen EG, Janbon G, Chaloupka J, Steegborn C, Fu MS, Moyrand F, et al. *Cryptococcus neoformans* senses CO<sub>2</sub> through the carbonic anhydrase Can2 and the adenylyl cyclase Cac1. *Eukaryot Cell*. 2006; 5(1):103-11.
35. Klutts JS, Doering TL. Cryptococcal xylosyltransferase 1 (Cxt1p) from *Cryptococcus neoformans* plays a direct role in the synthesis of capsule polysaccharides. *J Biol Chem*. 2008; 283(21):14327-34.
36. Kim MS, Ko YJ, Maeng S, Floyd A, Heitman J, Bahn YS. Comparative transcriptome analysis of the CO<sub>2</sub> sensing pathway via differential expression of carbonic anhydrase in *Cryptococcus neoformans*. *Genetics*. 2010; 185(4):1207-19.
37. Yazdanparast SA, Barton RC. Arthroconidia production in *Trichophyton rubrum* and a new ex vivo model of *onychomycosis*. *J Med Microbiol*. 2006; 55(Pt 11): 1577-81.
38. Coelho M, Belo I, Pinheiro R, Amaral A, Mota M, Coutinho J, et al. Effect of hyperbaric stress on yeast morphology: study by automated image analysis. *Appl Microbiol Biotechnol*. 2004; 66(3):318-24.
39. Shimoda M, Cocunubo-Castellanos J, Kago H, Miyake M, Osajima Y, Hayakawa I. The influence of dissolved CO<sub>2</sub> concentration on the death kinetics of *Saccharomyces cerevisiae*. *J Appl Microbiol*. 2001; 91(2):306-11.
40. Shimoda M, Yamamoto Y, Cocunubo-Castellanos J, Tonoike H, Kawano T, Ishikawa H, et al. Antimicrobial effects of pressured carbon dioxide in a continuous flow system. *J Food Sci*. 1998; 63(4):709-12.
41. Rhodes JC. *Aspergillus fumigatus*: growth and virulence. *Med Mycol*. 2006; 44(1):S77-81.
42. Mavridou E, Brüggemann RJ, Melchers WJ, Mouton JW, Verweij PE. Efficacy of posaconazole against three clinical *Aspergillus fumigatus* isolates with mutations in the cyp51A gene. *Antimicrob Agents Chemother*. 2010; 54(2):860-5.
43. Krishnan S, Manavathu EK, Chandrasekar PH. *Aspergillus flavus*: an emerging non *fumigatus* *Aspergillus* species of significance. *Mycoses*. 2009; 52(3):206-22.
44. Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14 $\alpha$ -demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob Agents Chemother*. 1998; 42(2):241-53.
45. Oshero N, Kontoyiannis DP, Romans A, May GS. Resistance to itraconazole in *Aspergillus nidulans* and *Aspergillus fumigatus* is conferred by extra copies of the A. *nidulans* P-450 14 $\alpha$ -demethylase gene, pdmA. *J Antimicrob Chemother*. 2001; 48(1):75-81.
46. Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M, Bille J. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother*. 1995; 39(11):2378-86.
47. Escribano P, Recio S, Peláez T, González-Rivera M, Bouza E, Guinea J. In vitro acquisition of secondary azole resistance in *Aspergillus fumigatus* isolates after prolonged exposure to itraconazole: presence of heteroresistant populations. *Antimicrob Agents Chemother*. 2012; 56(1):174-8.
48. Faria-Ramos I, Farinha S, Neves-Maia J, Tavares PR, Miranda IM, Estevinho LM, et al. Development of cross-resistance by *Aspergillus fumigatus* to clinical azoles following exposure to prochloraz, an agricultural azole. *BMC Microbiol*. 2014; 14:155.