Antifungal susceptibility testing of *Cryptococcus* strains from Kinshasa (DRC), intronic substitution of the ERG11 gene in one of the fluconazole resistant strains

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Introduction

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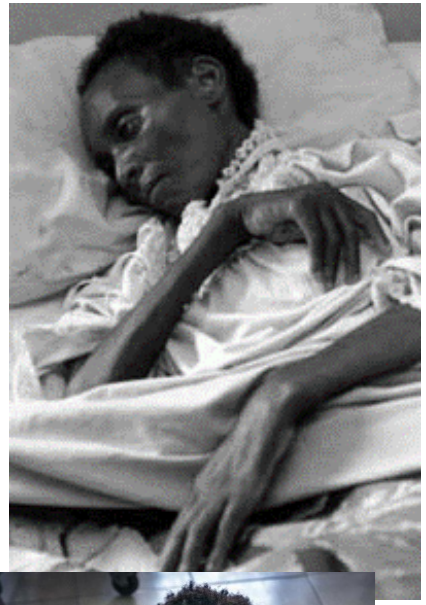
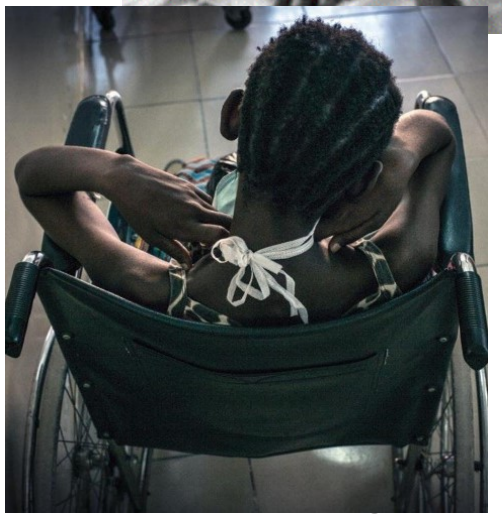
- Neuromeningeal cryptococcosis (NMC) is one of the most common opportunistic infections in people living with HIV (PLHIV).
- In 2014, its global burden was estimated at 223,100 incident cases with more than 80% (181,100) of deaths. It is therefore responsible for 15% of PLHIV deaths per year.
- About 75% of this burden falls on the sub-Saharan Africa.
- In DRC, the number of deaths due to NMC represents about 28.7% (4,883) of all PLHIV deaths (17,000).





Rajasingham et al. The Lancet Infectious Diseases. 2017
UNAIDS and AIDSinfo 2021)
Bive Zono et al. not yet published data.2021

Cryptococcal species in pathology (new taxonomy)

***Cryptococcus neoformans* species complex**	***Cryptococcus gattii* species complex**
▪ *Cryptococcus neoformans* (serotype A/ AFLP1/VNI/AFLP1A/VNB/VNII/AFLP1B/VNII) ▪ *Cryptococcus deneoformans* (serotype D/AFLP2/VNIV) ▪ *Cryptococcus deneoformans* X *Cryptococcus neoformans* (serotype AD/AFLP3/VNIII)	▪ *Cryptococcus gattii* (serotype B/AFLP4/VGI) ▪ *Cryptococcus bacillisporus* (serotype B & C/AFLP5/VGIII) ▪ *Cryptococcus deuterogattii* (serotype B/AFLP6/VGII) ▪ *Cryptococcus tetragattii* (serotype C/AFLP7/VGIV) ▪ *Cryptococcus decagattii* (serotype B/AFLP10/VGVI)

Non-*Cryptococcus* species

- Previously known as ***Cryptococcus* non-*neoformans/gattii* species**, are also involved in the development of cryptococcosis.
- *Papiliotrema laurentii* (previously named ***Cryptococcus laurentii***), *Cutaneotrichosporon curvatus* (***Cryptococcus curvatus***), *Naganishia albidus* (***Cryptococcus albidus***), *Naganishia diffluens* (***Cryptococcus diffluens***) etc.

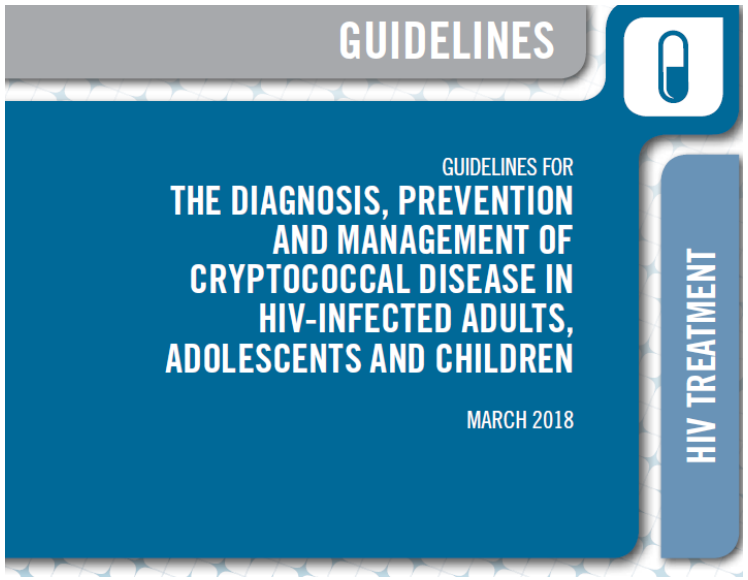
Hagen *et al.* 2015, Smith *et al.* 2017, Sugita, Cho, and Takashima 2017

Introduction

- The WHO guidelines on the management of NMC recommend the combined use of AMB + 5FC in the induction phase of treatment, followed by FCZ in the consolidation phase and later in the maintenance phase.

- In developing countries, the rarity and high cost of the first two antifungals, and the difficulty of managing the side effects of some of them, limit treatment to FCZ alone, a prone situation to resistance development.

- Several mechanisms explain the resistance of *Cryptococcus* spp. to azoles, including mutations in the ERG11 gene encoding the azole target protein sterol 14-α-demethylase.



World Health Organization 2018

Study objectives

- To determine the antifungal susceptibility profile of *Cryptococcus* spp. strains from meningeal PLHIV (from Kinshasa), and to explore genetic events in the ERG11 gene that would result in FCZ-resistant strains.

- In addition, we investigated whether strain susceptibility to antifungal agents was dependent on the ST-MLST profile of the *Cryptococcus neoformans* causing the meningitis.

Methods

Methods

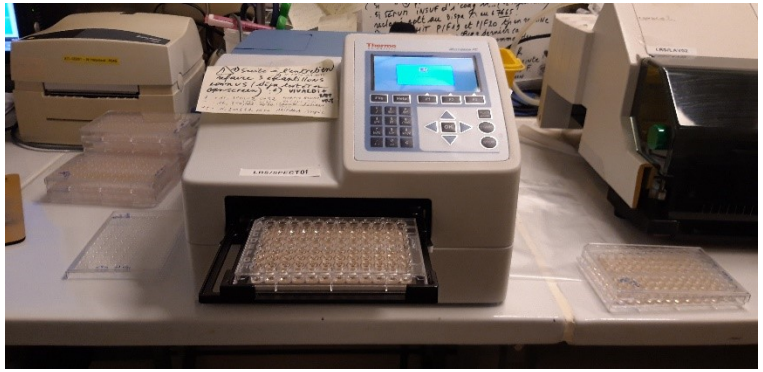
- We analyzed twenty-nine *Cryptococcus* spp. strains cross-sectionally isolated from cerebrospinal fluid (CSF) of PLHIV hospitalized in three hospitals in Kinshasa between February 2019 and February 2020.

- Molecular characterization of strains was previously performed using MALDI-TOF MS and ITS sequencing, with the following results: *Cryptococcus neoformans* (**n = 23**), *Cryptococcus curvatus* (**n = 5**), and *Cryptococcus laurentii* (**n = 1**).

- According to the ISHAM MLST scheme, *Cryptococcus neoformans* isolates were all identified as VNI, belonging to the following seven sequence types (ST): ST93 (**n = 15**), ST659 (**n = 2**), ST5 (**n = 2**), ST4 (**n = 1**), ST53 (**n = 1**), ST31 (**n = 1**), and ST69 (**n = 1**).

Methods

Antifungal susceptibility testing



- Carried out according to the EUCAST guidelines
- Interpretation criteria considered:
  - AMB (from EUCAST breakpoint tables) : S ≤ 1 mg/L; R > 1 mg/L.
  - FCZ and 5-FC (from ECOFFs CLSI) : S ≤ 8 mg/L; R > 8 mg/L.

EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING
European Society of Clinical Microbiology and Infectious Diseases

ERG11 gene sequence analysis

- Gene sequences were extracted from NGS data by mapping to the wt-gene.
- Nucleotide translation into protein and sequences alignment were carried out using Geneious Prime software.

Methods

Statistics

- *C. neoformans* ST-MLST profile were considered into two groups: major ST-MLST (ST93, n=15) versus less common ST-MLST (ST53, ST31, ST5, ST4, ST659, and ST69, n = 8).
- The Wilcoxon test was used to compare the mean MICs between *C. neoformans* ST-MLSTs groups.
- Pearson's chi-square test or Fisher's exact test was applied to estimate the association between strain susceptibility to antifungal agents and patient clinical outcomes.

Results

Results

Table: Antifungal susceptibility test

Species/ST	Amphoterin B-Resistant (%)	5-flucytosine-Resistant (%)	Fluconazole-Resistant (%)	Pejorotive outcome
Cryptococcus neoformans	0/23 (0)	**1/23 (4.3)**	**2/23 (8.7)**	13/23 (56.5)
ST93	0/15 (0)	1/15 (6.7)	1/15 (6.7)	6/15 (40.0)
Non-ST93	0/8 (0)	0/8 (0)	1/8 (12.5)	7/8 (87.5)
Cryptococcus curvatus	0/5 (0)	0/5 (0)	**2/5 (40.0)**	1/5 (25.0)
Cryptococcus laurentii	**1/1 (100)**	**1/1 (100)**	0/1 (0)	0/1 (0)
Total	**1/29 (3.4)**	**2/29 (6.9)**	**4/29 (13.8)**	**14/29 (48.3)**

Results

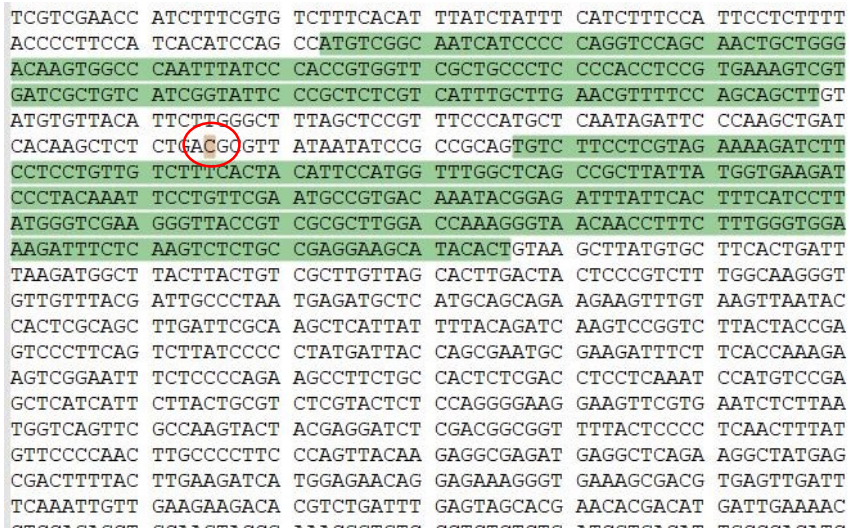
- Major *Cryptococcus neoformans* ST-MLST (ST93) isolates appeared to have better susceptibility to AMB than less common ST-MLST isolates.
- The reverse was found for FCZ and 5-FLU.
- In both groups of ST-MLST *Cryptococcus neoformans* isolates, the mean MIC values were not significantly different (0.68 versus 0.75 mg/L **for AMB**, 6.53 versus 4.90 mg/L **for FCZ**, and 3.78 versus 3.28 mg/L **for 5FC**, ST93 and others isolates respectively).
- While all cured patients were infected with sensitive strains to FCZ and 5FC (10/10), 3.4% of cured patients were infected with AMB-resistant strain (*C. laurentii*).
- No association was found between antifungal susceptibility and patient outcome.

Results

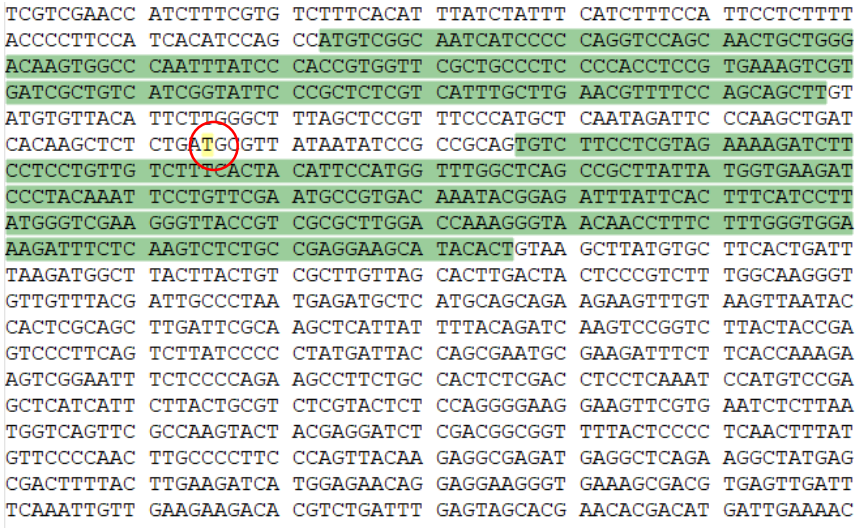
ERG11 gene sequence exploring

Of the two FCZ-resistant *C. neoformans* strains (**78RB**: MIC = **32mg/L**, **14LU** = **16mg/L**), genetic events were found in only one (**78RB**), including:

- Two silent point substitutions in the exonic sequences.
- One point substitution in intron1 (**g.315C > T**), at 22 nucleotides upstream from the exon1.
- This represents one of two potential sequences containing the branching site for pre-mRNA splicing.
- We assume that this mechanism could underlie the resistance of this strain to FCZ.



ERG11-wt sequence gene (part of)



ERG11-78RB sequence gene (part of)

Conclusions

Conclusions

- Resistant strains of *Cryptococcus* spp. to commonly used antifungals are reported from PLHIV in Kinshasa (DRC).
- Slight disparities in antifungals susceptibility were found between *Cryptococcus* species.
- No significant differences were observed in *C. neoformans* with different ST-MLSTs, either for MICs or patient outcome.
- We suspect that an intronic point substitution in a sequence that may be involved in pre-mRNA splicing of the ERG11 gene may induce fluconazole resistance in *C. neoformans*.
- More elaborate and in-depth investigations are needed to draw definitive conclusions.

Our research team

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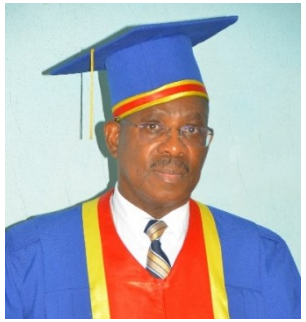

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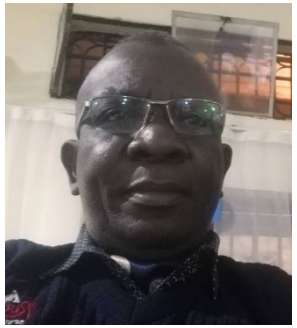

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Aksanti!

Thank you!

شكرا!

спасибо!

תודה!

Merci!