sektion für hygiene und medizinische mikrobiologie

MEDIZINISCHE UNIVERSITÄT INNSBRUCK

In vitro Susceptibility Testing of "**Ra**re **Mo**ulds"

RaMo

An ECMM-EC/EFISG/ECMM/ISHAM initiative

Convenor: Cornelia Lass-Flörl, MD (Innsbruck/Austria)

Division of Hygiene and Medical Microbiology
Innsbruck Medical University
6020 Innsbruck
Austria

Contact: Michaela Riedlsperger
Tel.: +43-512-70718
Michaela.Riedlsperger@i-med.ac.at

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Content

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 decades[1]. *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 phaeohyphomycoses[6]. 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 disease[6]. The use of antifungal agents, especially azole-type antifungals, has also been associated with the occurence of more resistant rare mould infections[7].

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 therapy[8]. 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 limited[9]. 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
Phaeohyphomycosis: localised infection	Voriconazole	L-AmB with or without echinocandins, or triazole	Isavuconazole	D-AmB	Isavuconazole, or posaconazole, or voriconazole
Phaeohyphomycosis: cutaneous or subcutaneous infection	Itraconazole or voriconazole	L-AmB with or without echinocandins, or triazole	Isavuconazole	D-AmB	Isavuconazole, or posaconazole, or voriconazole
Phaeohyphomycosis: 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
Phaeohyphomycosis: *Exserohilium rostratum*	Voriconazole with or without L-AmB	..	L-AmB plus triazoles other than voriconazole	D-AmB	..
Rasamsonia spp	Caspofungin, or micafungin	Caspofungin plus L-AmB or posaconazole, or micafungin plus L-AmB or posaconazole	..	Azole monotherapy	..
Schizophyllum commune	L-AmB; stepdown to posaconazole	..	Voriconazole	..	..
Schizophyllum spp other than *S commune* and other basidiomycetes (eg, *Coprinopsi cinereal*, *Hormographiella aspergillata*)	L-AmB with or without inhaled L-AmB, or L-AmB with or without voriconazole	..	Voriconazole	Echinocandins	L-AmB, or voriconazole
Scopulariopsis spp	Isavuconazole, or voriconazole	L-AmB with or without voriconazole	..	..	Posaconazole with or without micafungin with or without terbinafine
Penicillium spp: disseminated infection	L-AmB with or without other antifungals	..	..	..	Voriconazole
Penicillium spp: lung infection	Posaconazole	..	..	..	Voriconazole
Non-*marneffei Talaromyces* spp	L-AmB	..	..	..	Voriconazole, or echinacondine plus terbinafine
Paecilomyces spp	L-AmB	..	..	..	Itraconazole, or posaconazole
Purpureocillium spp	Voriconazole	..	Itraconazole or L-AmB or posaconazole	..	Itraconazole, or L-AmB, or posaconazole
Purpureocillium 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 infections[10]

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 strip[11]. 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.

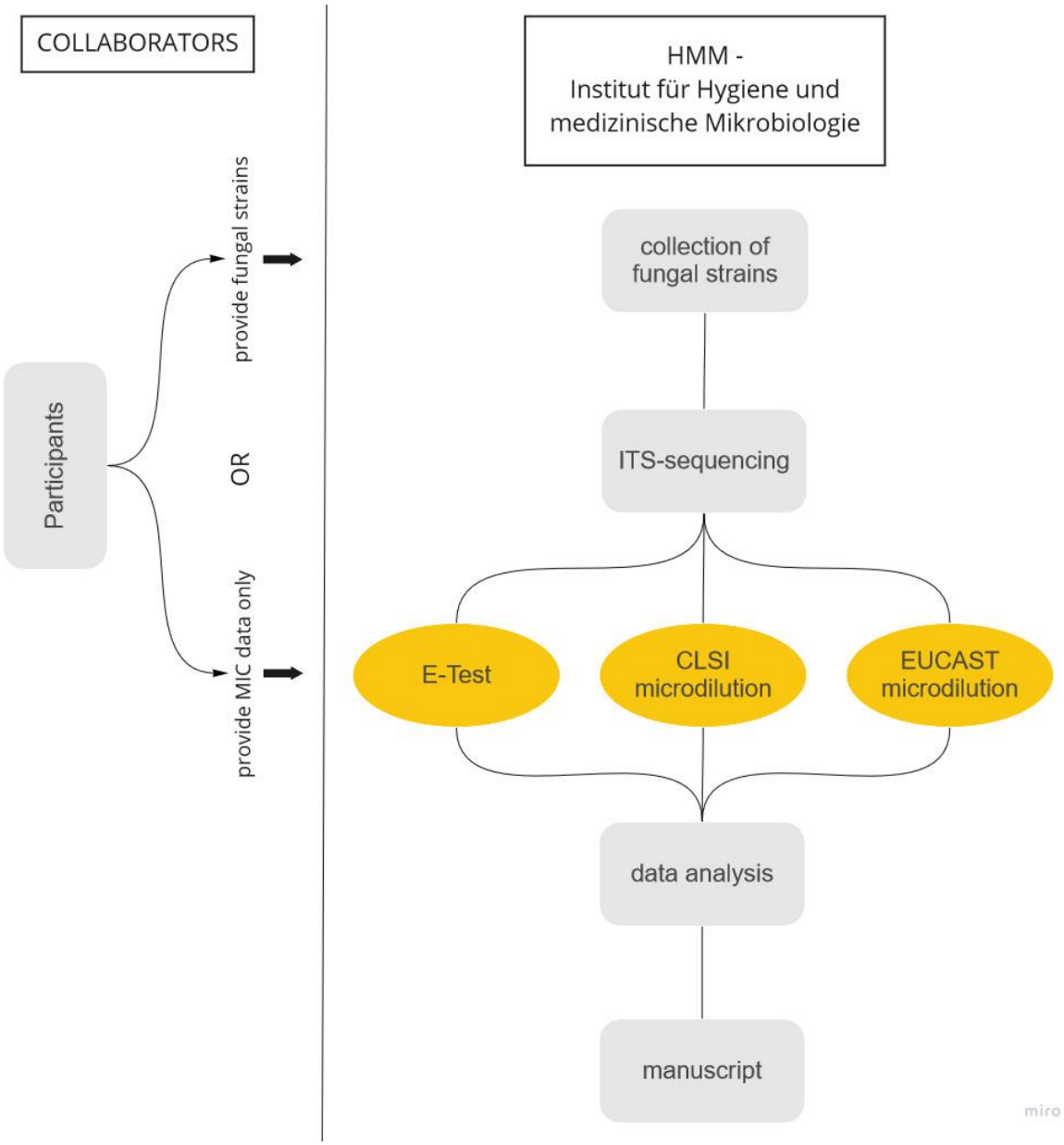

Figure 2: overview of workflow

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 Tests[11].

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 x 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 °C ± 2 °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 7.3.1[12].

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 x 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 °C ± 2 °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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10. Requested participants

Kontoyiannis	Dimitrios	dkontoyi@mdanderson.org
Lagrou	Katrin	katrien.lagrou@uzleuven.be
Oliveri	Salvatore	oliveri@unict.it
Peman	Javier	peman_jav@gva.es
Rezusta	Antonio	arezusta@unizar.es
Willinger	Birgit	birgit.willinger@meduniwien.ac.at
Alastruey-Izquierdo	Ana	anaalastruey@isciii.es
Alanio	Alexandre	alexandre.alanio@pasteur.fr
Chowdhary	Anuradha	chowdhary.anuradha@gmail.com
Arikan-Akdagli	Sevtap	sarikanakdagli@gmail.com
Meletiadis	Joseph	jmeletiadis@med.uoa.gr
Arendrup	Maiken	maca@ssi.dk
Richardson	Riina	riina.richardson@manchester.ac.uk
Gangneux	Jean-Pierre	jean-pierre.gangneux@univ-rennes1.fr
Arsic Arsenijevic	Valentina	mikomedlab@yahoo.com
Guinea Ortega	Jesus	jguineaortega@yahoo.es
Chakrabarti	Arunaloke	arunaloke@hotmail.com
Cornely	Oliver	oliver.cornely@zks-koeln.de
Rath	Peter	Peter-Michael.Rath@uk-essen.de
Steinmann	Jörg	joerg.steinmann@uk-essen.de
Lamoth	Frederic	Frederic.Lamoth@chuv.ch
Mares	Mihai	mycomedica@gmail.com
Sabino	Raquel	raquel.sabino@insa.min-saude.pt
Sanguinetti	Mauritio	maurizio.sanguinetti@unicatt.it
Segal	Esther	segale@post.tau.ac.il
Friberg	Nathalie	nathalie.friberg@hus.fi
Hamal	Petr	petr.hamal@fnol.cz
Krause	Robert	robert.krause@medunigraz.at
Verweij	Paul	paul.verweij@radboudumc.nl
Queiroz-Telles	Flavio	queiroz.telles@uol.com.br
Colombo	Arnaldo	colomboal@terra.com.br
Suzana	Hadina	suzana.hadina@vef.hr
Roilides	Emmanuel	roilides@gmail.com
Fernandez	Mariana	mariana_f19@hotmail.com
Martin-Gomez	Teresa	maitestc@gmail.com
Badali	Hamid	badalii@yahoo.com
Roudbary	Maryam	m_roudbary@yahoo.com
Grancini	Anna	anna.grancini@libero.it
Klingspor	Lena	lena.klingspor@ki.se
Lewis	Russel L.	russeledward.lewis@unibo.it

Seidel	Daniela	daniela.seidel@uk-koeln.de
Hamprecht	Axel	axel.hamprecht@uk-koeln.de
Bader	Oliver	obader@gwdg.de
Kurzai	Oliver	okurzai@hygiene.uni-wuerzburg.de
Farina	Claudio	ifarinadibergamo@gmail.com
Sanchez-Reus	Ferran	fsancez@santpau.es
Özenci	Volkan	volkan.ozenci@ki.se
Johnson	Elizabeth	elizabeth.johnson@uhbristol.nhs.uk
Lo Cascio	Giuliana	giuliana.locascio@ospedaleuniverona.it
Marekovic	Ioana	imarekov@kbc-zagreb.hr
Gow	Neil	n.gow@exeter.ac.uk
Meyer	Wieland	wieland.meyer@sydney.edu.au
Tortorano	Anna Maria	annamaria.tortorano@unimi.it
Seyedmousavi	Amir	seyedmousavi@nih.gov
Tullio	Vivian	vivian.tullio@unito.it
Wiederhold	Nathan	wiederholdn@uthscsa.edu
Rodriguez-Iglesias	Manuel	manuel.rodriguezigelsias@uca.es
White	Lewis	lewis.white@wales.nhs.uk
Drogari-Apiranthitou	Miranda	mdrogari@hotmail.com
Chen	Sharon	sharon.chen@health.nsw.gov.au
Pasqualotto	Alessandro	acpasqualotto@hotmail.com
Ben-Ami	Ronen	ronen.benami@gmail.com
Mer	Mervyn	mervyn.mer@wits.ac.za
Kanj	Souha	sk11@aub.edu.lb
Oladele	Rita	oladelerita@gmail.com
Patel	Atul	atulpatel65@gmail.com
Soman	Rajeev	rajeev.soman@yahoo.com
Ban Hock	Tan	tan.ban.hock@singhealth.com.sg
Araj	George	garaj@aub.edu.lb

Table 1: list of people who will be contacted