***Pre-meeting workshop:***
***Aspergillus Speciation in the 21st Century.***

6th ADVANCES AGAINST ASPERGILLOSIS
Madrid, Spain
27 Feb – 1 March 2014
Meliá Castilla Conference and Convention Centre

# Study of antifungal resistance mechanisms in cryptic species

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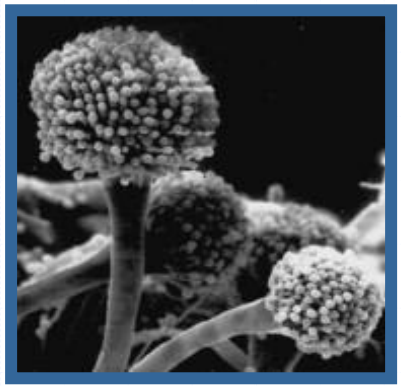
**Micology Unit**
**Centro Nacional Microbiologia**
**Instituto de Salud Carlos III (ISCIII)**
**Majadahonda, Madrid, Spain**



# *Aspergillus* Section *Fumigati*

- *Aspergillus lentulus*
- *A. novofumigatus*
- *A. fumigatiaffinis*
- *A. fumisynnematus*
- *A. viridinutans*
- *A. neoellipticus*
- *A. thermotolerans*

- *Neosartorya hiratsukae*
- *N. psudofischeri*
- *N. udagawae*
- *N. glabra*
- *N. espinosa*
- *N. fischeri*
- *N. primulina*

- *A. waksmanii sp.*
- *A. marvanovae sp.*

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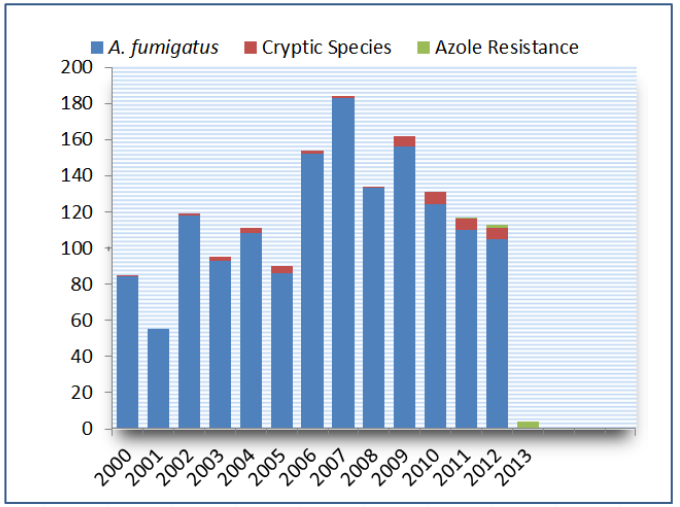


*Teresa Pelaez. Personal Communication*

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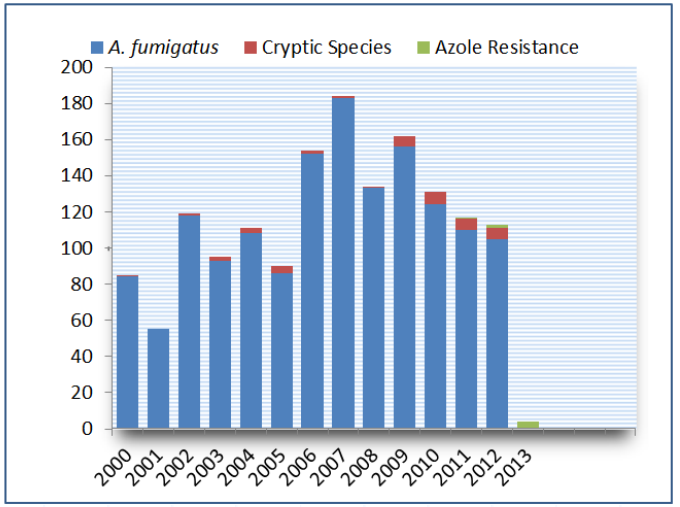


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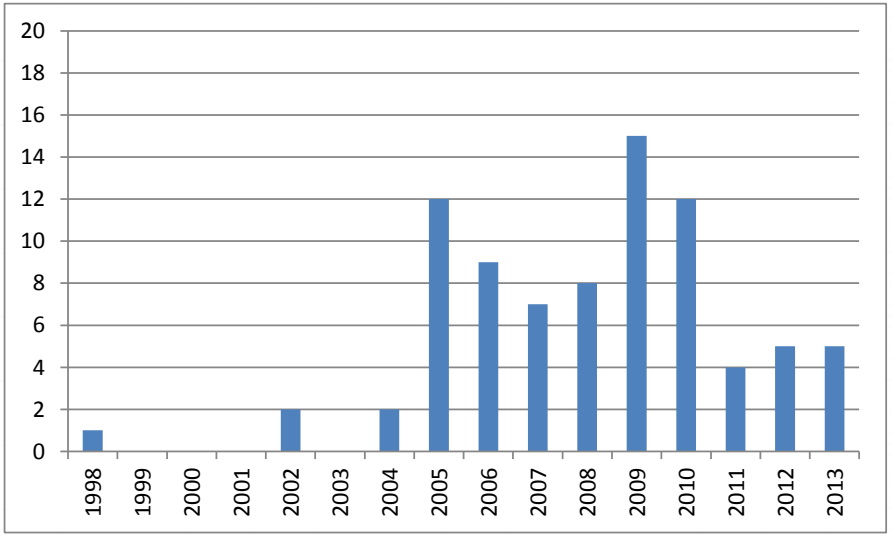


**Clinical samples**

**Also, enviromental**

# Antifungal Drug Resistance

| Strains n = 82 | MICs (μg/ml)* | | | | |
|---|---|---|---|---|---|
| | ITC | VRC | RVC | POS | AmB |
| *A. fumigatus* | 0.25 | 0.50 | 0.50 | 0.12 | 0.5 |
| *A. lentulus* (40) | 0.5 - 8 | 2 - 4 | 2 - 4 | 0.40 | 2 - 8 |
| *A. fumigatiaffinis* (9) | 8 | 4 - 8 | 4 - 8 | 0.5 - 1 | 4 - 16 |
| *N. pseudofischeri* (6) | 8 | 2 - 4 | 2 - 4 | 0.25 - 0.5 | 0.25 |
| *A. viridinutans* (5) | 8 | 4 | 4 - 8 | 0.25 - 0.5 | 0.25 - 1 |
| *N. udagawae* (9) | 8 | 2 | 2 | 0.25 | 2 - 8 |
| *A. novofumigatus* (2) | 8 | 8 | 8 | 0.5 - 1 | 0.5 |
| *N. hiratsukae* (9) | 0.12 - 0.25 | 0.5 - 2 | 0.5 - 1 | 0.06 - 0.12 | 0.5 - 1 |
| *A. fumisynnematus* (1) | 0.25 | 0.50 - 1 | 0.50 - 1 | 0.12 | 1 |

- All from clinical origin. Most from respiratory specimen. Clinical relevance unknown
- Important due to their multiple resistance patterns including to AmB
- they were identify on bases: ITSs, cytB, b-tubulin, *rod*A, Cyp51A and Cyp51B.
- Also, its azole resistance seems to be **intrinsic** and not secondary

# Why we Study the resistance mechanisms ?

- Frequency of their isolation is increasing
- Need to know if these strains have clinical relevance?
- Why are they resistant? Molecular bases
- If we know that, we should be able to avoid it
- Help in the developing of new antifungal
- Screening Test of the new antifungals as they appear

# Why we Study the Resistance Mechanisms ?

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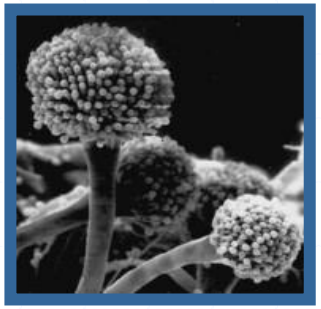
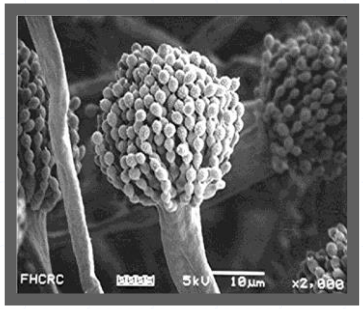
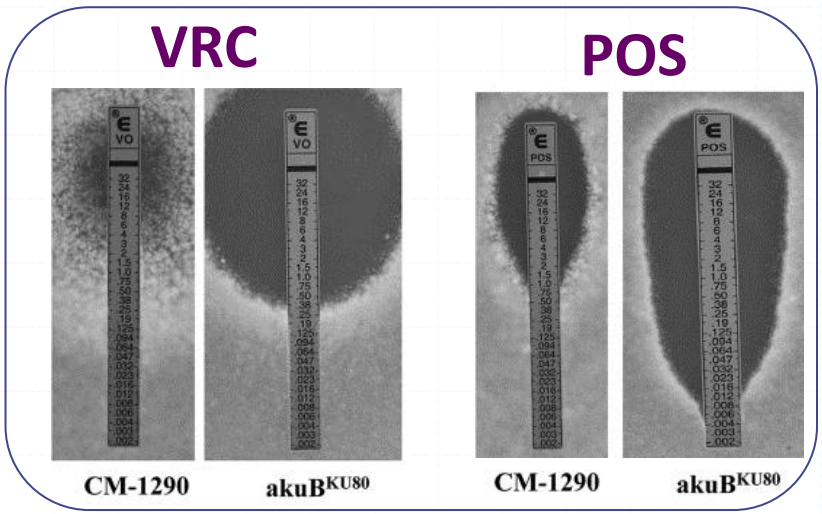
- Study the molecular bases of antifungal resistance
- Correlation in vitro - in vivo: Alternative models of infection

# *A. fumigatus* vs. *A. lentulus*



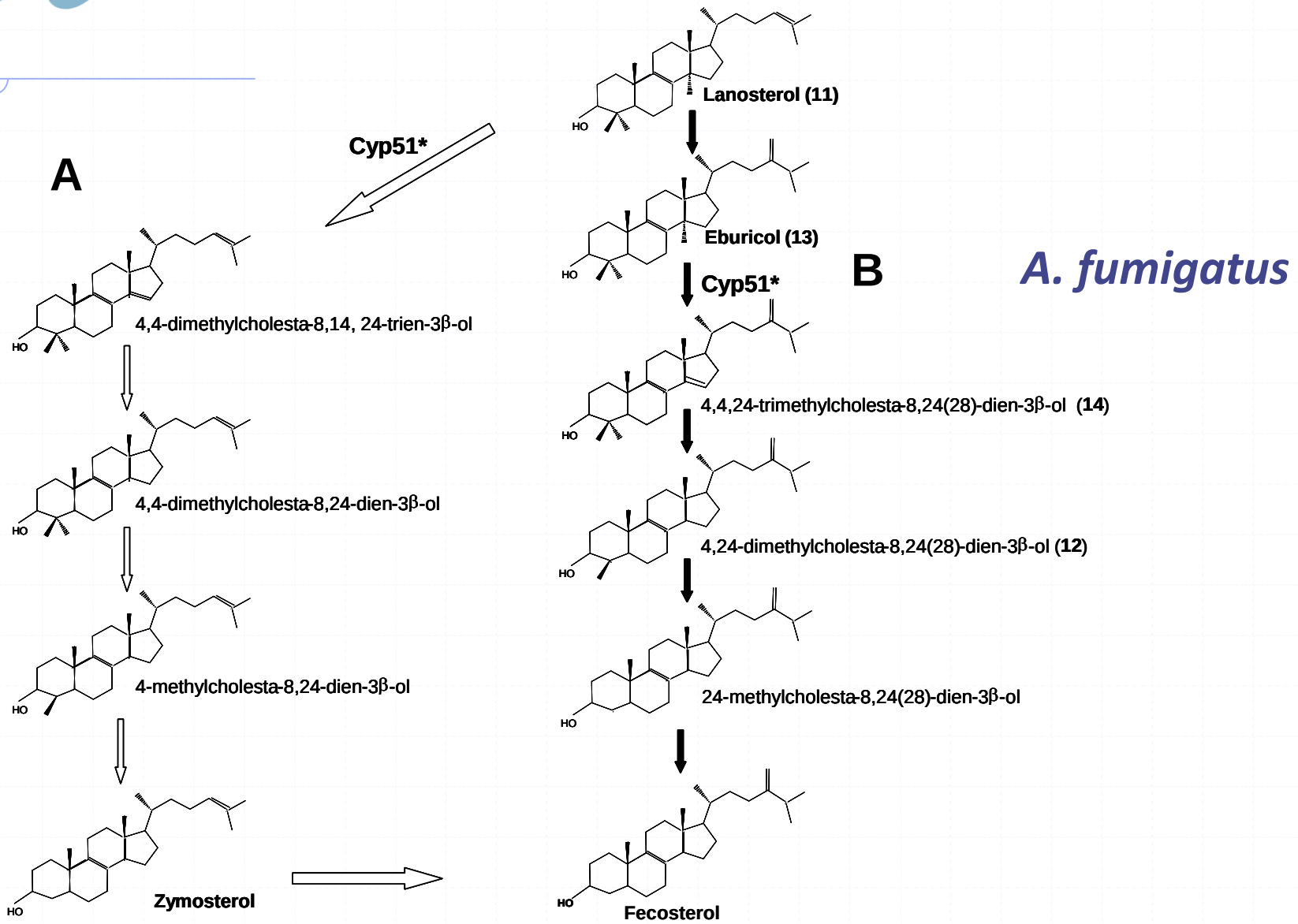



| Strains | MICs (µg/ml)* | | | | |
|---|---|---|---|---|---|
| | ITC | VRC | RVC | POS | AmB |
| *akuB*$^{KU80}$ *A. fumigatus* | 0,25 | 0,50 | 0,50 | 0.12 | 0.5 |
| CM-1290 *A. lentulus* | 1 - 8 | 4 | 4 | 0.12 | 2 - 4 |




Intrinsic azole and AmB Resistance

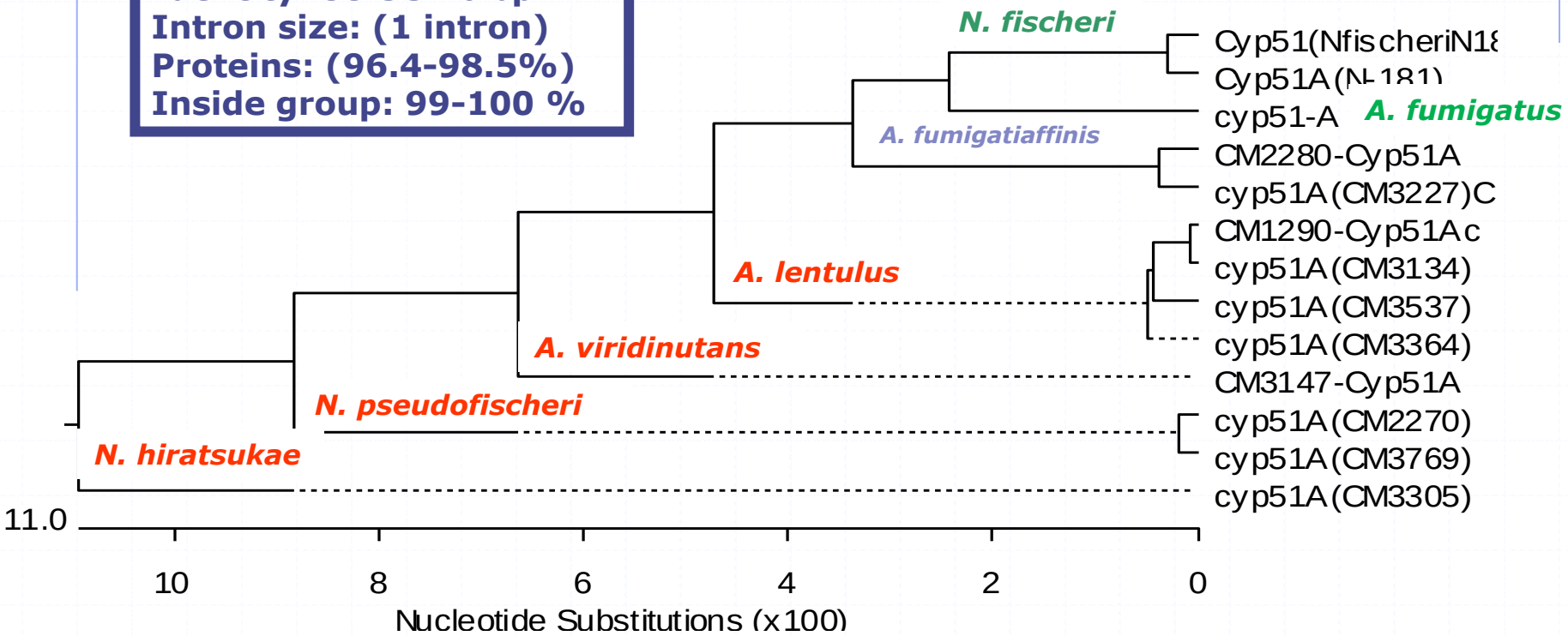
# Target: Ergosterol or its biosynthesis



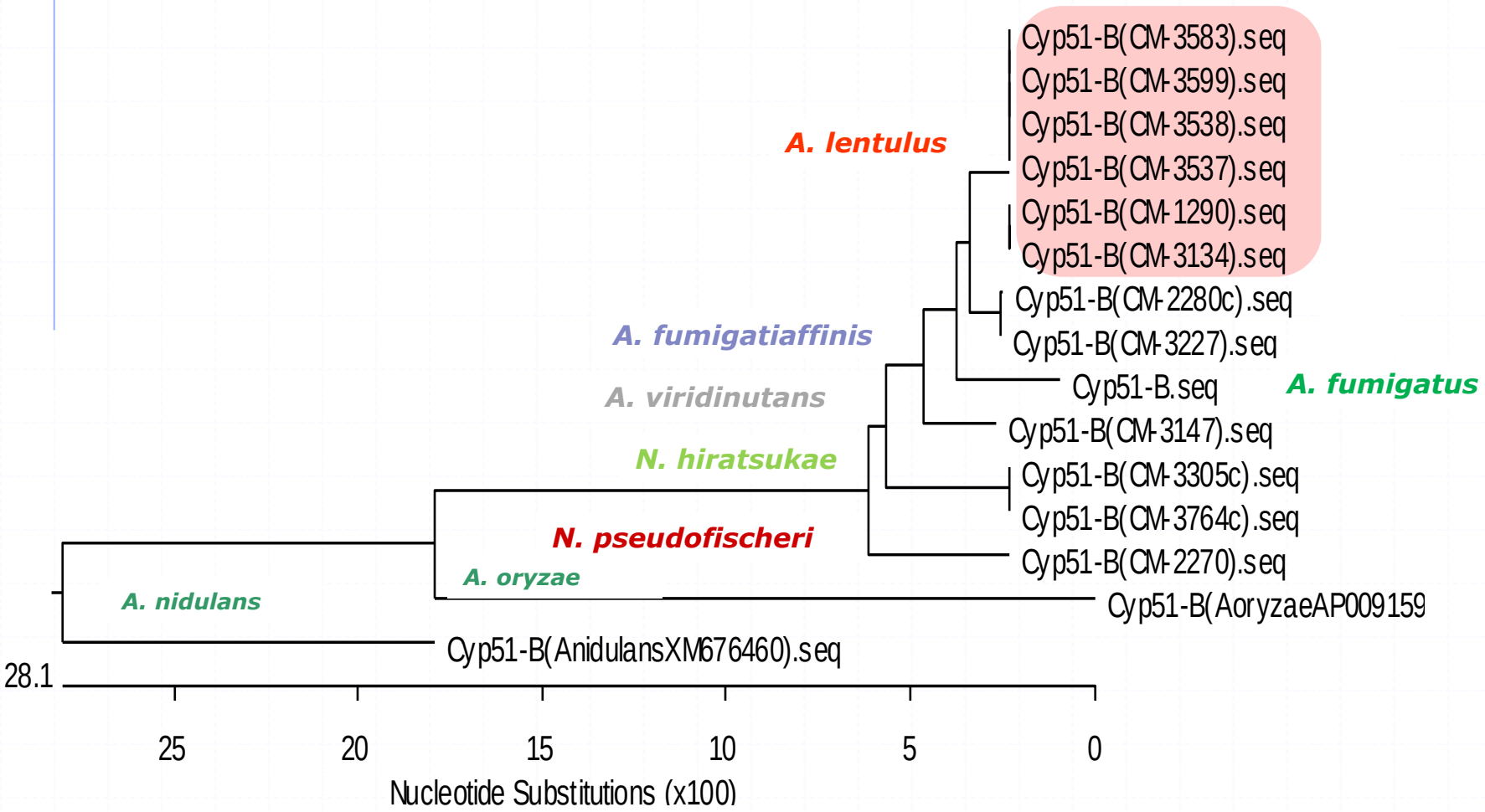

*A. fumigatus*

# Azole target: 14-α sterol demethylase Cyp51A (*cyp*51A gene and Protein)

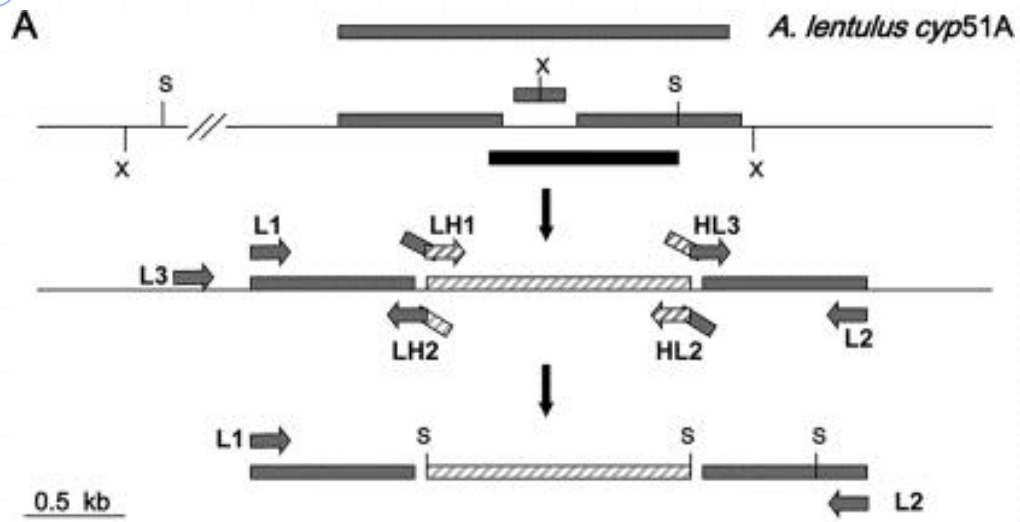
**Identity: 88-95 % bp**
**Intron size: (1 intron)**
**Proteins: (96.4-98.5%)**
**Inside group: 99-100 %**



# Azole target: 14-α sterol demethylase Cyp51B (*cyp*51B gene)



# *A. lentulus* Azole Resistance




*A. lentulus*, CM1290

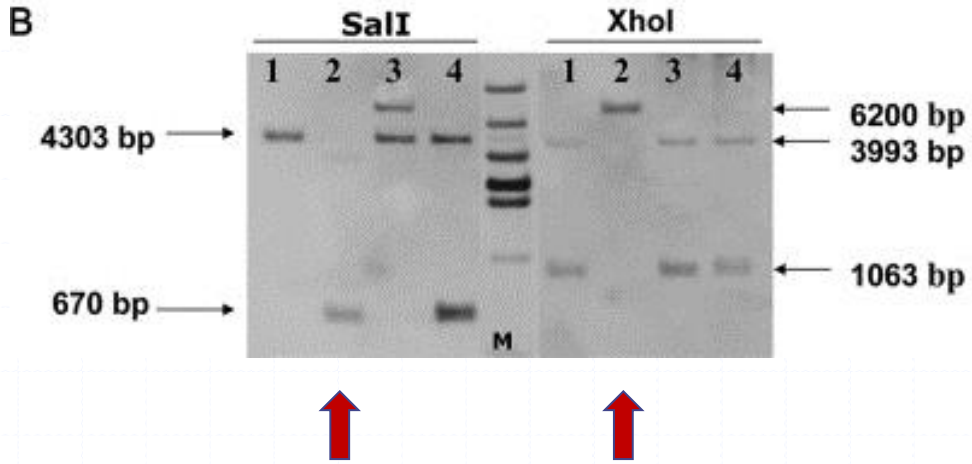
$10^7$/ml

Electroporation


2-7 days

37ºC

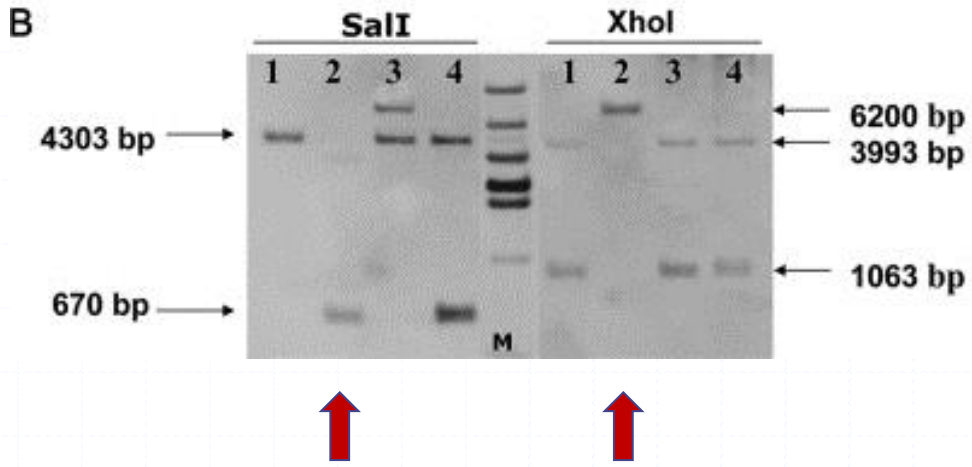
# *A. lentulus* Azole Resistance




**Mutant T29.18 Δ*cyp*51A**

| Strains | MICs (µg/ml)* | | | | |
|---|---|---|---|---|---|
| | ITC | VRC | RVC | POS | AmB |
| CM-237 | 0,25 | 0,50 | 0,50 | 0.12 | 2.00 |
| CM-1290 | 1 - 8 | 4 | 4 | 0.12 | 2 - 4 |
| T29.18 | 0.06 | 0.25 | 0.25 | 0.015 | 4.00 |

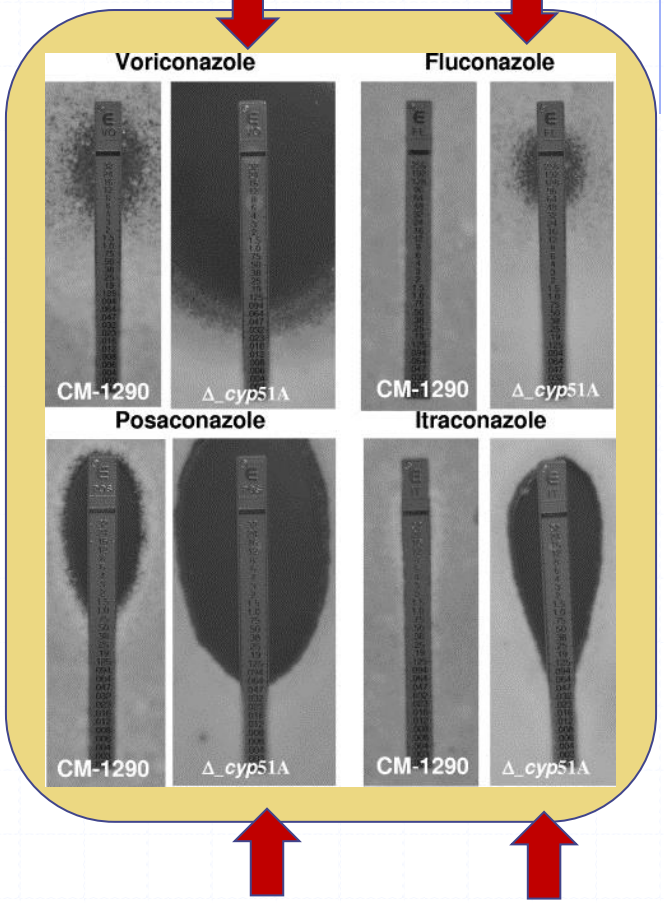
# *A. lentulus* Azole Resistance




**Mutant T29.18**

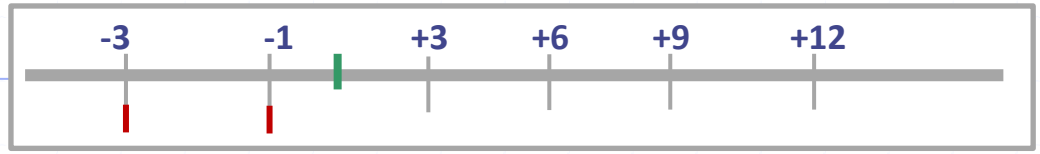
| Strains | MICs (µg/ml)* | | | | |
|---|---|---|---|---|---|
| | ITC | VRC | RVC | POS | AmB |
| CM-237 | 0,25 | 0,50 | 0,50 | 0.12 | 2.00 |
| CM-1290 | 1 - 8 | 4 | 4 | 0.12 | 2 - 4 |
| T29.18 | 0.06 | 0.25 | 0.25 | 0.015 | 4.00 |

**Mutant T29.18-CR050**

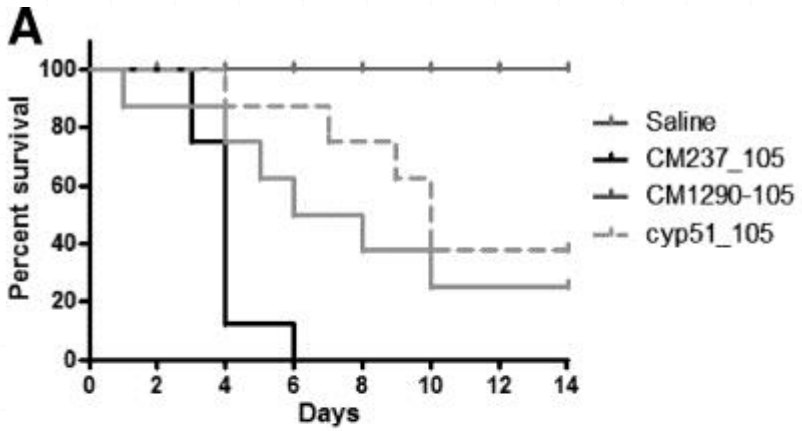


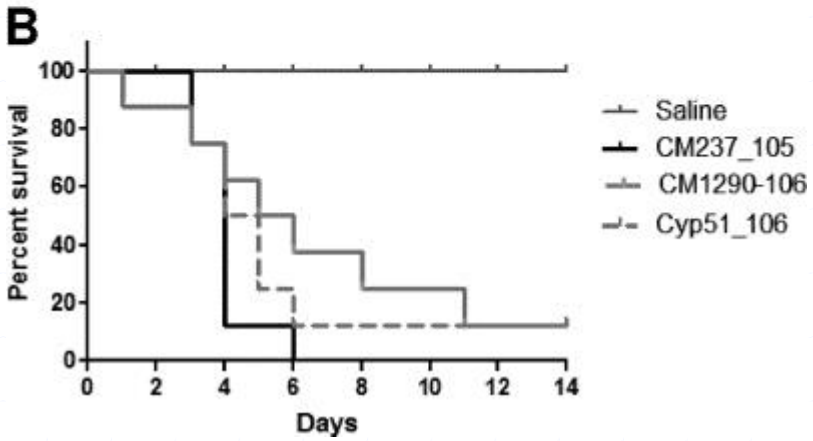

**Mutant T29.18-CR050**

# *A. lentulus* pathogenicity




**Murine Model: ciclofosfamida + Cortisone**



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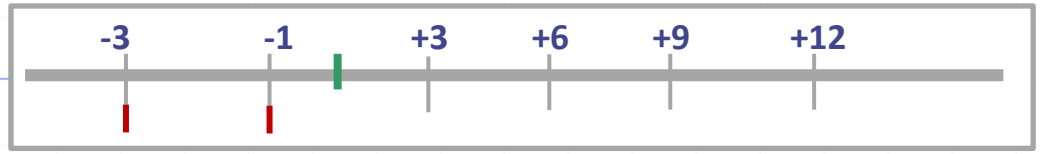


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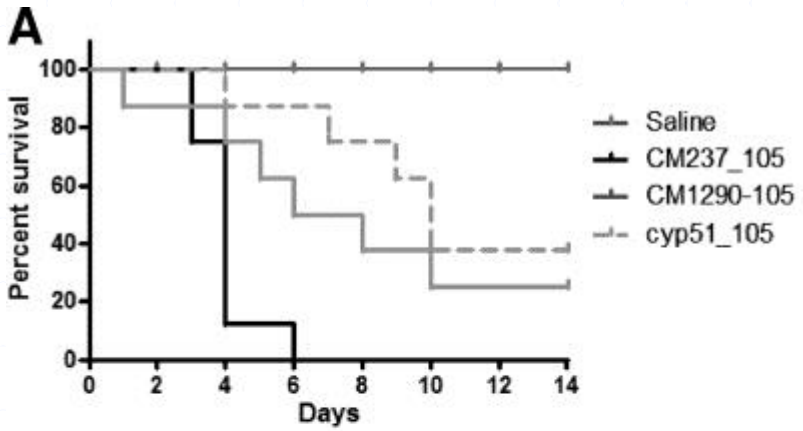
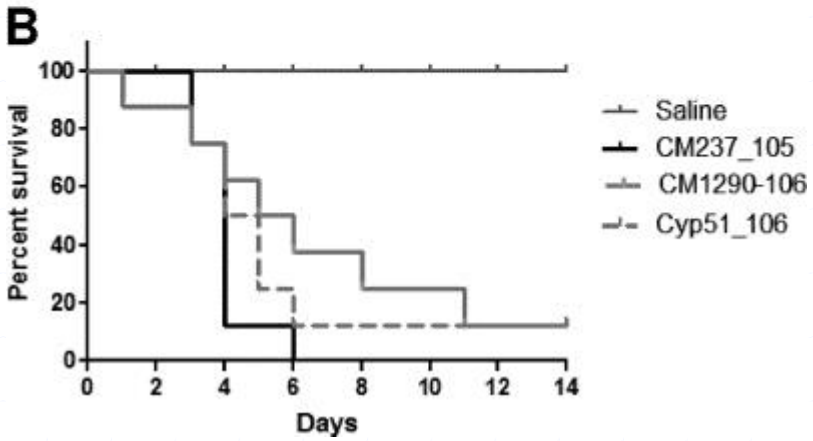
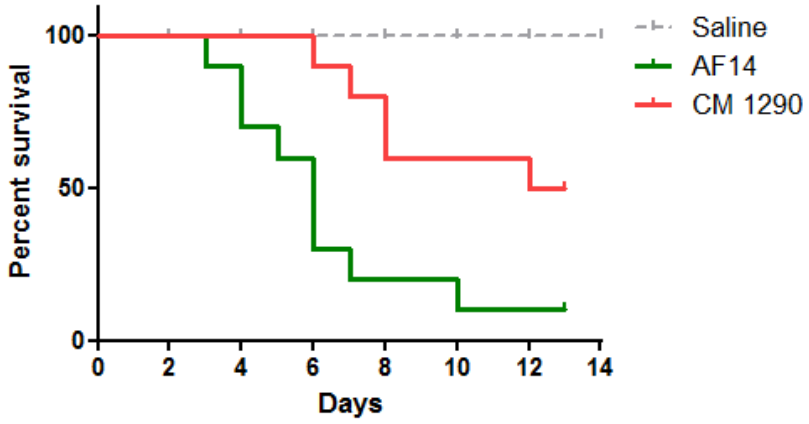


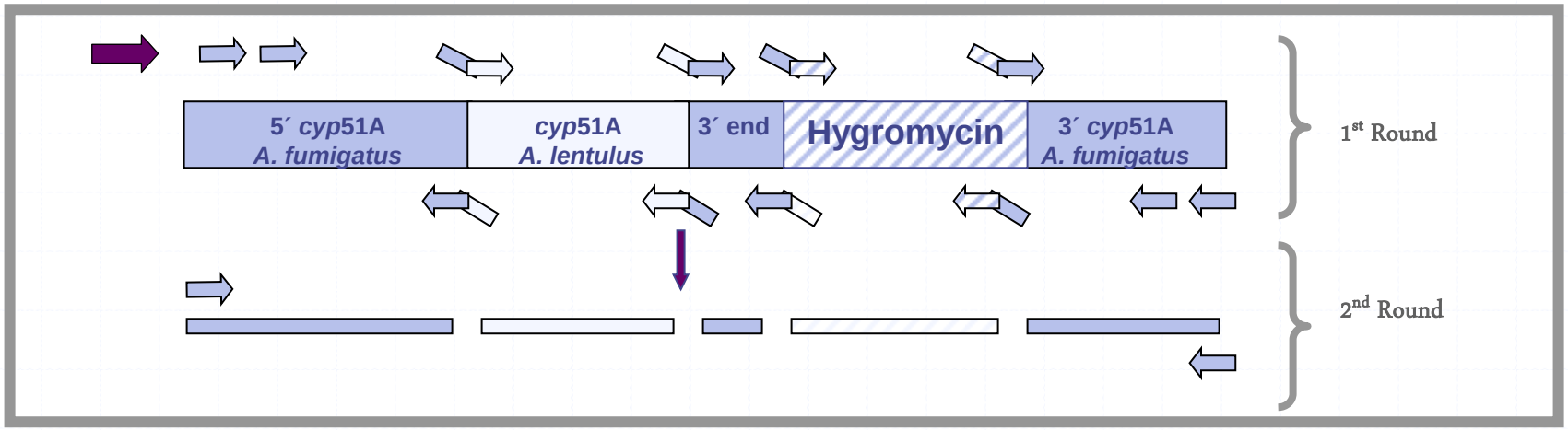




## ID by Cortisone




**Inoculum: $10^5$**

# *Aspergillus fumigatus:* Azole Resistance with the *A. lentulus* copy of *cyp*51A?




*A. fumigatus, akuB*$^{KU80}$

$10^7$/ml

Electroporation


2-7 days

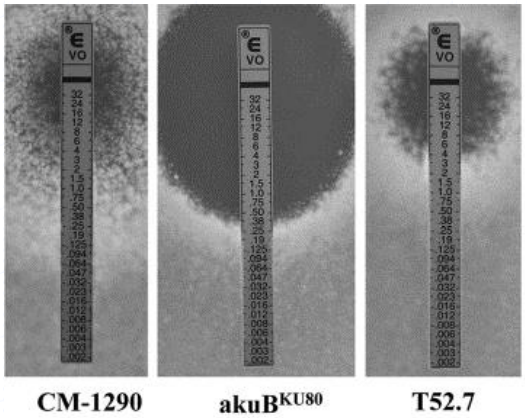
37°C

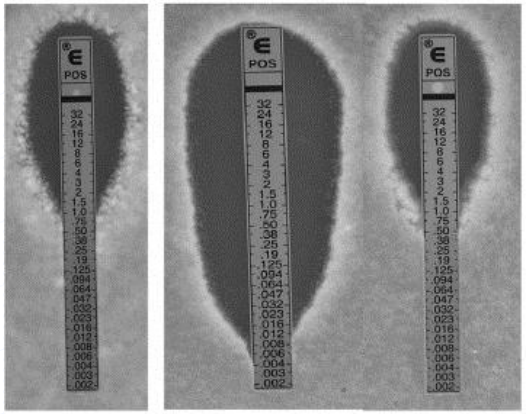
# *Aspergillus fumigatus:* Azole Resistance with the *A. lentulus* copy of *cyp*51A?

**Alcyp51A**

**AfΔcyp51A_Alcyp51A**

*A. lentulus*



*A. fumigatus*

**T52.7**

AZL-R

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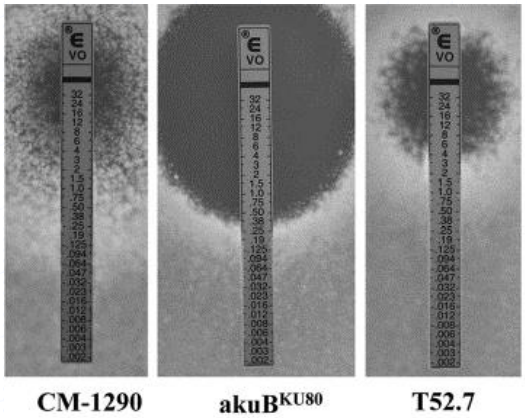
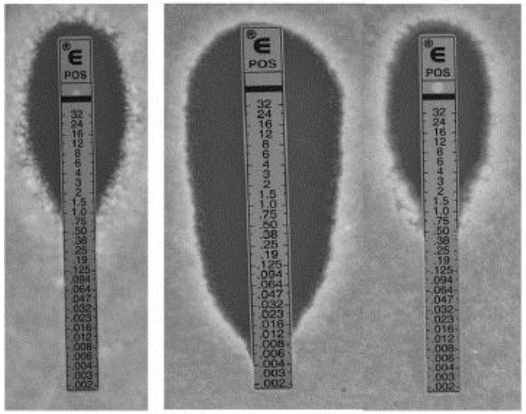
**Alcyp51A**

**AfΔcyp51A_Alcyp51A**

*A. lentulus*



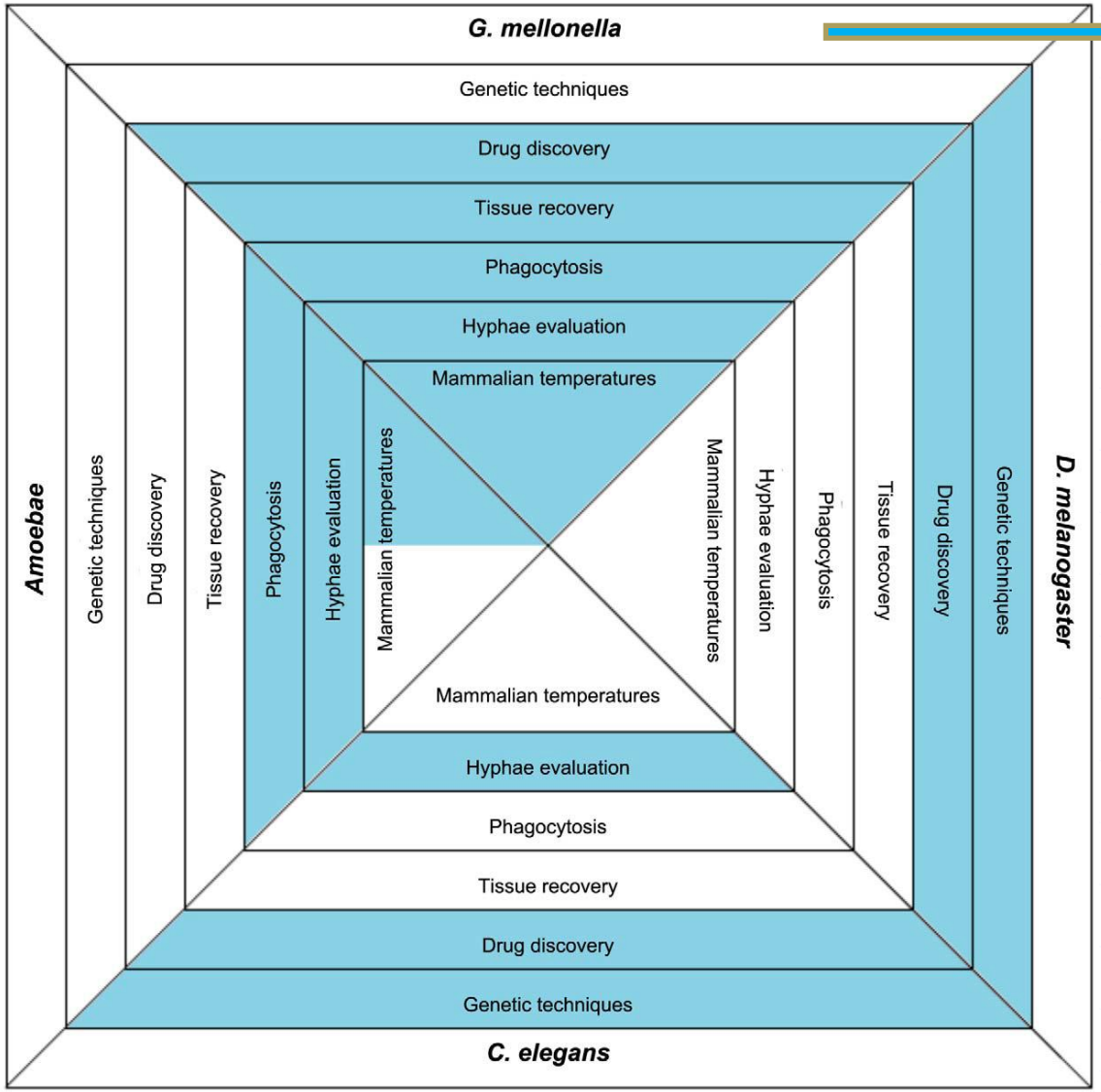
*A. fumigatus*

**T52.7**

AZL-R

*In vitro – in vivo*
Correlation
alternative model host

**The mini-host models provide an opportunity of** exploring the molecular mechanisms of **fungal pathogenicity** and candidate agents with **antifungal activity**.




**More Advantages:**

*Galleria mellonella*

Desalermos *et al.*
Plos Pathogen 8:e102451, 2012

# Fungal infections in *Galleria mellonella*

## Advantages:

- Relatively large size (about 2 cm and 5 cm long):
- easy handling,
- low cost and maintenance
- allows for the injection of standardized fungal inoculum and drug dosage
- in-vivo studies of phagocytic cell function

- Their optimum temperature of growth and maintenance is 29°C:
  - but are able to survive at the mammalian physiological temperature (37°C)

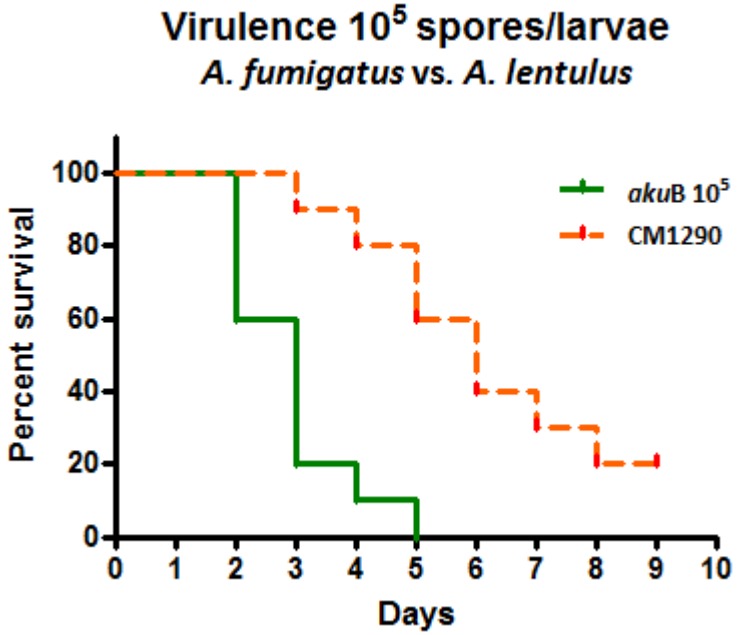
- Important, because it could allow for the expression of certain temperature regulated virulence factors of fungal pathogens

## Disadvantages

- Effect of temperatures on the *G. mellonella* immune response has not been studied. It seems that *G. mellonella* exhibits increased susceptibility to pathogenic fungi at 37º.

- the absence of methods for genetic analysis and lack of genome sequencing.

# *Galleria mellonella* , an alternative host to test *Aspergillus lentulus* virulence and drug response.




Inoculum: $10^5$/ larva

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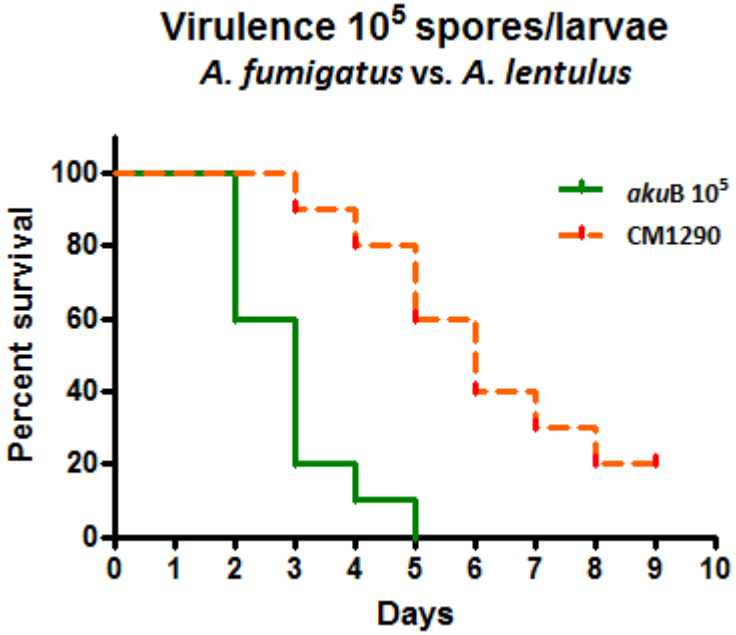
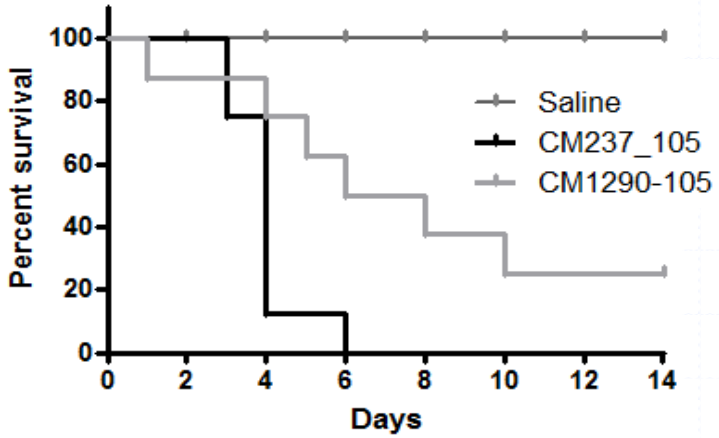
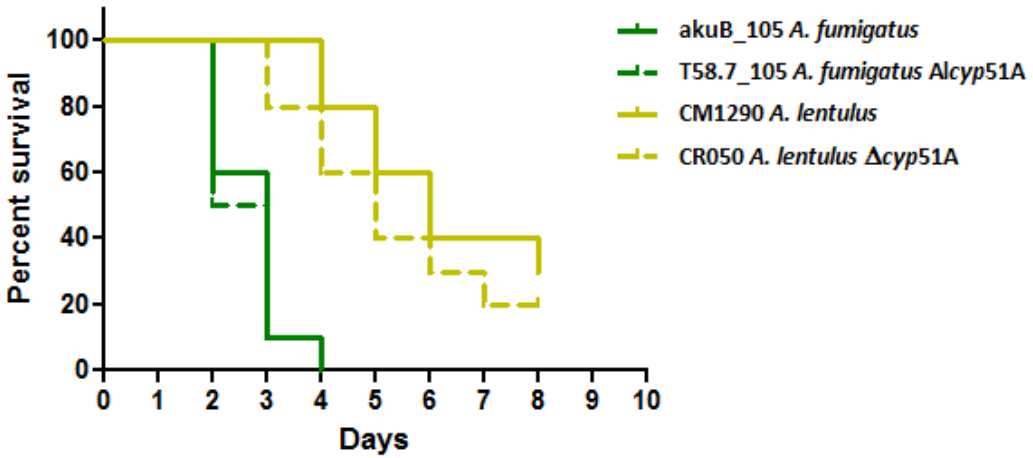



Inoculum: $10^5$/ larva

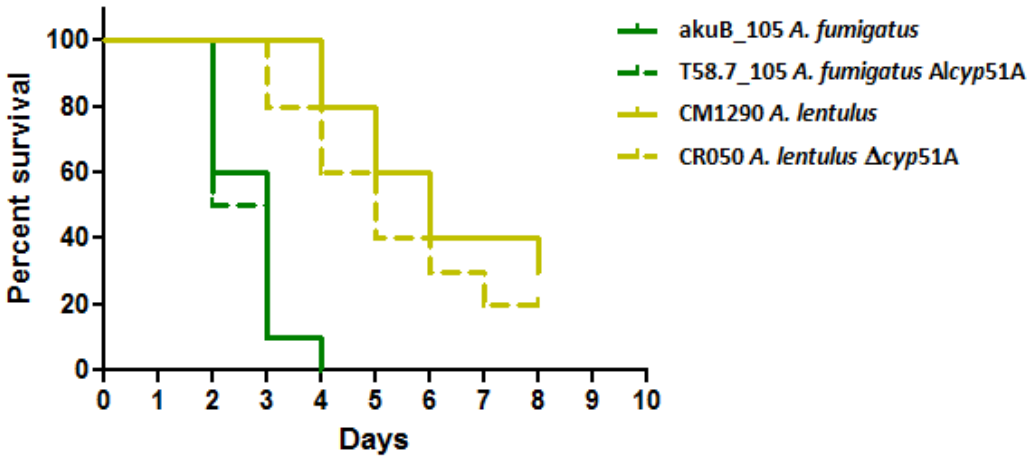



Inoculum: $10^5$/mouse

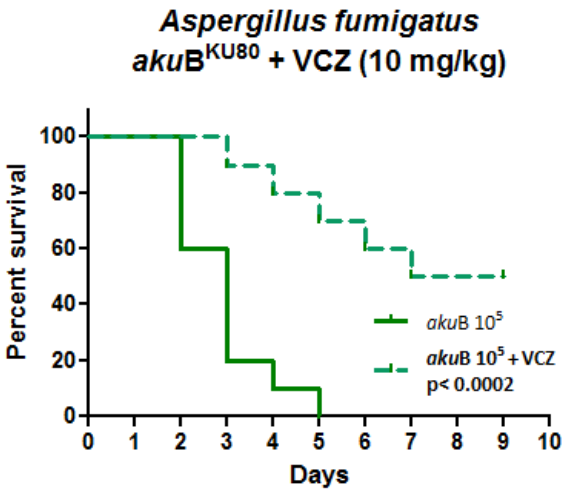
# *Galleria mellonella*: **mutants virulence.**



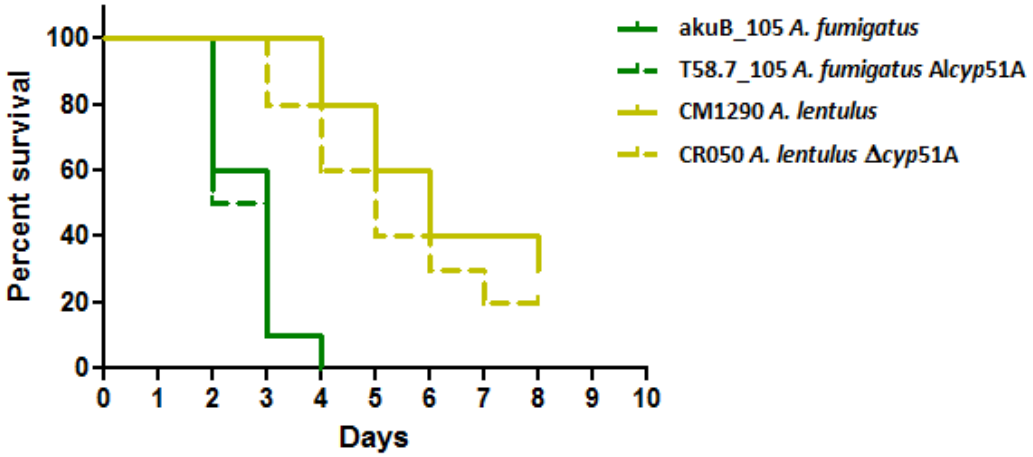

## *Galleria mellonella*: **mutants virulence.**




## *Galleria mellonella*: **drug response.**

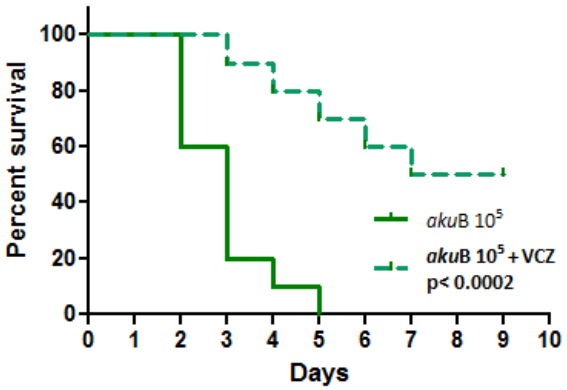



# *Galleria mellonella*: **mutants virulence.**

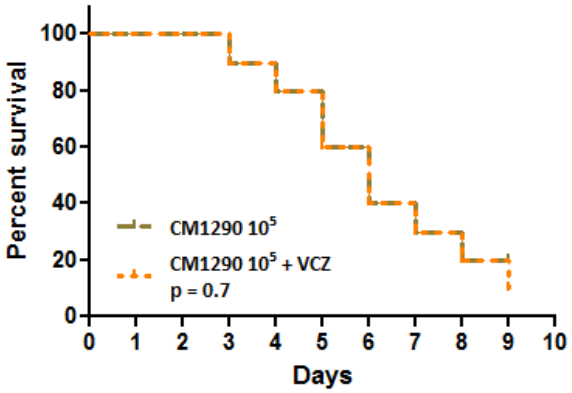



# *Galleria mellonella*: **drug response.**

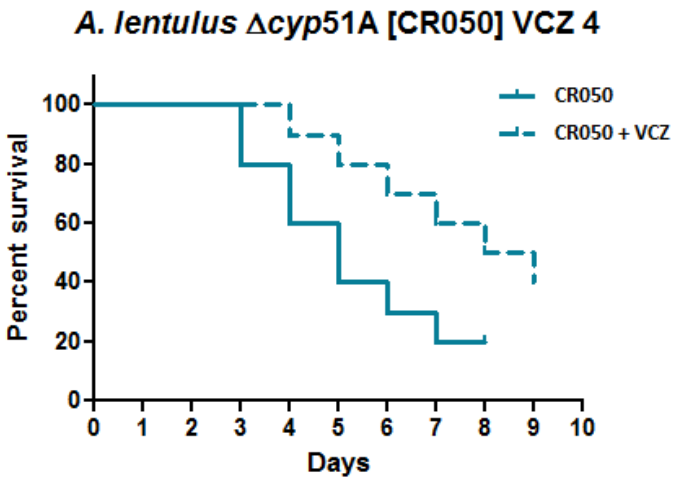
***Aspergillus fumigatus***
***akuB*$^{KU80}$ + VCZ (10 mg/kg)**

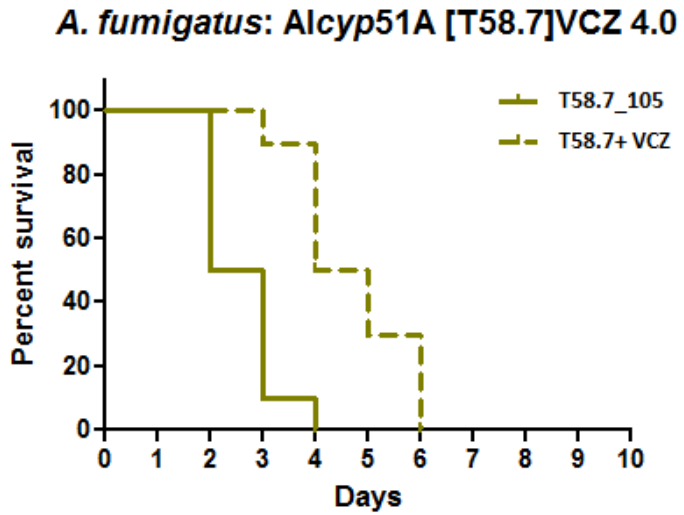



***Aspergillus lentulus***
**CM1290 + VCZ (10 mg/kg)**




# *Galleria melllonella*: mutants **drug response.**



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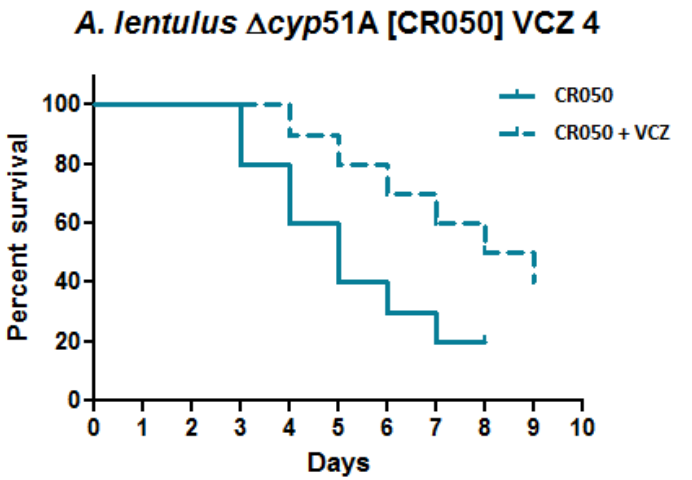
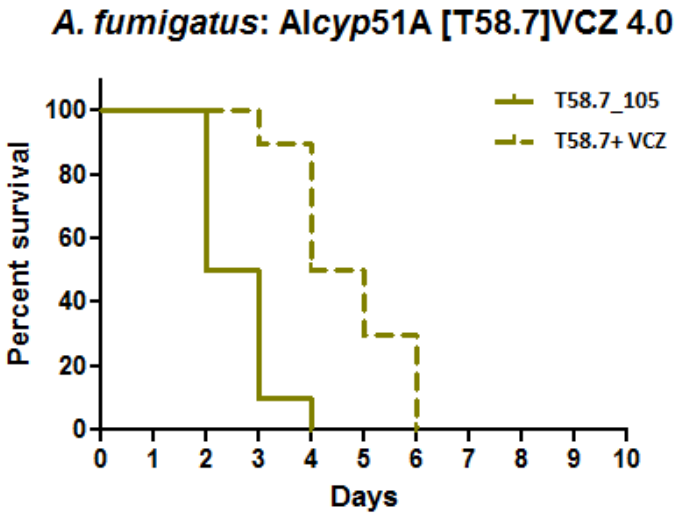


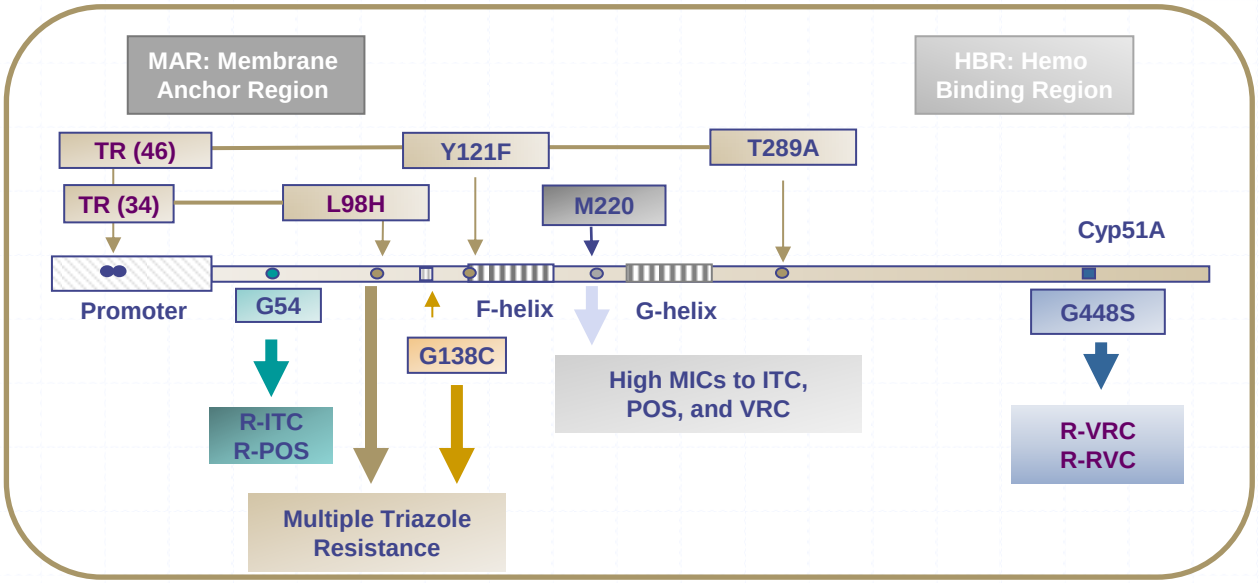




- ***Aspergillus lentulus*** **AZL resistance was demonstrated in vivo**

- **Dependant of the azole target: *cyp*51A**

- **Explore the *A. lentulus* protein Cyp51A**

# Azole Resistance Mechanisms in *A. fumigatus*



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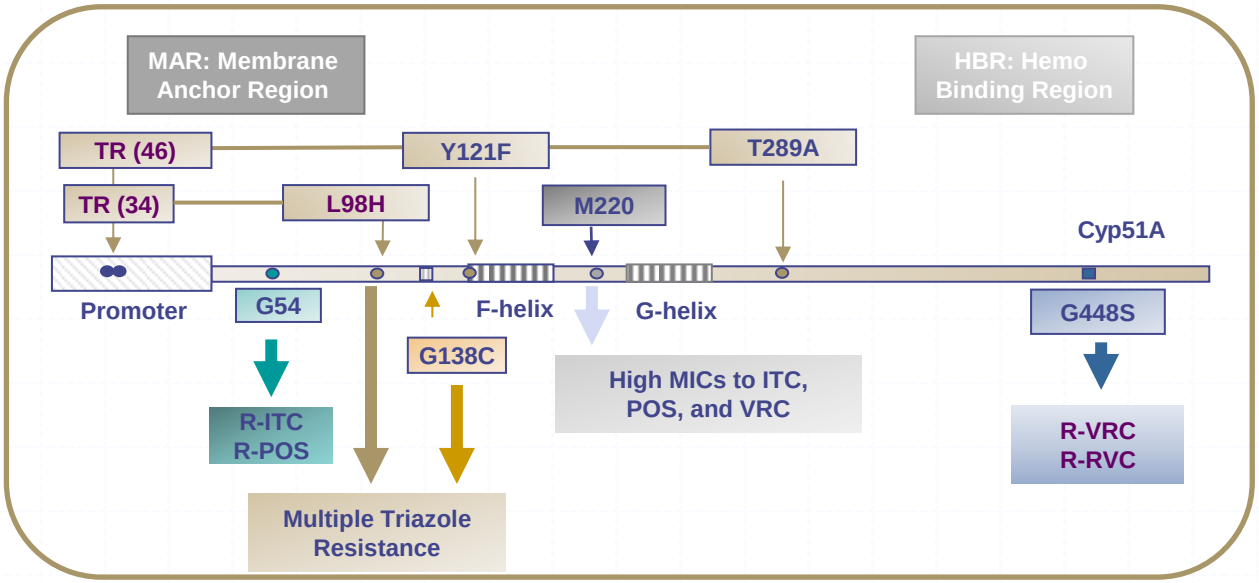
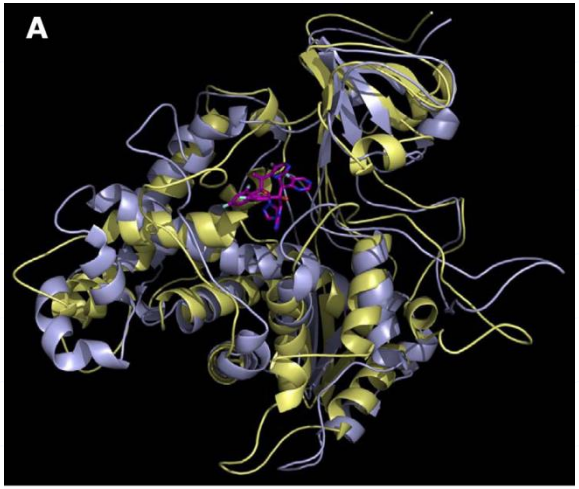


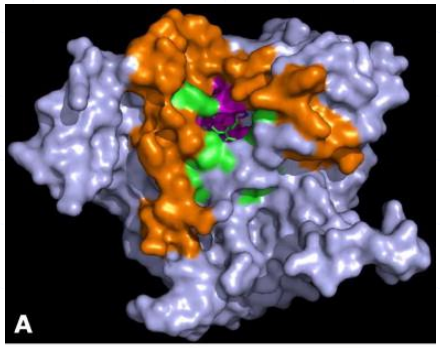

A 3-D homology model was constructed to explore possible structural differences in voriconazole interaction between Cyp51A from *A. fumigatus* (*Af*Cyp51A) and *A. lentulus* (*Al*Cyp51A). (sequence identity between AlCyp51A and AfCyp51A proteins is 95%)

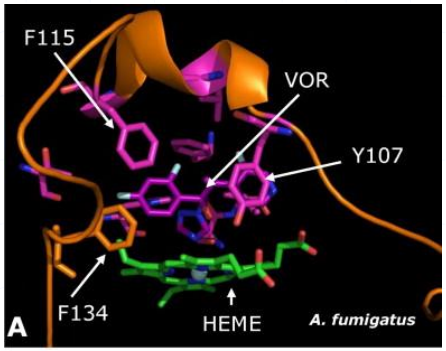
The few amino acid changes could not explain, a priori, the differences in azole susceptibility, the next step was an analysis of the structural conformation of these closely related proteins.



**Molecular dynamics (MD)** was applied to both 3D protein models to explore possible differences in the Cyp51A–voriconazole interaction.

Some critical differences were observed in the putative closed form adopted by the protein upon voriconazole binding.




***A. fumigatus***

The BC loop conformation shows that the occlusion of the catalytic centre upon VCZ binding could be mediated by the residues Y107, L110, F115 and F134 with the azole.

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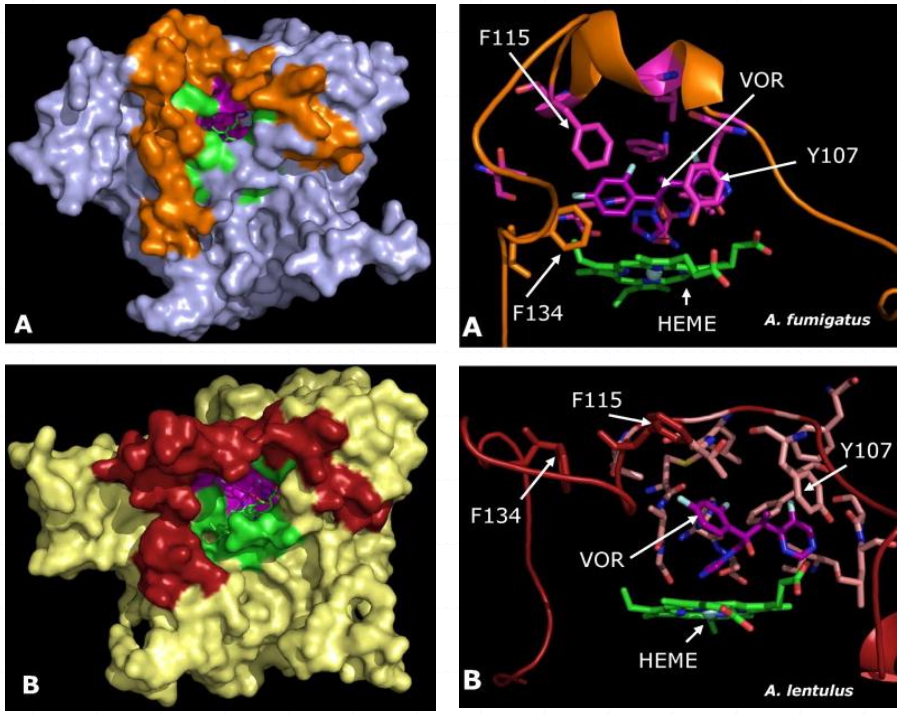


***A. fumigatus***

The model for *Af*Cyp51A BC loop conformation shows that the occlusion of the catalytic centre upon VCZ binding could be mediated by the residues Y107, L110, F115 and F134 with the azole.

***A. lentulus***

The more relaxed *Al*Cyp51A BC loop structure would behave differently with respect to azole interactions since the residues 4 Å around voriconazole are Y107, L110 and T111 and V114.

Suggested that some major differences in the protein's BC loop could differentially affect the lock-up of VCZ, in correlatetion with their different azole susceptibility profiles.

# *Aspergillus lentulus:* AmB Resistance

| Strains n = 82 | MICs (μg/ml)* | | | | |
|---|---|---|---|---|---|
| | ITC | VRC | RVC | POS | AmB |
| *A. fumigatus* | 0.25 | 0.50 | 0.50 | 0.12 | 0.5 |
| *A. lentulus* (40) | 0.5 - 8 | 2 - 4 | 2 - 4 | 0.40 | 2 - 8 |

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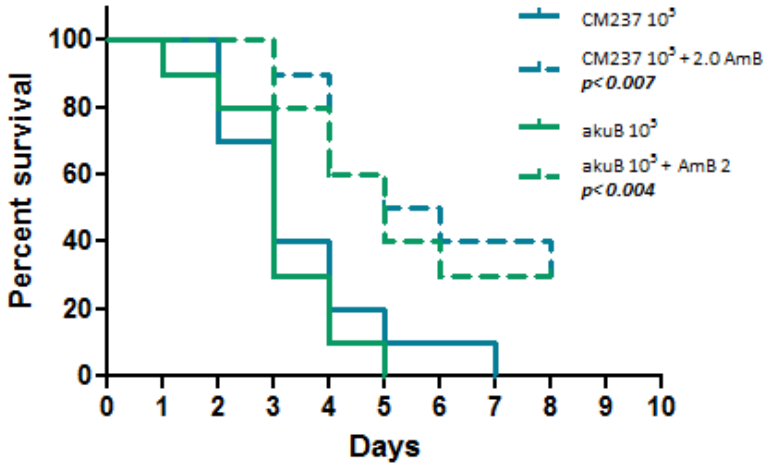
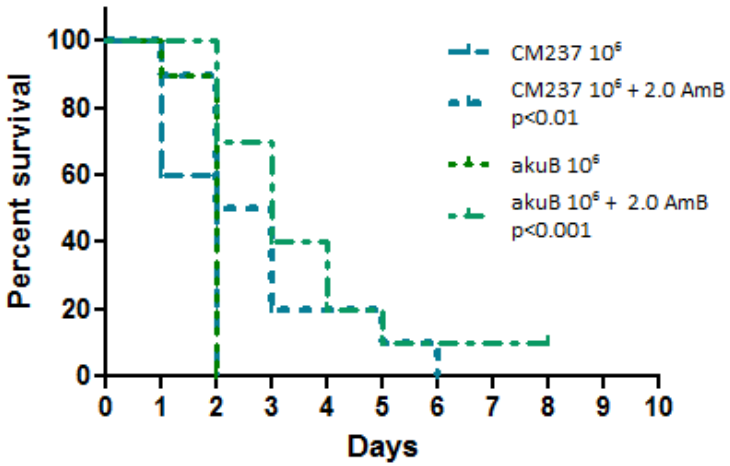
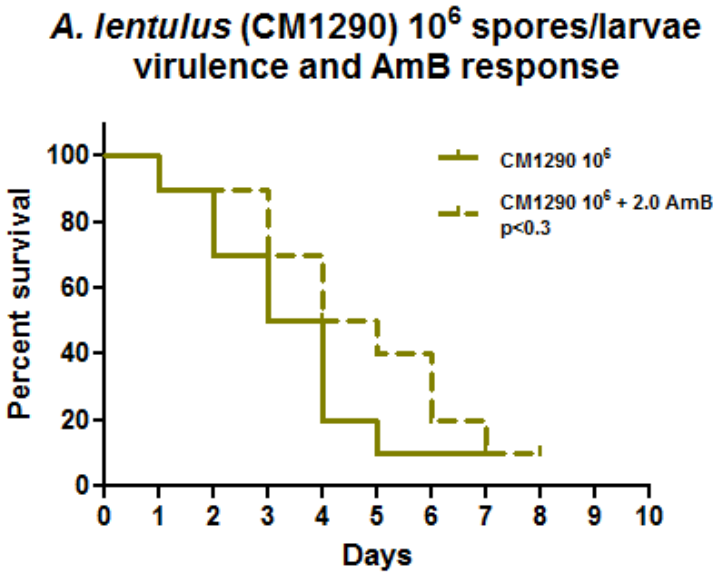
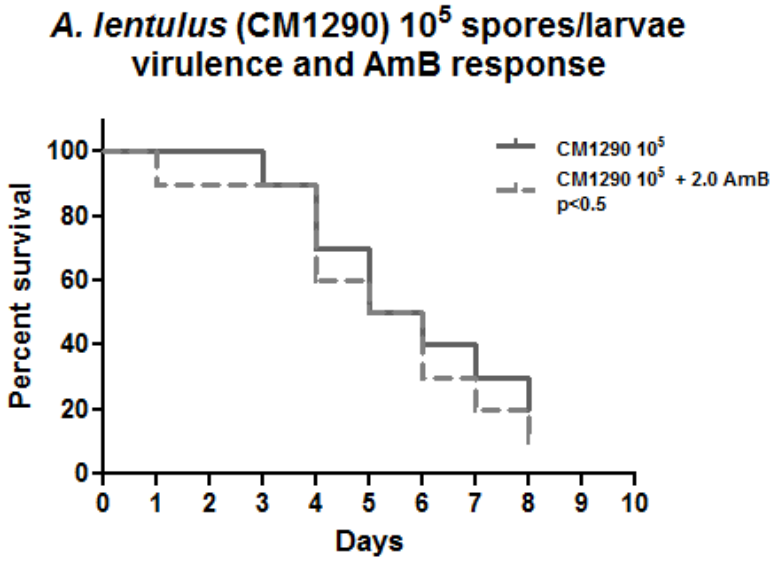



***A. fumigatus*** **(CM237 vs akuB) $10^6$ spores/larvae virulence and AmB response**



# *Galleria melllonella* , an alternative host to test *Aspergillus spp* drug response.



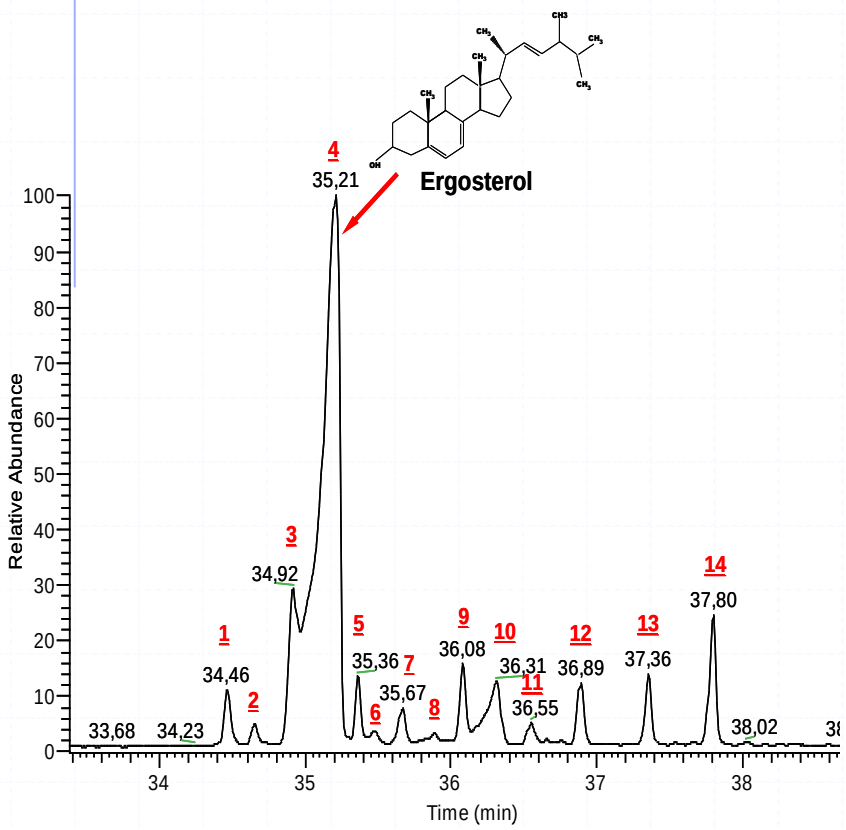



# Amphotericin B Resistance

- Amphotericin B (AmB) is known to bind to ergosterol in fungal cell membranes, but the precise mechanism of its toxicity to cells is yet poorly understood.
- In first place we investigated the ergosterol biosynthetic pathway serching for an alteration leading to decreased ergosterol content.
- AmB autoxidizes, and it is possible that its antifungal effects could result from oxidative damage. ROS production.

# Ergosterol Biosynthesis in *A. fumigatus*

## Ergosterol precursors of the *A. fumigatus* wild type strain CM-237 (GC- MS)




1. 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol
2. 24-methylcholesta-5,8,22-trien-3β-ol
3. 24-methylcholesta-5,7,9,22-tetraen-3β-ol
4. 24-methylcholesta-5,7,22-trien-3ß-ol (Ergosterol)
5. 24-methylcholesta-7,22-dien-3β-ol
6. 24-methylcholesta-5,7,22,24(28)-tetraen-3β-ol
7. 24-methylcholesta-7,22,24(28)-trien-3β-ol
8. 24-methylcholesta-5,7,24(28)-trien-3β-ol
9. 24-methylcholesta-7,24(28)-dien-3β-ol
10. 24-Ethylcholesta-5,7,22-trien-3β-ol
11. 4,4,14-trimethylcholesta-8,24-dien-3β-ol
12. 4α,24-dimethylcholesta-8,24(28)-dien-3β-ol
13. 4,4,14,24-tetramethylchoesta-8,24(28)-dien-3β-ol
14. 4,4,24-trimethylcholesta-8,24(28)-dien-3β-ol

# Ergosterol Biosynthesis in *A. fumigatus*

## Ergosterol precursors of the *A. lentulus* strains CM-1290 and CM4415

| Sterols | | | Strain | | |
|---|---|---|---|---|---|
| | Systematic name | Common name | CM-237 | CM-1290 | CM-4415 |
| 1 | Squalene | | - | 0.95 | 0.17 |
| 2 | Cholest-5-en-3β -ol | | - | 0.11 | 0.15 |
| 3 | 24-methylcholesta-5,8,22-trien-3β -ol | | 2.31 | 1.64 | 1.76 |
| 4 | 24-methylcholesta-5,7,9,22-tetraen-3β -ol | Neoergosterol | 0.01 | 0.01 | 0.01 |
| **5** | **24-methylcholesta-5,7,22-trien-3ß-ol** | Ergosterol | **75.44** | **87.03** | **86.17** |
| 6 | 24-methylcholesta-7,22-dien-3β -ol | | 2.34 | 2.50 | 2.75 |
| 7 | S28:4 (unknown) | | 0.24 | 0.10 | 0.14 |
| 8 | 24-methylcholesta-5,7,22,24(28)-tetraen-3β -ol | | 0.36 | 0.40 | 0.34 |
| 9 | 24-Methylcholesta-8,24(28)-dien-3β -ol | Fecosterol | 0.09 | 0.10 | 0.11 |
| 10 | 24-methylcholesta-7,22,24(28)-trien-3β -ol | | 0.46 | 0.32 | 0.36 |
| 11 | 24-methylcholesta-5,7,24(28)-trien-3β -ol | | 0.55 | 0.61 | 0.30 |
| 12 | 24-Methylcholesta-5,7-dien-3β -ol | | 0.72 | 0.67 | 0.44 |
| 13 | 24-methylcholesta-7,24(28)-dien-3β -ol | Episterol | 2.12 | 1.40 | 1.52 |
| 14 | **24-Ethylcholesta-5,7,22-trien-3β -ol** | | **9.78** | **1.18** | **3.84** |
| 15 | 4α,4β,14-trimethylcholesta-8,24-dien-3β -ol | Lanosterol | 0.75 | 0.59 | 0.27 |
| 16 | 4α,24-dimethylcholesta-8,24(28)-dien-3β -ol | | 1.26 | 0.78 | 1.06 |
| 17 | 4α,4β,14,24-tetramethylcholesta-8,24(28)-dien-3β-o | Eburicol | 1.06 | 0.89 | 0.12 |
| 18 | 4α,24-dimethylcholesta-7,24(28)-dien-3β -ol | | 0.40 | 0.32 | 0.31 |
| 19 | S30:2 (unknown) | | 0.03 | 0.02 | 0.01 |
| 20 | S30:3 (unknown) | | 0.14 | 0.14 | 0.03 |
| 21 | 4α,4β,24-trimethylcholesta-8,24(28)-dien-3β -ol | | 1.93 | 1.20 | 0.31 |

# Ergosterol Biosynthesis in *A. fumigatus*

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| 4 | 24-methylcholesta-5,7,9,22-tetraen-3β -ol | Neoergosterol | 0.01 | 0.01 | 0.01 |
| **5** | **24-methylcholesta-5,7,22-trien-3ß-ol** | Ergosterol | **75.** | | |
| 6 | 24-methylcholesta-7,22-dien-3β -ol | | 2.3 | | |
| 7 | S28:4 (unknown) | | 0.2 | | |
| 8 | 24-methylcholesta-5,7,22,24(28)-tetraen-3β -ol | | 0.3 | | |
| 9 | 24-Methylcholesta-8,24(28)-dien-3β -ol | Fecosterol | 0.0 | | |
| 10 | 24-methylcholesta-7,22,24(28)-trien-3β -ol | | 0.4 | | |
| 11 | 24-methylcholesta-5,7,24(28)-trien-3β -ol | | 0.5 | | |
| 12 | 24-Methylcholesta-5,7-dien-3β -ol | | 0.7 | | |
| 13 | 24-methylcholesta-7,24(28)-dien-3β -ol | Episterol | 2.1 | | |
| 14 | **24-Ethylcholesta-5,7,22-trien-3β -ol** | | **9.7** | | |
| 15 | 4α,4β,14-trimethylcholesta-8,24-dien-3β -ol | Lanosterol | 0.7 | | |
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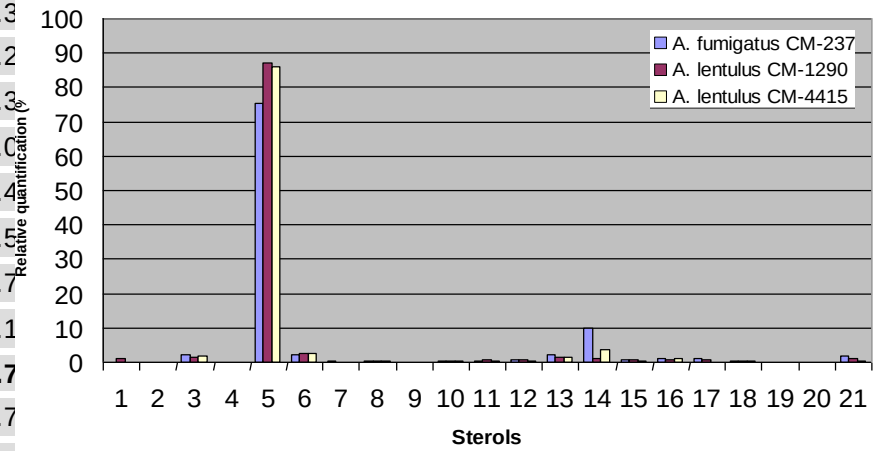
**Sterol composition**



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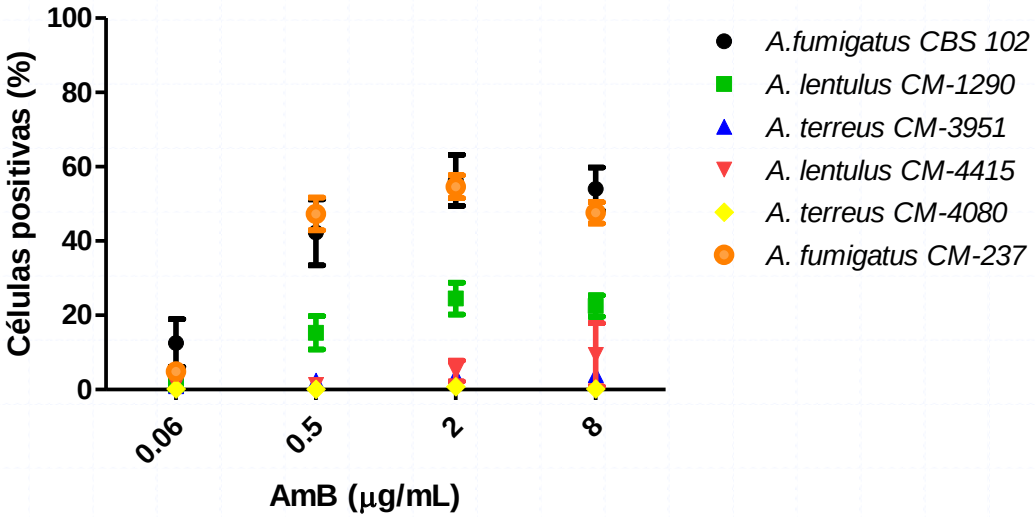
- **AmB autoxidizes, and it is possible that its antifungal effects could result from oxidative damage. ROS production.**

# ROS PRODUCTION: FLOW CYTOMETRY DHF

- Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen.
- ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis.
- However, during stress (AmB) ROS levels can increase dramatically. This may result in significant damage to cell structures.




Still lacking:

- Antioxidant response: Catalase, SOD production
- Cell wall: structure and composition

# CONCLUSIONS

- *A. lentulus* AZL and AmB resistance was demonstrated in vivo
- AZL-R is dependent of the azole target: *cyp*51A
- *A. lentulus* AmB resistance is not dependent of Ergosterol amount
- There is a reduction in ROS production compared to *A. fumigatus* that could explain, at least in part, the AmB resistance
- We can not exclude other concomitant factors that could be responsible for the AmB-R
- The alternative model host in *Galleria mellonella* could be use in the screening of the response of *A. lentulus* to new antifungals

6th ADVANCES AGAINST ASPERGILLOSIS
Madrid, Spain
27 Feb – 1 March 2014
Meliá Castilla Conference and Convention Centre

# THANK YOU !

In especial to: Ana Cecilia Mesa
Gema del Rio

**Members of Mycology Reference Lab**

GILEAD