A mutation in the Class III Histidine-Kinase Bos1 is responsible for *Aspergillus fumigatus* dicarboximide resistance

J. LUCIO[1], I. GONZALEZ-JIMENEZ[1] and E. MELLADO[1,2]
[1]Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, Spain.
[2]Spanish Network for Research in Infectious Diseases (REIPI RD16/CIII/0004/0003), ISCIII, Majadahonda, Madrid, Spain.

10th Advances Against Aspergillosis and Mucormycosis

Poster Nº 52

INTRODUCTION

Aspergillus fumigatus is a worldwide opportunistic fungal pathogen that causes aspergillosis, a highly lethal broad spectrum of pathologies in immunocompromised individuals[1]. Invasive aspergillosis is the most critical clinical manifestation of aspergillosis regarding its high morbidity and mortality rates. Triazole drugs are the first line antifungal treatment for aspergillosis, targeting the 14-α sterol demethylases (Cyp51A/Cyp51B), key role enzymes in the ergosterol biosynthesis pathway. However, the rising isolation of azole-resistant *A. fumigatus* strains in the last decade is imposing a great challenge in the management of patients with aspergillosis[2].

The continuous exposure of *A. fumigatus* to environmental fungicides, such as demethylase inhibitors (DMIs), used for crop protection against other fungal plant pathogens, is believed to be selecting multi-drug resistant strains. Besides the use of DMI fungicides in the environmental side, there is a parallel exposure to fungicides of single mode of action[3], which is leading to the selection of mutations in the genes encoding the target site protein and therefore, decreasing the effectiveness of these antifungals. This situation applies, not only to a diverse amount of fungal plant pathogens but also to non-targeted fungi, including *A. fumigatus*[4], via the selection of antifungal resistant strains that are unaffected by multiple fungicides and favoring their spread and dissemination.

Recently, we have described that azole resistant *A. fumigatus* isolates bearing the TR_{34}/L98H Cyp51A mutations showed resistance to several other environmental single sites antifungal classes such as benzimidazoles (MBCs), strobilurins (QoIs) and succinate dehydrogenase inhibitors (SDHIs)[5]. A whole-genome sequencing previously performed with a collection of 163 *A. fumigatus* strains indicates a common evolution pattern and a genetic relationship among fungicide multiresistant strains, grouping into subclusters where all the *A. fumigatus* TR_{34}/L98H azole-resistant isolates grouped.

In this study, a collection of azole-susceptible and resistant *A. fumigatus* strains with different mutations in Cyp51A, β-tubulin, cytochrome b and SdhB were susceptibility tested against dicarboximide (iprodione) and phenylpirrole (fludioxinil) antifungals.

MATERIAL AND METHODS

To carry out this study we selected sixty *A. fumigatus* isolates, 19 azole-susceptible strains and 41 azole-resistant strains, harboring different mutations in Cyp51A, β-tubulin, cytochrome b and SdhB **(Table 1)**.

The susceptibility of these isolates to two nonazole fungicides, dicarboximide (iprodione) and phenylpirrole (fludioxonil) was tested. Susceptibility testing was assessed spotting 3 µL, containing $3x10^3$ spores, on three sets of minimal medium plates, one of them containing 8 mg/L fludioxonil, another plate containing 32 mg/L iprodione and a growth control plate.

The target gene *bos1* was PCR amplified, sequenced and analyzed in all selected strains. In addition, the *bos1* gene was analyzed in a collection of 163 *A. fumigatus* genomes from different countries with a variety of azole resistant mechanisms.

CONCLUSIONS

1. In this work we described for the first time the *A. fumigatus* dicarboximide resistance in clinical isolates from Spain.

2. The Bos1 mutation I399N is responsible for *A. fumigatus* resistance to the dicarboximide iprodione but not to the phenylpirrole fludioxonil.

3. A strong association between the azole resistance mechanism TR_{34}/L98H and the resistance phenotype to several environmental fungicides reinforced the environmental resistance origin of these resistant strains. These results suggest a selection of multi-drug resistant strains in crops.

FUNDING

This work was supported by Fondo de Investigacion Sanitaria (FIS PI18CIII/00045) and Plan Nacional de I+D+i 2013-2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD16/CIII/0004/0003), co-financed by European Development Regional Fund ERDF "A way to achieve Europe", Operative program Intelligent Growth 2014-2020. J. Lucio holds a predoctoral fellowship from the Fondo de Investigación Sanitaria (F17CIII/00037).

SEQUENCE ANALYSIS OF Bos1 AND ANTIFUNGAL SUSCEPTIBILITY TESTING

Three azole-resistant strains harbored a point mutation, I399N **(Table 1)**, in the HAMP 3 domain of Class III Histidine-Kinase Bos1 coding region **(Figure 1)**.

```
HAMP1  237                          LREIGGIITQVANGDLSMKVQIHPLEMDPEIATFKRTINTMMDQLQVFGSEVSRVAREVGTEGILGGQA
HAMP2  305 QITGVHGIWKELTENVNIMAKNLTDQVREIAAVTTAVAHGDLSQKIESRAQGEILELQQTINTMVDQLRTFATEVTRVARDVGTEGVLGGQA
HAMP3  398 QIEGVQGMWNELTVNVNAMANNLTTQVRDIATVTKAVAKGDLTQKVQANCKGEIABLKNIINSMVDQLRQFAQEVTKIAKEVGTDGVLGGQA
HAMP4  490 TVNDVEGTWKDLTENVNRMANNLTTQVREIADVTTAVAKGDLTKKVTANVQGEILDLKSTINGMVDRLNTFAFEVSKVAREVGTDGTLGGQA
HAMP5  582 KVDNVEGKWKDLTDNVNTMAQNLTSQVRSISDVTQAIAKGDLSKKIEVHAQGEILTLKVTINHMVDRLAKFATELKKVARDVGVDGKMGGQA
HAMP6  674 NVEGIAGTWKEITEDVNTMAENLTSQVRAFGEITDAATDGDFTKLITVNASGEMDELKRKINKMVSNLRDSIQRNTAAREAAELANRTKSE
```

Figure 1. Representation of the six HAMP domains of *A. fumigatus* Class III Histidine-Kinase Bos1. The amino acid sequences were aligned with Clustal W. The amino acids identical over 80% are in bold. Isoleucine at position 399 (**I399**) in Hamp 3 domain is marked in red and highlighted.

These strains showed a iprodione resistant phenotype, considering resistance when the growth observed in the minimal medium plates containing 32 mg/L of iprodione was similar to the growth control **(Figure 2A and 2C).**

None of the *A. fumigatus* strains harboring the point mutation I399N or the rest of strains included in the study showed a resistant phenotype to the phenylpyrrrole fludioxonil **(Figure 2B)**. Iprodione resistant strains showed concomitant resistance to DMIs and MBCs or SDHIs.

Table 1. *A. fumigatus* Cyp51A, β-tub, CytB, SDHB and Bos1 sequence analysis. WT: Wild type; S, susceptible; R, resistant; DMI, Demethylase inhibitors; MBC, benzimidazoles; QoIs, strobilurins; SDHI, succinate dehydrogenase inhibitors.

# of isolates	Amino acid substitutions					Susceptibility to agricultural antifungal drugs									
						DMIs [3]		MBCs [1]		QoIs [11]		SDHIs [7]		Dicarboximides [2]	Phenylpirrole [12]
	Cyp51A	β-tub	CytB	SDHB	Bos1	Imidazole	Triazole	BNY	CBZ	AZB	PYB	BCL	FLP	Iprodione	Fludioxinil
Azole-susceptible strains with no mutations (19)															
19	WT	WT	WT	WT	WT	S	S	S	S	S	S	S	S	S	S
Azole-resistant strains with mutations only in Cyp51A (10)															
10	TR_{34}/L98H	WT	WT	WT	WT	S	R	S	S	S	S	S	S	S	S
Azole-resistant with mutations in Cyp51A, and β-tub (14)															
4	TR_{34}/L98H	E198A	WT	WT	WT	S	R	R	R	S	S	S	S	S	S
3	TR_{34}/L98H	E198Q	WT	WT	WT	S	R	R	R	S	S	S	S	S	S
7	TR_{34}/L98H	F200Y	WT	WT	WT	S	R	R	R	S	S	S	S	S	S
Azole-resistant with mutations in Cyp51A, β-tub and Bos1 (1)															
1	TR_{34}/L98H	F200Y	WT	WT	I399N	S	R	R	R	S	S	S	S	R	S
Azole-resistant strains with mutations in Cyp51A and CytB (2)															
2	TR_{34}/L98H	WT	G143A	WT	WT	S	R	S	S	R	R	S	S	S	S
Azole-resistant strains with mutations in Cyp51A, β-tub and CytB (5)															
1	TR_{34}/L98H	F200Y	F129L	WT	WT	S	R	R	R	R	S	S	S	S	S
3	TR_{34}/L98H	F200Y	G143A	WT	WT	S	R	R	R	R	R	S	S	S	S
1	TR_{34}/L98H	E198A	G143A	WT	WT	S	R	R	R	R	R	S	S	S	S
Azole-resistant strains with mutations in Cyp51A, β-tub and SDHB (3)															
3	TR_{34}/L98H	F200Y	WT	H270R	WT	S	R	R	R	S	S	R	S	S	S
Azole-resistant strains with mutations in Cyp51A, β-tub, SDHB and Bos1 (2)															
2	TR_{34}/L98H	F200Y	WT	H270R	I399N	S	R	R	R	S	S	R	S	R	S
Azole-resistant strains with mutations in Cyp51A, β-tub, CytB and SDHB (4)															
1	TR_{46}/F121Y/T289A	F200Y	G143A	H270R	WT	R	R	R	R	R	R	R	R	S	S
3	TR_{46}/F121Y/T289A	F200Y	G143A	H270Y	WT	R	R	R	R	R	R	R	R	S	S

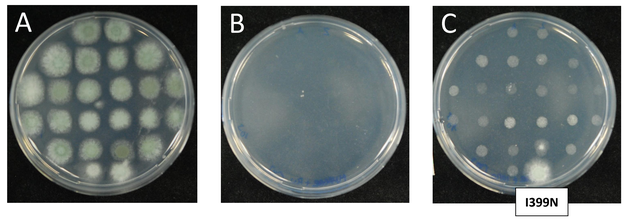

Figure 2. Susceptibility of *A. fumigatus* strains to the nonazole fungicides tested. 3 µL spotted ($3x10^4$ spores) on minimal medium plates: growth control, **A**; fludioxonil 8 mg/L, **B** and iprodione 32 mg/L, **C**.

RESULTS

PHYLOGENETIC TREE REPRESENTATION

The phylogenetic tree showed that *A. fumigatus* strains harboring the point mutation I399N in Bos1, resulting in iprodione resistance, grouped together in one sub-cluster where all strains were azole resistant, with TR_{34}/L98H mutation in Cyp51A, and have a variety of different patterns of cross-resistance to other environmental fungicides such as imidazoles and triazoles (DMIs), MBCs, QoIs and SDHIs **(Figure 3)**.

These results confirmed the existence of a strong association between azole resistant strains harboring TR_{34}/L98H and their environmental resistance origin.

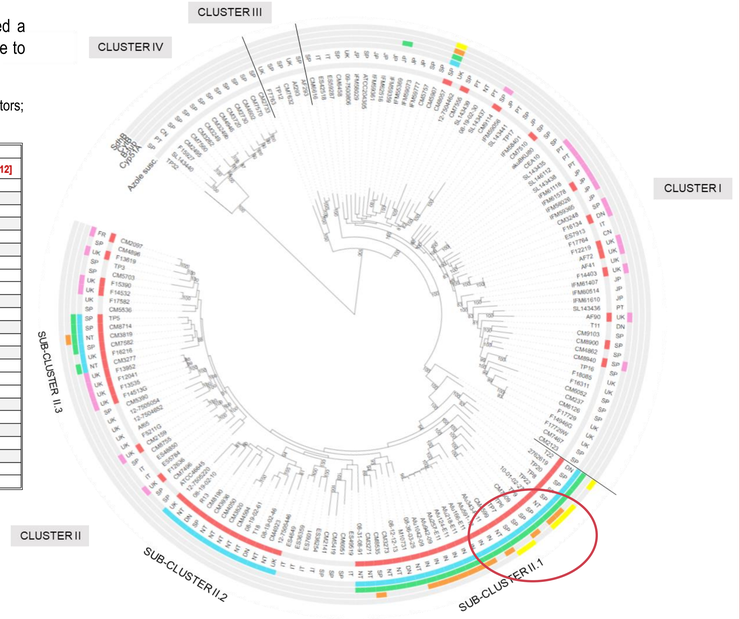

Figure 3. Phylogenic tree representation of a whole-genome alignment of a collection of 163 *A. fumigatus* genomes clustered according to their genetic proximity. SP, Spain; PT, Portugal; CN, Canada; UK, United Kingdom; IT, Italy; JP, Japan; NT, The Netherlands; DN, Denmark; IN, India; FR, France. Azole resistance is marked in red, strains harboring azole resistance mechanisms based on tandem-repeat insertions in the promoter of the *cyp51A* gene are marked in blue, and azole resistance mechanisms based on point mutations in the *cyp51A* gene are marked in pink. Mutations in the three fungicide targets are also color-coded: green for *benA*, orange for *cytB*, and yellow for *sdhB*.

CONTACT INFORMATION

Jose Lucio Vicente
Phone: +34 91 8223661 **e-mail:** jose.lucio@isciii.es
Corresponding Author: Emilia Mellado, emellado@isciii.es

REFERENCES

1. Latgé JP & Chamilos G. 2019. *Aspergillus fumigatus* and aspergillosis in 2019. Clin Microbiol Rev 33:e00140-18. https://doi.org/10.1128/CMR.00140-18.
2. Lestrade et al.2019. Triazole resistance in *Aspergillus fumigatus*: recent insights and challenges for patient management. Clin Microbiol Infect ;25(7):799–806.
3. Brauer et al. 2019. Antifungal agents in agriculture: friends and foes of public health. Biomolecules 9:521. https://doi.org/10.3390/ biom9100521.
4. Fraaije et al. 2020. The multi-fungicide resistance status of *Aspergillus fumigatus* populations in arable soils and the wider European environment. Front Microbiol 11:599233. https:// doi.org/10.3389/fmicb.2020.599233.
5. Gonzalez-Jimenez, et al. 2021. Multiresistance to nonazole fungicides in *Aspergillus fumigatus* TR34/L98H azole resistant isolates. Antimicrob Agents Chemother 65:e00642-21. https://doi.org/10 .1128/AAC.00642-21