



In vitro Susceptibility Testing of “Rare Moulds”

RaMo

An ECMM-EC/EFISG/ECMM/ISHAM initiative

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1. Background

Invasive fungal infections (IFI) are commonly caused by *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* species. However, an increased incidence of fungal infections caused by rare moulds has been observed for several decades¹. *Mucorales*, *Aspergillus*, *Scedosporium*, and *Fusarium* species are among the most abundant rare moulds in this regard. But thus even rarer fungi occur with increased prevalence^{2,3}. These include so-called hyaline moulds such as *Acremonium*, *Paecilomyces*, and *Trichoderma* which have similar clear hyphae to *Aspergillus*^{4,5}, but also melanin-containing moulds, the so-called dematiaceous moulds or phaeohyphomycoses⁶. The frequent emergence of such infections occurs as a result of several factors and is due to an increasing number of immunocompromised patients and prolonged survival of patients with previously fatal disease⁶. The use of antifungal agents, especially azole-type antifungals, has also been associated with the occurrence of more resistant rare mould infections⁷.

2. Hypothesis

Studies indicated that rare moulds have an intrinsic resistance and are therefore less susceptible or even resistant to general antifungal agents, limiting the success of therapy⁸. In this study, we would like to evaluate whether this observation is true for various rare moulds tested against AmB (amphotericin B), Voriconazole, Isavuconazole, Posaconazole and Micafungin.

3. Aim of this project

Because of the relatively rare occurrence of rare fungal pathogens so far, less data are available on the in vitro susceptibility of rare fungi, hence treatment recommendations are limited⁹. This makes infections caused by rare moulds a major clinical challenge, so the goal of this project is

- i. to collect as many of rare moulds of a specific species/genus as possible to provide antifungal susceptibility testing data
- ii. to perform E-test and broth microdilution standardized by EUCAST and CLSI
- iii. to compare the data from EUCAST/CLSI broth microdilution and concentration gradient strip (Etest)

Because of the heterogeneity of the term rare moulds, we hereby classify that we are not interested in any species of *Aspergillus*, *Mucorales*, *Fusarium* und *Scedosporium*. Figure 1 from “Global guideline for the diagnosis and management of rare mould infections: an initiative of the European Confederation of Medical Mycology in cooperation with the International Society for Human and Animal Mycology and the American Society for Microbiology”¹⁰ gives an overview of interested species/genus (the grayed area includes fungi/genus that we are not interested in). The fungi finally being considered for this study depend on “your strain collection”, the origin of the strains should be “clinically”; no other medical information is not necessary.

■ Strongly recommended
■ Moderately recommended
■ Marginally recommended
■ Recommended against

	First-line	First-line alternative	Second-line	Treatments to avoid	Salvage treatments
Fusariosis	Voriconazole, or voriconazole plus L-AmB, or voriconazole plus ABLC	L-AmB, or ABLC	Isavuconazole, or posaconazole	D-AmB	Posaconazole
Lomentosporosis	Voriconazole plus terbinafine	Voriconazole	Isavuconazole, or posaconazole	L-AmB	Voriconazole
Scedosporiosis	Voriconazole	Voriconazole in combination with L-AmB, ABLC, echinocandins, or terbinafine	Isavuconazole, or posaconazole, or itraconazole	L-AmB	Voriconazole echinocandins, or posaconazole
Phaeohiphomycosis: localised infection	Voriconazole	L-AmB with or without echinocandins, or triazole	Isavuconazole	D-AmB	Isavuconazole, or posaconazole, or voriconazole
Phaeohiphomycosis: cutaneous or subcutaneous infection	Itraconazole or voriconazole	L-AmB with or without echinocandins, or triazole	Isavuconazole	D-AmB	Isavuconazole, or posaconazole, or voriconazole
Phaeohiphomycosis: disseminated infection	Posaconazole, or voriconazole plus echinocandins, or voriconazole plus terbinafine	L-AmB with or without echinocandins, or triazole	Isavuconazole	D-AmB	Isavuconazole, or posaconazole, or voriconazole
Phaeohiphomycosis: <i>Exserohilium rostratum</i>	Voriconazole with or without L-AmB	..	L-AmB plus triazoles other than voriconazole	D-AmB	..
<i>Rasamsonia</i> spp	Caspofungin, or micafungin	Caspofungin plus L-AmB or posaconazole, or micafungin plus L-AmB or posaconazole	..	Azole monotherapy	..
<i>Schizophyllum commune</i>	L-AmB; stepdown to posaconazole	..	Voriconazole
<i>Schizophyllum</i> spp other than <i>S commune</i> and other basidiomycetes (eg, <i>Coprinopsis cinerea</i> , <i>Hormographiella aspergillata</i>)	L-AmB with or without inhaled L-AmB, or L-AmB with or without voriconazole	..	Voriconazole	Echinocandins	L-AmB, or voriconazole
<i>Scopulariopsis</i> spp	Isavuconazole, or voriconazole	L-AmB with or without voriconazole	Posaconazole with or without micafungin with or without terbinafine
<i>Penicillium</i> spp: disseminated infection	L-AmB with or without other antifungals	Voriconazole
<i>Penicillium</i> spp: lung infection	Posaconazole	Voriconazole
Non- <i>marseffei</i> <i>Talaromyces</i> spp	L-AmB	Voriconazole, or echinacondine plus terbinafine
<i>Paecilomyces</i> spp	L-AmB	Itraconazole, or posaconazole
<i>Purpureocillium</i> spp	Voriconazole	..	Itraconazole or L-AmB or posaconazole	..	Itraconazole, or L-AmB, or posaconazole
<i>Purpureocillium</i> spp: cutaneous or subcutaneous infection	Voriconazole plus terbinafine	..	Itraconazole or L-AmB or posaconazole	..	Itraconazole, or L-AmB, or posaconazole

Figure 1: Recommended systemic antifungal treatment for adults with rare mould infections¹⁰

4. Methods

4.1 Fungal strain collection and workflow

Our goal is to perform testing of at least 10 strains per species or genus in order to get a sufficient set of susceptibility data. Your participation in this study could consist of (i) providing us with rare mould strains to perform AFST (antifungal susceptibility testing) OR (ii) providing us with already evaluated MICs of rare moulds, preferably with data from Etest and CLSI/EUCAST broth microdilution. After receiving the strains, they will be molecularly characterized by ITS-sequencing and tested for in vitro susceptibility using Etest and CLSI/EUCAST broth microdilution methods. Figure 2 gives an overview of the workflow. After the data have been evaluated, they are to be published. Your active participation (providing strains or adequate MICs) supports co-authorship.

4.2 Concentration Gradient Strip (Etest)

An agar plate (medium: RPMI 1640 MOPS with 2 % glucose) is homogeneously inoculated with the prepared cell suspension of the desired mould. A plastic strip with a predefined exponential gradient of the antifungal agent is applied to the agar surface. The antimycotic is released and forms a concentration gradient in the agar medium which, after incubation at 37 °C, leads to possible growth inhibition, seen by ellipse formation. The MIC value is determined by the intersection of the ellipse with the scale on the top of the strip¹¹. The first reading should happen at 20-24 hours and the second at 48 hours. Since the reading time depends on the growth of the fungus, slow growing fungi can also be read after 72 hours.

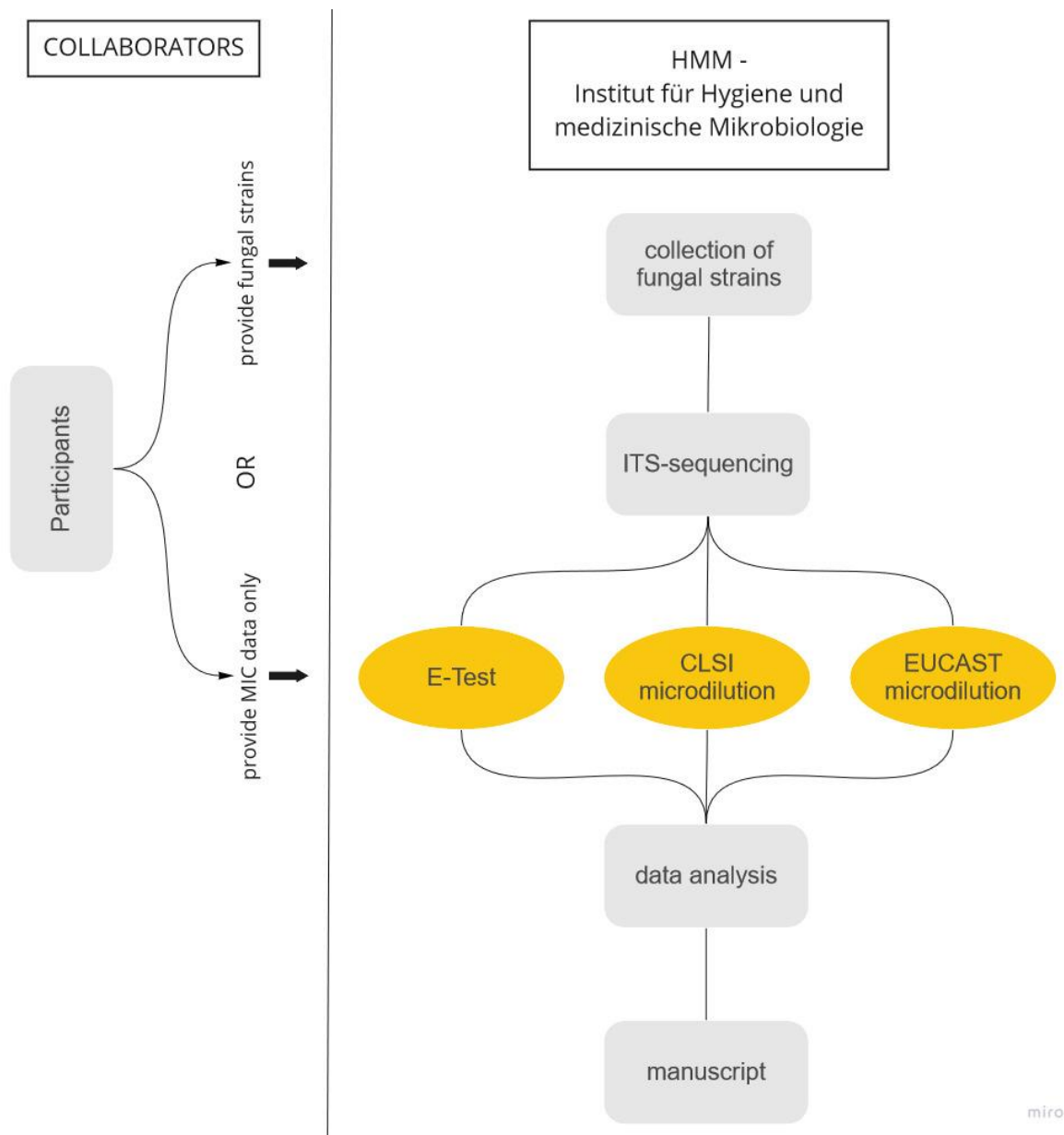


Figure 2: overview of workflow

4.3 Broth microdilution – CLSI

The CLSI guidelines for broth microdilution Antifungal Susceptibility testing for filamentous fungi are described in the M38 document of the CLSI Subcommittee on Antifungal Susceptibility Tests¹¹.

For the testing one requires 96-well microdilution plates of untreated polystyrene with U-shaped wells. The medium is RPMI 1640 culture medium buffered with MOPS and a glucose concentration of 0.2 %. An antifungal stock solution of at least 1,280 µg/mL or 10 times the highest concentration to be tested, should be prepared. From this, dilutions are prepared, of which 0.1 mL is pipetted into each well of the 96-well plate. The colonies of the desired fungus, previously cultured on agar plates, are washed off with sterile saline or water. The density of the cell suspension is determined by hemocytometer and

the final inoculum size should be between $0.4 - 5 \times 10^4$ cells/mL for Nondermatophytes. Each well of the 96-well plate should be inoculated with 0.1 mL of the diluted inoculum suspension. After drug plates are inoculated, they are incubated at $35 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$. Since there are no reading guidelines for rare moulds, the plates are read after 24 hours. Plates with insufficient growth may be held for a further 24-48 hours. All plates are read visually using a mirror viewer. Wells should be scored for growth compared to that of the drug-free control well.

4.4 Broth microdilution – EUCAST

The EUCAST guidelines for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds are described in the EUCAST document E.DEF 7.3.1¹².

In contrast to the CLSI, EUCAST recommends the use of tissue-treated plates with flat-bottomed wells for testing. The medium is RPMI 1640 culture medium buffered with MOPS and a glucose concentration of 2 %. An antifungal stock solution with concentrations of at least 200 times higher than the highest concentration to be tested, should be prepared. From this, dilutions are prepared, of which 0.1 mL is pipetted into each well of the 96-well plate. The colonies of the desired fungus, previously cultured on agar plates, are washed off with sterile distilled water. The density of the cell suspension is determined by hemocytometer and the final inoculum size should be between $2 - 5 \times 10^5$ cells/mL for filamentous fungi. Each well of the 96-well plate should be inoculated with 0.1 mL of the diluted inoculum suspension. After drug plates are inoculated, they are incubated at $35 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$. Since there are no reading guidelines for rare moulds, the plates are read after 24 hours. Plates with insufficient growth may be held for a further 24-48 hours. All plates are read visually using a mirror viewer. Wells should be scored for growth compared to that of the drug-free control well.

4.5 Timetable

Task	Month									
	2022				2023					
	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Collection of fungal strains	x	x								
ITS sequencing			x	x						
E-Test			x	x	x	x				
CLSI microdilution			x	x	x	x				
EUCAST microdilution			x	x	x	x				
Data analysis							x	x		
Paper / manuscript									x	x

Figure 3: timetable

5. Participants

A list of definite participants will be created after acceptance of participation in this study, the list of requested colleagues is given under chapter 8.

6. Funding

Funding is supported by HMM (Institut für Hygiene und medizinische Mikrobiologie) – HOROS project.

7. Ethical aspects

Ethical considerations and data privacy protections does not come into force, as we need only “fungal strains”.

8. Conclusion

As already mentioned before, rare moulds are causing more and more challenging problems, so the main goal of this project is to collect as many rare moulds as possible to obtain data on MICs. A number of different rare moulds have already been provided from our routine laboratory, yet it would be essential for our project to obtain additional rare fungal pathogens or already evaluated MIC data of them.

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