Prof. Dr. Dominique Sanglard
Institut de Microbiologie
de l'Université de Lausanne
Rue du Bugnon 44
1011 Lausanne
dominique.sanglard@chuv.
hospvd.ch

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Prof. Dr. Jacques Bille
Institut de Microbiologie de
l'Université de Lausanne
Jacques.bille@chuv.hospvd.ch

Resistance of the fungal pathogen Aspergillus fumigatus to antifungal agents

Objectives *Aspergillus fumigatus* is a widespread fungal species in the natural environment that is pathogenic to plants, animals and humans. As *A. fumigatus* infections are on the rise in humans and the use of antifungal agents is increasing, antifungal resistance is emerging as well. Since mechanisms of resistance have been poorly investigated up to now, this project aims to i) identify the genes involved in *A. fumigatus* antifungal resistance and investigate their involvement in drug resistance in sample isolates, and ii) collect *A. fumigatus* strains from Swiss hospitals and the environment in order to establish the present status regarding antifungal drug resistance in Switzerland.

Conclusions For the first time in Switzerland, the status of antifungal drug resistance in *A. fumigatus* isolates originating from clinical centres and from different environmental sites was established. Despite extensive use of azoles in hospital as well as in environmental settings (agriculture), few *A. fumigatus* isolates exhibited resistance to azoles. The observation that high MIC (Minimum Inhibitory Concentration) values (MIC ≥ 2 µg/ml) for itraconazole or voriconazole in clinical isolates are not accompanied with high MIC across all azoles reinforces the idea of a lack of cross-resistance among tested azoles, which may be due to the different chemical structures of these drugs acting differentially on cellular targets. No clear cross-resistance patterns with azoles used in agriculture (imazalil, prochloraz and propiconazole) were observed.
Thanks to the new analytical tool that was designed to study specific mechanisms in resistance to antifungal drugs, several azole resistance genes, among them multidrug transporter genes, could be found. However, even if their involvement in azole resistance in *A. fumigatus* could not be clearly demonstrated, the identification of other antifungal resistance genes (i.e. *cyp51A*) helped to elucidate mechanisms by which resistance can develop.

Main results and findings

Antifungal susceptibility of clinical and environmental *A. fumigatus* isolates originating from Switzerland The antifungal susceptibility of Swiss clinical (336) and environmental (184) *A. fumigatus* isolates (for a total of 520 isolates) was assessed. The results can be summarised as follows:

- *A. fumigatus* isolates are intrinsically resistant to iprodione (dicarboximide), benalaxyl (phenylamid) or cyprodinil (MIC_{90} ≥ 32 µg/ml).
- Caspofungin susceptibility in *A. fumigatus* was not undertaken due to known technical difficulties.
- For the tested drugs, the results are summarised in the following table:

Drug	MIC range (µg/ml)		MIC distribution (µg/ml)			
	Clinical isolates	Environmental isolates	Clinical isolates		Environmental isolates	
			MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Itraconazole	0.03-4	0.06-16	0.5	1	0.5	2
Voriconazole	0.03-4	0.06-16	0.25	1	0.25	0.5
Amphotericin B	0.06-8	0.5-8	1	4	2	4
Imazalil	0.08-2	0.02-8	0.13	0.5	0.06	0.13
Prochloraz	0.02-8	0.02-16	0.13	1	0.06	0.25
Propiconazole	0.03-16	0.5-32	2	8	4	8
Azoxystrobin	0.25-≥16	1-≥16	≥16	≥16	≥16	≥16

- Even if MIC ranges for the clinical and environmental isolates were not totally overlapping, they were in the same concentration range.
- The MIC_{50} results obtained demonstrate that among the antifungals tested and used in medicine (i.e. amphotericin B, itraconazole and voriconazole), voriconazole is the most active agent on both clinical and environmental isolates. These results confirm that voriconazole has a broader coverage than its predecessors (i.e. itraconazole).

– Among the 336 clinical isolates, the following particularities were identified:
  - Only 10 isolates were found with itraconazole MIC ≥ 2 µg/ml (referenced itraconazole MICs: susceptible ≤0.25 µg/ml, resistant ≥ 8 µg/ml). Generally, high itraconazole MIC values are not correlated with high MIC across all azoles, suggesting absence of cross-resistance.
  - Only 11 isolates were found with high MIC values for voriconazole (MIC ≥ 2 µg/ml). Again, high voriconazole MIC values are not accompanied with high MIC across all azoles, reinforcing the idea of a lack of cross-resistance among tested azoles.
– Among the 184 environmental isolates, the following particularities were identified:
  - For itraconazole, 7 isolates showed MIC ≥ 4 µg/ml. However, taking MICs ≥ 2 µg/ml as threshold, the number of isolates increased to 49, which is largely above the number observed for clinical strains.
  - For voriconazole, only 1 isolate showed a MIC of 16 µg/ml.
  - One isolate (Env51) was particularly interesting, with itraconazole and propiconazole resistance (MIC ≥ 16 µg/ml) as well as high voriconazole and prochloraz MICs (1 µg/ml).
  - Fifteen percent of the isolates showed amphotericin B MICs of 4 µg/ml.

Cloning of *A. fumigatus* genes involved in antifungal drug resistance Several cDNAs conferring antifungal resistance were isolated. Some clones showed strong homology to ABC transporters from fungal species, to MFS transporters and to YAP-1 like transcription factors. Screening a cDNA library in *S. cerevisiae* with fluconazole allowed the isolation of a new ABC transporter, named *AtrH,* and a B-Zip transcription factor, named *YapA.* Screening with voriconazole allowed the isolation of a gene similar to transporters of the class of the major facilitator Superfamily (MFS), named *MdrA.* The expression of isolated genes into *S. cerevisiae* and the isolation on selective media containing antifungals and metabolic inhibitors showed that *AtrH* conferred resistance to almost all of the tested azoles and to inhibitors like cerulinin and cycloheximide, and *AtrF* and *MdrA* conferred resistance to a more restricted panel of azoles.

Involvement of cloned genes (*AtrF*, *AtrH*, *MdrA* and *YapA*) in the development of antifungal drug resistance in *A. fumigatus* A complex pattern of expression levels of the different transporters could be observed. However, no correlation between itraconazole resistance in *A. fumigatus* isolates and overexpression of the investigated genes could be observed, suggesting that other resistance mechanisms could be involved in the itraconazole resistance of these isolates.

***A. fumigatus* mutations in Cyp51A and resistance to azole antifungal agents** In clinical isolates it was observed that some Cyp51A mutations are correlated with itraconazole resistance. In general, Cyp51A mutations from itraconazole-resistant isolates contained the expected mutations, whereas cDNA from itraconazole susceptible strains do not contain mutations. The Cyp51A mutations so far investigated conferred only itraconazole resistance in *A. fumigatus.* The results show that itraconazole resistance is due to the presence of a mutation at position 54 rather than a mutation at position 220.
In environmental isolates, none of the mutations found in clinical isolates were recovered, but several other mutations (as compared to wild type *Cyp51A* sequence) were identified.

Publications of the NRP 49 project

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