In vitro susceptibility testing for black grain eumycetoma causative agents

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Eumycetoma is a neglected tropical implantation mycosis characterized by large subcutaneous swellings. Inside the infected tissue, the causative agents are found in grains. The most common causative agents form black grains and are sterile upon isolation. In vitro susceptibility assays were developed for eumycetoma causative agents. They were based on the Clinical and Laboratory Standards Institute M38A protocol and modified to enable the use of hyphae as a starting inoculum. To ease endpoint reading, viability dyes such as resazurin or XTT have been used. So far the in vitro susceptibility assays developed have mainly been used to establish if causative agents are inhibited in growth by various antifungal agents, but not for clinical decision making. For drug discovery, the assay proved useful in determining which compounds were able to prevent hyphal growth. However, a clear correlation between in vitro inhibition in terms of the half maximal inhibitory concentration or 50% minimum inhibitory concentration (MIC50) and therapeutic efficacy assayed in a novel model system in terms of Galleria mellonella larval survival was not found. For clinical decision making, a range of MICs were found for each antifungal agent. However, no clinical breakpoints have been established for any of the causative agents. For itraconazole, the MIC50 of most causative agents was below the attainable serum levels, which might indicate that they are susceptible. However, before in vitro susceptibility can be used in clinical decision making for mycetoma, a correlation between MIC and clinical outcome needs to be made.

Introduction

Mycetoma is a chronic granulomatous infectious disease of the subcutaneous tissue. It is characterized by tumorous lesions and malformation of the infected limbs. It can be caused by a large number of various agents. These causative agents can be bacteria (actinomycetoma) or fungi (eumycetoma). Characteristic of mycetoma is that the causative agent organizes itself in a grain inside the human tissue and the colour of the grain is dependent on the causative agent. In this grain, the bacterial filaments or fungal hyphae are packed tightly together. Depending on the causative agent, cement material can be found surrounding the filaments or hyphae that protects them from the hostile environment in the host. Globally, eumycetoma is more common than actinomycetoma and the most common eumycetoma causative agents are Madurella mycetomatis (75.1%), Falciformispora senegalensis (6.2%), Trematosphaeria grisea (4.3%), Scedosporium apiospermum complex (3.1%) and Medicopsis romeroi (0.8%). Of these causative agents, M. mycetomatis, F. senegalensis, T. grisea and M. romeroi form black grains while S. apiospermum complex forms white grains. Eumycetoma is treated with a combination of antifungal agents and surgery. The treatment chosen is not dependent on the species identification. To treat eumycetoma, most commonly the regimen from the Mycetoma Research Centre in Khartoum, Sudan is used. In this regimen, the patient is treated with 200–400 mg itraconazole daily for 6 months to create a good fibrous capsule around the lesion, followed by wide local excision. After surgery the patient continues on 200–400 mg itraconazole daily until cure is achieved. In regions where itraconazole is not widely available, terbinafine is used as an alternative. In that case, eumycetoma is treated with 500 mg terbinafine twice daily and then surgery. For fungal infections caused by Candida, Cryptococcus and Aspergillus species, antifungal susceptibility assays are routinely used to support clinicians in improving patient management and predicting treatment response. A correlation between the minimal inhibitory concentration (MIC) and therapeutic outcome has been established for each of these species. For Candida, this correlation is often called the ‘90–60’ rule, which means that infections due to susceptible Candida strains respond to appropriate antifungal therapy in approximately 90% of cases, whereas
infections due to resistant strains respond in approximately 60% of cases. For *Aspergillus*, a correlation between voriconazole resistance and therapeutic failure has been found, therefore in vitro susceptibility assays are used to guide patient management. For mycetoma, routine antifungal susceptibility assays are not performed and no correlation between the MIC and clinical outcome has been established to date. However, efforts are under way to determine if antifungal susceptibility assays can be of value in mycetoma management. Here I will review the development and use of the current antifungal susceptibility assays for eumycetoma causative agents.

### International guidelines for antifungal susceptibility testing

For antifungal susceptibility testing, two international reference methods have been developed. These are the reference method of the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These methods are quite similar, as they are both performed in 96-well plates, use Roswell Park Memorial Institute (RPMI) 1640 culture medium and spores or conidia as starting points and have identical incubation temperatures and times. In both assays, growth is read visually by the naked eye. Between these two different methods, only minor differences are found. These include the concentration of glucose within the RPMI medium (0.2% in CLSI and 2% in EUCAST), the type of microdilution plate used (U-shaped in CLSI and flat bottom in EUCAST) and the fungal inoculum used (0.4×10⁵–5×10⁴ cfu/ml in CLSI and 1×10⁵–2.5×10⁵ cfu/ml in EUCAST). For most fungal species, good agreement between the two standard reference methodologies has been found. For eumycetoma causative agents, the CLSI-based reference method has been used as a template in the development of an antifungal susceptibility assay.

### Hyphae vs conidia

As mentioned above, for in vitro susceptibility testing, conidia or spores are used as inocula. For *M. mycetomatis*, the most common causative agent of eumycetoma, only sporulates in very rare circumstances and sterile hyphae are usually obtained. Therefore, to use conidia as a starting point is not feasible. For that reason, an alternative approach for generating a starting inoculum was needed. To assess the effect of the nature of the starting inoculum on the MIC, conidia and hyphal fragments were compared. Two different approaches have been reported in the literature to generate hyphal fragments direct from a colony. The first is to generate hyphal fragments via sonication, the second is by rubbing the fungal colony with a sterile scalpel or transfer tip. When hyphal fragments were generated from *Aspergillus fumigatus* by sonication and used as a starting inoculum (70–80% transmission at 660 nm) in the CLSI antifungal susceptibility assay, similar MICs for amphotericin B and voriconazole were obtained compared with the conidia starting inoculum (5×10⁴ cfu/ml). The MICs for itraconazole were one dilution step higher. When hyphal fragments were generated by rubbing the fungal colony and used as a starting inoculum (2–5×10⁴ cfu/ml) in the EUCAST-based antifungal susceptibility assay, again similar MICs for amphotericin B, posaconazole and voriconazole were obtained. This was not only demonstrated for *Aspergillus*, but also for species of *Absidia*, *Acremonium*, *Emericella*, *Fusarium*, *Microsporum*, *Mucor*, *Paecilomyces*, *Rhizomucor*, *Rhizopus*, *Scedosporium*, *Trichoderma* and *Trichophyton*. Therefore hyphal fragments seem a good substitute for conidia. In contrast, when hyphal clumps instead of hyphal fragments were used as a starting inoculum, a significant increase in 50% minimum inhibitory concentration (MIC50) from 1 µg/ml (conidia) to 8 µg/ml (hyphal clumps) was noted for amphotericin B and from 0.5–1 µg/ml (conidia) to >16 µg/ml (hyphal clumps) for the azoles. This indicated that only MICs obtained for hyphal fragments were comparable to those obtained with conidia, not those obtained with fungal clumps. The manner in which hyphal fragments were generated seemed less important as long as the same cfu/ml or density was used. Therefore, for mycetoma agents, hyphal fragments are currently generated by sonication.

### Viability dyes

Since both the CLSI and EUCAST reference methods use visual endpoint reading, this becomes more complicated when the starting inoculum is already turbid. In the case of a hyphal suspension, this is often the case. In such cases, viability dyes detecting metabolic activity can be used as an alternative method for endpoint reading even though growth and metabolic activity are not necessarily equivalent. This is because any drug that has a direct influence on the metabolic rate could have an effect on the reduction of viability dyes even if the biomass remains the same.

There are several viability dyes that have been used for endpoint reading in in vitro antifungal susceptibility assays, including resazurin, tetrazolium salts and the luciferin–luciferase bioluminescence assay (Table 1). The most common dyes are resazurin and tetrazolium salts (Figure 1). Of these, resazurin is the oldest in use and also the cheapest. Furthermore, its derivative, alamarBlue, is also used in the commercial CLSI-based antifungal susceptibility assay YeastOne. Resazurin is non-fluorescent and deep blue coloured and will be metabolically reduced by 1,4-dihydroroticinamide adenine dinucleotide (NADH) in viable cells to the fluorescent pink-coloured resorufin and upon prolonged incubation to the colourless hydroresorufin. It can be dissolved directly in culture media and is often added at the beginning of an antifungal susceptibility assay and incubated during the assay. To measure the metabolic activity, resazurin and resorufin are measured fluorometrically ($\lambda_{em}$ 579 nm, $\lambda_{em}$ 584 nm) or colourimetrically (resazurin, 605 nm; resorufin, 573 nm). There is no need for cell lysis prior to spectrophotometric measurement, as both resazurin and resorufin diffuse freely through the fungal cell membrane. Although resazurin is relative cheap, it has only limited linear range that is highly dependent on the temperature, pH and initial resazurin concentration. Due to the prolonged incubation and the secretion of pyomelanin by *M. mycetomatis*, growth was
Table 1. Viability dyes used in fungal in vitro susceptibility assays

| Viability dye               | Full name                                                                 | Active mechanism          | Incubation during or after assay | Absorbance wavelength (nm) |
|-----------------------------|---------------------------------------------------------------------------|---------------------------|----------------------------------|----------------------------|
| Resazurin/alamarBlue        | 7-hydroxy-3H-phnoxazin-3-one-10-oxide sodium salt                         | Reduction by NADH         | During                           | 600/620                    |
| MTT                         | 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide              | Reduction by NADH         | After                            | 550                        |
| XTT                         | 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide | Reduction by NADH         | After                            | 450                        |
| WST-8                       | 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt | Reduction by NADH         | After                            | 460                        |
| Luciferase/luciferin        |                                                                           | ATP                       |                                   | 560                        |

Figure 1. Viability dyes used with M. mycetomatis. As an example, an in vitro susceptibility assay with itraconazole is used. (A) The visual appearance of the wells and the calculated growth percentages obtained with alamarBlue, a resazurin derivative. The well on the far left indicates the growth control, followed by various concentrations of itraconazole. The isolates used produced pyomelanin, therefore a brown colour is seen. The well on the far left is the negative control, which is blue in the case of amphotericin B or light purple in the case of itraconazole. Different shades of brown or pink indicate different percentages of growth. These differences are also noted when the growth is calculated relative to the growth and negative controls. Typically growth percentages range from 0 to 100%. The MIC is considered the first well, which has a growth percentage <20%. (B) The visual appearance of the wells and the calculated growth percentages obtained with XTT. The well on the far left indicates the growth control. A dark orange colour can be seen. The well on the far right is the negative control, in which a light orange colour is seen. Between the negative controls, different concentrations of amphotericin B or itraconazole are seen. Different shades of orange indicate different percentages of growth. Typically growth percentages range from 0 to 100%. The MIC is considered the first well, which has a growth percentage <20%.

observed as brown instead of pink (Figure 1A). The brown pigmentation did not influence the visual endpoint reading of the MIC (Figure 1A), however, it influenced the wavelength at which reliable readings could be obtained. Therefore, for M. mycetomatis, absorbance was measured at 620 nm when alamarBlue was used and at 600 nm when resazurin was used.

The other most commonly used viability dyes are the tetrazolium salt solutions. These salt solutions are weakly coloured and change to a strong coloured solution when forming the formazan product (Figure 1B). Over the years, several tetrazolium salts have been developed. For antifungal susceptibility testing, 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) have been used. Of these tetrazolium salts, MTT is the only one able to pass the cell membrane, due to its lipophilic side groups and positive net charge. XTT and WST-8 cannot. Tetrazolium salts are reduced by mitochondrial or cell plasma enzymes like oxidoreductases, dehydrogenases, oxidases and peroxidases using NADH, dihydronicotinamide-adenine dinucleotide phosphate (NADPH), succinate or pyruvate as electron donors. With MTT as substrate, purple needle-like
| Species                                      | Prevalence | Starting inoculum   | Incubation temperature (°C) | Incubation time (h) | Viability dye         |
|---------------------------------------------|------------|---------------------|-----------------------------|--------------------|-----------------------|
| Madurella mycetomatis                       | C          | Hyphal fragments    | 35–37                       | 144                | XTT or resazurin      |
| Falciformispora senegalensis                | O          | Hyphal fragments    | 30                          | 144                | Resazurin             |
| Trematosphaeria grisea                      | O          | Hyphal fragments    | 30                          | 48                 | Resazurin             |
| Medicago romeroi                           | O/R        | Hyphal fragments    | 30                          | 48                 | Resazurin             |
| Nigrograna mackinnonii                      | O/R        | Hyphal fragments    | 30                          | 48                 | Resazurin             |
| Madurella pseudomycetomatis                 | R          | Hyphal fragments    | 35–37                       | 144                | Resazurin             |
| Madurella tropicana                         | R          | Hyphal fragments    | 35–37                       | 144                | Resazurin             |
| Madurella fahalii                           | R          | Hyphal fragments    | 35–37                       | 144                | Resazurin             |
| Falciformispora tompkinsii                  | R          | Hyphal fragments    | 35–37                       | 144                | Resazurin             |
| Emarellia grisea                            | R          | Hyphal fragments    | 35                          | 48, 96             | No viability dye      |
| Emarellia paragrisea                        | R          | Hyphal fragments    | 35                          | 48, 96             | No viability dye      |
| Exophiala dermatitidis                      | R          | conidia             | 35                          | 48, 96             | No viability dye      |
| Exophiala jeanselmei                        | R          | conidia             | 35                          | 48, 96             | No viability dye      |
| Pseudochaetosphaerona larense               | R          | Hyphal fragments    | 30                          | 144                | Resazurin             |

*Prevalence as calculated in Ahmed et al. and indicated as common (C), occasional (O) or rare (R).

Formazan crystals are produced in the cell that will destroy the cell's integrity and eventually lead to cell death because they are unable to pass the cell membrane. To quantify the formazan production, a 30-min cell lysis step with isopropanol containing 5% 1M hydrochloric acid is needed to release the formazan dye. To overcome this lysis step, tetrazolium derivatives that produce water-soluble products such as XTT and WST-8 have been developed. The solubility was achieved by introducing negative-charged sulfone groups to the phenyl rings. Due to this modification, most of the dyes are unable to pass through cell membranes and the reduction of these dyes is therefore mainly performed extracellularly. The electron transfer necessary for reduction of the tetrazolium salts is transduced by electron mediators like menadione and 2-methyl-1,4-naphthoquinone. The only tetrazolium salt used in the *in vitro* susceptibility assays for mycetoma causative agents is XTT (Figure 1B).

To assess the effect of a drug on the metabolic percentage of the fungus, the absorbance of the negative control (NC; well containing the culture media and the solvent but not the fungus) and the positive control (PC; the well containing the culture media, the solvent and the fungus but no antifungal agent) are used to normalize. The following formulas are used:

For alamarBlue:

\[
\text{Percentage metabolic activity} = \left( \frac{\text{Absorbance}_{620\text{nm}} \text{ test} - \text{Absorbance}_{620\text{nm}} \text{ NC}}{\text{Absorbance}_{620\text{nm}} \text{ GC} - \text{Absorbance}_{620\text{nm}} \text{ NC}} \right) \times 100
\]

For resazurin:

\[
\text{Percentage metabolic activity} = \left( \frac{\text{Absorbance}_{600\text{nm}} \text{ test} - \text{Absorbance}_{600\text{nm}} \text{ NC}}{\text{Absorbance}_{600\text{nm}} \text{ GC} - \text{Absorbance}_{600\text{nm}} \text{ NC}} \right) \times 100
\]

### Assays developed for mycetoma causative agents

Using the methodology described above, *in vitro* susceptibility assays have been developed for several black grain eumycetoma causative agents. These include the common causative agent *M. mycetomatis*, the occasional causative agents *F. senegalensis*, *T. grisea*, *M. romeroi* and *Nigrograna mackinnonii* and the rare causative agents *Madurella pseudomycetomatis*, *Madurella fahalii*, *Madurella tropicana*, *Falciformispora tompkinsii*, *Emarellia grisea*, *Emarellia paragrisea*, *Exophiala dermatitidis* and *Exophiala jeanselmei*. For most of these causative agents, a hyphal inoculum was prepared using sonication or scraping.

Due to differences in growth rate and growth temperature, incubation times and temperatures are dependent on the causative agent (Table 2). For slow-growing species, an incubation time of 144 h is needed before endpoints can be read. For faster-growing species, endpoints can be read after 48 h.
the order Sordariales usually grow well at 37°C and therefore in vitro susceptibility assays are performed at that temperature. For some of the mycetoma causative agents belonging to the order Pleosporales, in vitro susceptibility needs to be performed at 30°C due to a lack of growth at 37°C.

**Antifungal agents tested**

The antifungal susceptibility assays developed for mycetoma causative agents have been used to screen antifungal agents indicated for the treatment of other fungal infections, antifungal combinations, herbal medicine and drug screening purposes.

**Antifungal agents indicated for other fungal infections**

Using the CLSI-based assays, several antifungal agents have been assessed for their activity against mycetoma causative agents. These include the polyene amphotericin B; the azoles ketoconazole, itraconazole, posaconazole, fluconazole, voriconazole, isavuconazole and ravuconazole; the allylamine terbinafine; the pyrimidine analogue 5-flucytosine; the echinocandins caspofungin, anidulafunin and micafungin; and the orotomide olorofim (Table 3). Of these, the polyene amphotericin B forms pores in the ergosterol membrane, the azoles and the allylamines inhibit ergosterol synthesis at different stages, the pyrimidine analogue 5-flucytosine competes with pyrimide and the orotomide olorofim inhibits pyrimidine biosynthesis. The echinocandins inhibit 1,3-β-glucan synthesis (Figure 2). As can be seen in Table 3, the most common causative agent, *M. mycetomatis*, has low MICs for the azoles (median MIC50 0.03 µg/ml) and olorofim (MIC50 0.016 µg/ml), slightly higher MICs for amphotericin B (MIC50 0.5 µg/ml) and terbinafine (MIC50 8 µg/ml) and is not inhibited by 5-flucytosine and the echinocandins. The lowest MICs were obtained for ravuconazole, the drug currently clinically investigated. The sibling species *M. pseudomyctomatis* and *M. tropicana* have a similar susceptibility profile, while *M. fahalii* has higher MIC50s for itraconazole (MIC > 16 µg/ml) and fluconazole (MIC >256 µg/ml).

Of the mycetoma causative agents belonging to the order Pleosporales, low MICs are also found for amphotericin B and the azoles and higher concentrations for 5-flucytosine and caspofungin (Table 3). However, the MICs found for the azoles are in general three to four two-fold dilution steps higher than those found for *M. mycetomatis*. In contrast, although *Madurella* sp. are not inhibited at all by 5-flucytosine, growth inhibition was noted for the Pleosporales causative agents (Table 3).

**Combining antifungal agents**

As demonstrated in the previous paragraph, *M. mycetomatis* is inhibited by antifungal agents belonging to the polyenes, azoles, allylamines and orotomides. The next question was if enhanced growth inhibition occurs when itraconazole is combined with an antifungal agent belonging to a different class of antifungal agents. Therefore the combinations ketoconazole–terbinafine, itraconazole–terbinafine and itraconazole–olorofim have been tested. All combinations appeared to be indifferent according to the calculated Fractional inhibitory concentration index (FICI) and interaction ratio’s (IR) (Table 4). This indifference was confirmed in vivo in *Galleria mellonella* larvae. Treatment with a combination of itraconazole and terbinafine did not enhance larval survival compared with treatment with itraconazole or terbinafine alone. Combining amphotericin B with either itraconazole or terbinafine was antagonistic in the in vivo *G. mellonella* grain model.

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**Figure 2.** *M. mycetomatis* cell demonstrating the compounds with antifungal activity. The green antifungal agents are the agents that have been able to inhibit growth at the concentrations tested. These include amphotericin B, the azoles, terbinafine and olorofim. The compounds not able to inhibit growth are the echinocandins and 5-flucytosine.
Table 3. In vitro susceptibilities of eumycetoma causative agents

| Species            | MIC50 (µg/ml) (range, n) | References |
|--------------------|--------------------------|------------|
| Madurella          |                          |            |
| mycetomatis        | 0.5 (0.016–4; 34)        | 4,17,22,28 |
| AMB                | 0.06 (0.03–4; 38)        | 31,32,35,40,42 |
| KTZ                | 0.03 (0.03–4; 38)        | 31,32,35,40,42 |
| ITZ                | 0.03 (0.03–4; 38)        | 31,32,35,40,42 |
| PCZ                | 0.03 (0.125–32; 34)      | 31,32,35,40,42 |
| FLZ                | 0.03 (0.125–32; 34)      | 31,32,35,40,42 |
| V CZ               | 0.06 (0.125–32; 34)      | 31,32,35,40,42 |
| ISA                | 0.06 (0.06–2)            | 31,32,35,40,42 |
| RVZ                | 0.03 (0.06–2)            | 31,32,35,40,42 |
| TBF                | 8 (1–6; 34)              | 31,32,35,40,42 |
| 5-FC               | 8 (1–6; 34)              | 31,32,35,40,42 |
| CAS                | 8 (1–6; 34)              | 31,32,35,40,42 |
| ANI                | 8 (1–6; 34)              | 31,32,35,40,42 |
| MCF                | 8 (1–6; 34)              | 31,32,35,40,42 |
| OLO                | 8 (1–6; 34)              | 31,32,35,40,42 |
| Madurella          | 0.5 (0.03–4; 38)         | 36,43      |
| pseudomyce-        | 0.03 (0.03–4; 38)        | 36,43      |
| tomatis            | 0.03 (0.03–4; 38)        | 36,43      |
| Madurella          | 0.125 (0.03–1; 7)        | 36         |
| fahalii            | 0.03 (0.03–1; 7)         | 36         |
| Madurella          | 0.5 (0.125–2; 14)        | 36         |
| fahalii            | 1 (0.125–2; 14)          | 36         |
| Scedosporum        | 0.25 (0.03–4; 21)        | 4,44,45    |
| baydii             | 1 (ND; 30)               | 4,44,45    |
| Falciformispora     | 0.5 (0.125–0.25; 4)      | 4,19       |
| senegalensis       | 2 (0.06–0.25; 4)         | 4,19       |
| Falciformispora     | 2 (0.125–0.25; 4)        | 4,19       |
| tomphini            | 1 (0.25–0.25; 4)         | 4,19       |
| Trematosphaeria     | 0.125 (0.03–0.25; 11)    | 4,19,46    |
| graeza             | 8 (0.25–0.25; 11)        | 4,19,46    |
| Medicago           | 4 (0.125–2; 10)          | 4,19,46    |
| romeria            | 1 (0.125–2; 10)          | 4,19,46    |
| Nigrograna          | 0.25 (0.125–0.25; 2)     | 19         |
| macriniomni        | 0.25 (0.125–0.25; 2)     | 19         |
| Emorellia           | 0.5 (0.25–0.5; 5)        | 38         |
| griesea            | 0.25 (0.06–0.5; 5)       | 38         |
| Emorellia           | 1 (0.125–0.25; 5)        | 38         |
| paragriseae        | 1 (0.125–0.25; 5)        | 38         |
| Exophiala           | 0.25 (0.003–4; 51)       | 47         |
| dermatitidis       | 1 (0.003–4; 51)          | 47         |
| Exophiala           | 0.06 (0.125–0.25; 17)    | 4,44,45    |
| jeaneselmei        | 0.06 (0.125–0.25; 17)    | 4,44,45    |

Antifungal agents abbreviated as follows: amphotericin B (AMB), ketoconazole (KTZ), itraconazole (ITZ), posaconazole (PCZ), fluconazole (FLZ), voriconazole (VCZ), isavuconazole (ISA), ravuconazole (RVZ), terbinafine (TBF), 5-flucytosine (5FC), caspofungin (CAS), anidulafungin (ANI), micafungin (MCF), olorofim (OLO).

^{a}Therapeutic goal according to Andes et al. 49

^{b}Serum level modelled by Dalton et al. 50
Antiseptic solutions

The XTT-based antifungal susceptibility assay was also used to determine the in vitro activity of the antiseptic solutions. From the MICs of 1% povidone iodine, 0.5% taurolidine Ringer’s solution, 0.02% chlorhexidine and 1% hydrogen peroxide against 10 M. mycetomatis isolates, MICs ranged from 1:100 to 1:10 for 0.02% chlorhexidine and 1:200 to 1:20 for 1% hydrogen peroxide (H₂O₂). However, the killing time of the solutions was different. The 1% povidone iodine was able to kill M. mycetomatis within 5 min, while 2 h was needed when 0.02% chlorhexidine or 1% H₂O₂ was used. A killing time of >6 h was needed when 0.5% taurolidine Ringer’s solution was used. Since the killing time for antiseptic solutions is more important than the killing concentration, probably the most active antiseptic solution against M. mycetomatis is 1% povidone iodine.

Herbal compounds

In vitro susceptibility assays can also be used to determine the activity of medicinal plants against mycetoma causative agents. So far the activity of fractions or essential oils from the following plants have been determined: Acacia nubica, Boswellia papyrifera, Cinnamomum verum, Croton zambesiensis, Cumminium cymimum, Cymbopogon nervatus, Cymbopogon proximus, Cyperus rotundus, Eucalyptus camaldulensis, Eugenia caryophillus, Melaleuca alternifolia, Mentha spicata, Nigella sativa, Ocimum basilicum, Piper nigrum, Xylopia aethiopica and Zingiber officinalis (Table 5). In general, relatively low MICs were obtained for the essential oils (MIC50 ranging from 0.063 to 0.25% v/v). The crude methanol extracts of A. nubica (MIC50 1 µg/ml), N. sativa (MIC50 1 µg/ml) and B. papyrifera (MIC50 1 µg/ml) were more active than those from E. caryophillus (MIC50 25 µg/ml), C. verum (MIC50 25 µg/ml), P. nigrum (MIC50 25 µg/ml) and Z. officinalis (MIC50 12.5 µg/ml).

Drug screening

The in vitro susceptibility assay for M. mycetomatis has been used to screen 1200 drug-like compounds gathered in the Medicines for Malaria Venture (MMV) Pathogen, Stasis and Pandemic boxes in the open source drug discovery program MycoCOS (https://github.com/OpenSourceMycetoma/General-Start-Here). From the 1200 compounds screened, 287 inhibited the growth of M. mycetomatis at a concentration of 100 µM and 29 had a half maximal inhibitory concentration (IC50) <5 µM. These included the azoles ketoconazole, itraconazole, posaconazole, ravuconazole, isavuconazonium (isavuconazole), eberconazole, luliconazole, miconazole, bitertanol and difenoconazole; the benzimidazoles fenbendazole and carbendazim; and the strobilurins azoxystrbin and triflloxystrobin. Two of the potent hits obtained from these screenings were olofloxin and the fenarimol EPL-BS1246. For the fenarimols, 35 additional analogues were screened and an additional 4 potent fenarimols were identified: EPL-BS0178, EPL-BS0495, EPL-BS0800 and EPL-BS1025. The hits obtained in the screenings of the MMV boxes are currently further evaluated as potential new drugs for mycetoma.

Correlation of in vitro susceptibility data with in vivo efficacy in animal models

To assess the therapeutic efficacy in experimental animal models, only two mouse studies52,54 and three invertebrate studies34,31,33 have been performed. All five studies investigated the therapeutic efficacy of antifungal agents against M. mycetomatis. This has not been assessed for any of the other causative agents. In each of these studies, only a single M. mycetomatis isolate was investigated. This makes it impossible to correlate the MIC of a drug with the therapeutic efficacy in an animal model.

In the two M. mycetomatis intraperitoneal murine grain models developed by Murray et al.,56 and Ahmed et al.,59 the therapeutic efficacy of diamidinodiphenylamine, aphthorin B and itraconazole52 were determined. With the CLSI-based in vitro susceptibility assay, the MIC50s for amphotericin B and itraconazole were 0.5 µg/ml and 0.06 µg/ml, respectively (Table 3). Murray and Colichon54 demonstrated that treatment of 15 weeks with 200 µg/day diamidinodiphenylamine did not result in a reduced number of grains compared with the non-treated control. Ahmed et al.,57 demonstrated that in mice treated with 20 mg/kg itraconazole twice daily, grains were still present in five of six mice at day 21. In mice treated with 0.5 mg/kg amphotericin twice daily, no grains were detected in the treated mice. The therapeutic superiority was confirmed in an invertebrate G. mellonella grain model.58 When G. mellonella larvae were infected with 4 mg/larvae and treated with 5.7 mg/kg of either ketoconazole, itraconazole, voriconazole or posaconazole, no enhanced activity was observed. In addition, Ahmed et al.,57 demonstrated that the MICs for the drugs were not altered when the drugs were used in combination. This made it impossible to correlate the MIC of a drug with the therapeutic efficacy in an animal model.

Table 4. Combinations of antifungal agents tested for M. mycetomatis

| Combination | Number of strains tested | FICI rangea | IR rangeb | Reference |
|-------------|--------------------------|-------------|-----------|-----------|
| KTZ/TBF     | 8                        | 0.30–2.40 (I) 0.91–1.02 (I) | 37        |          |
| ITZ/TBF     | 8                        | 0.82–2.45 (I) 0.93–1.00 (I) | 37        |          |
| ITZ/OLO     | 1                        | 3.2 (I)     0.91 (I)      | 35        |          |

aFractional inhibitory concentration index (FICI) was calculated with the following formula: \[ \text{FICI} = \text{MIC}_A + \text{MIC}_B = \frac{\text{MIC}_{\text{combi}}}{\text{MIC}_A} + \frac{\text{MIC}_{\text{combi}}}{\text{MIC}_B} \], where \( \text{MIC}_{\text{combi}} \) and \( \text{MIC}_{\text{combi}} \) are the inhibitory concentrations of the drugs A and B in the combinations and \( \text{MIC}_A \) and \( \text{MIC}_B \) are the inhibitory concentrations of drugs A and B when acting individually. A FICI ≤0.5 is considered synergistic (S), a FICI >0.5 ≤4 is indifferent (I) and a FICI >4 is antagonistic (A).

bThe interaction ratio (IR) was calculated with the following formula: \[ \text{IR} = \frac{i}{o} \], where \( i \) is considered the observed percentage of inhibition and \( o \) is the expected percentage of inhibition for a certain combination. \( i \) is calculated as follows: \( i = A + B - (AB) \), where A and B are the inhibition observed for compounds A and B alone. An IR >1.5 is considered synergistic (S), an IR between 0.5 and 1.5 is indifferent (I) and an IR <0.5 is antagonistic (A).
larval survival was noted compared with the phosphate-buffered saline–treated control. Grains were still present after 10 days.55 When larvae were treated with 1 mg/kg amphotericin B or 7.14 mg/kg terbinafine or 14 mg/kg posaconazole, enhanced survival was noted.34,55 This indicated that, at least for posaconazole, a concentration-dependent effect was noted. Combining amphotericin B with either itraconazole or terbinafine resulted in antagonism.51

Table 5. In vitro activity of essential oil and plant extracts against M. mycetomatis

| Plant                  | Part of plant | Number of strains tested | MIC50     | References |
|------------------------|---------------|--------------------------|-----------|------------|
| Acacia nubica          | Crude methanol extract | 13                     | 1 µg/ml   | 30         |
| Boswellia papyrifera   | Crude methanol extract | 13                     | 1 µg/ml   | 30         |
| Boswellia papyrifera   | Essential oil  | 10                      | 0.063% v/v| 52         |
| Cinnamomum verum       | Crude methanol extract | 13                     | 25 µg/ml  | 30         |
| Croton zambesicus      | Essential oil  | 10                      | 0.063% v/v| 52         |
| Curmin cyminum         | Essential oil  | 10                      | 0.125% v/v| 52         |
| Cymbopogon nervus      | Essential oil  | 10                      | 0.063% v/v| 52         |
| Cymbopogon proximus    | Essential oil  | 10                      | 0.063% v/v| 52         |
| Cyperus rotundus       | Essential oil  | 10                      | 0.125% v/v| 52         |
| Eucalyptus camaldulensis| Essential oil  | 10                      | 0.125% v/v| 52         |
| Eugenia caryohiulus    | Crude methanol extract | 13                     | 50 µg/ml  | 30         |
| Melaleuca alternifolia | Essential oil  | 34                      | 0.063% v/v| 33         |
| Mentha spicata         | Essential oil  | 10                      | 0.063% v/v| 52         |
| Nigella sativa         | Crude methanol extract | 13                     | 1 µg/ml   | 30         |
| Ocimum basilicum       | Essential oil  | 10                      | 0.25% v/v | 52         |
| Piper nigrum           | Crude methanol extract | 13                     | 25 µg/ml  | 30         |
| Xylopia aethiopica     | Essential oil  | 10                      | 0.063% v/v| 52         |
| Zingiber officinalis   | Crude methanol extract | 13                     | 12.5 µg/ml| 30         |

indicated that the grain was difficult to penetrate by antifungal agents.

Characteristic of the M. mycetomatis grain is the presence of melanin and an extracellular matrix (cement material) that surrounds the hyphae in the grain. M. mycetomatis 1,8-dihydroxynaphthalene–melanin did interfere with susceptibility to antifungal agents.59 A four to five twofold dilution step increase in MIC was obtained when 250 µg/ml M. mycetomatis melanin was added in the Sensititre YeastOne in vitro susceptibility assay. This increase in MIC was only observed for ketoconazole and itraconazole. This was not found for voriconazole, fluconazole or amphotericin B.59 For ketoconazole and itraconazole, this increase in MIC was likely due to binding of the drug to melanin. This binding most likely prevented the drug from reaching its intracellular target.59 Along with melanin, the extracellular matrix can protect against antifungal agents. This matrix consists of proteins, chitin and polysaccharides,60–63 components also found in fungal biofilms. Functionally the extracellular matrix can serve as a protective barrier against chemical and biological antimicrobial agents.64 Some of the compounds inside the fungal extracellular matrix, such as β-1,3-D-glucan and extracellular DNA, can physically bind antifungal agents and thereby prevent the drugs from reaching the intended target at the surface or within the fungal cell.64 Along with the physical barrier, the upregulation of efflux pumps and the presence of metabolically dormant cells have been implicated in the increased resistance.64 Biofilms have been studied for the rare mycetoma causative agent Exophiala dermatitidis in vitro. For E. dermatitidis it was demonstrated that despite the use of susceptible planktonic cells in generating biofilms, the biofilm itself was completely resistant to itraconazole, voriconazole and posaconazole.65,66 The only
antifungal agent tested that did have some activity against the *E. dermatitidis* biofilm was amphotericin B. A 50% reduction in fungal metabolic activity was obtained when *E. dermatitidis* was incubated with 1–2 µg/ml amphotericin B, a concentration similar to the MIC obtained with planktonic cells.65

Besides looking at correlations between *in vitro* activity of standard antifungal agents and *in vivo* efficacy, the *in vitro* susceptibility assays were also used to discover novel drugs for mycetoma. For this, 1200 compounds were screened and 14 were tested in the *G. mellonella* grain model for therapeutic efficacy at a fixed concentration of 20 µM/larvae. The compounds evaluated *in vivo* were bitertanol, difenconazole, azoxytrobin, trifloxystrobin, MMV006357, MMV675968, MMV687807, MMV022478, MMV689244 (EPL-BS1246), EPL-BS0178, EPL-BS0495, EPL-BS0800 and EPL-BS1025. From these compounds, only bitertanol, MMV006357, MMV675968, MMV022478, EPL-BS0178, EPL-BS0495 and EPL-BS1025 significantly enhanced larval survival.34 As can be seen in Figure 3, no correlation between either IC50 or MIC50 and larval survival was found. This indicated that the currently used *in vitro* susceptibility assay can determine which compounds are able to inhibit fungal growth but the inhibiting concentration is not predictive for *in vivo* activity using fixed concentrations. For the fenarimol compounds, the logD value at pH 7.4 was more indicative than the IC50 or MIC50.34

**Correlation with therapeutic data in humans**

Although *M. mycetomatis* is by far the most common causative agent for mycetoma and has more strains tested than the other species, no epidemiologic cut-off values or clinical breakpoints have been set for this or any of the other common causative agents, neither by the CLSI or the EUCAST. Therefore there is currently no correlation between the MIC or clinical failure or success. The CLSI developed clinical breakpoints only for *Candida*, while the EUCAST defined different method-based clinical breakpoints for both *Candida* and *Aspergillus*.7 For itraconazole, clinical breakpoints as determined by the EUCAST are 0.06 µg/ml for *Candida albicans* and *Candida dubliniensis*, 0.125 µg/ml for *Candida parapsilosis* and *Candida tropicalis* and 1 µg/ml for *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus nidulans* and *Aspergillus terreus*.67 For posaconazole, the clinical breakpoints are 0.06 µg/ml for *C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *C. tropicalis* and 0.125 µg/ml for *A. fumigatus* and *A. terreus*.67 The MIC50s obtained for most causative agents of mycetoma are below the clinical breakpoints for the filamentous aspergilli.

Another parameter that might offer some prediction is to assess if the MICs found are above or below the serum levels obtained for each antifungal agent. For most of the antifungal agents, attainable serum levels have been determined (Table 3).49 For itraconazole, the therapeutic goal for invasive fungal infections is to reach a blood trough level >1–2 µg/ml itraconazole.69 This is a concentration above the MIC50 for *M. mycetomatis*, *M. pseudomycetomatis*, *M. tropicana*, *F. senegalensis*, *F. tomkinsii*, *T. grisea*, *N. mackinnonii*, *E. grisea*, *E. paragrisea* and *E. jeanselmi*, but not for *M. fahalii* and *M. romerai* (Table 3). *M. fahalii* and *M. romerai* both have an MIC50 for posaconazole that is attainable in serum (Table 3). For terbinafine, a serum level of 2.8–3 µg/ml was modelled at the dosages used to treat mycetoma. This concentration is lower than the MICs determined for *M. mycetomatis* (Table 1).

Currently there are no linked data between MICs obtained for *M. mycetomatis* and clinical outcome. Furthermore, since surgery is always a component of mycetoma treatment, direct comparison between MICs and outcomes might be complicated. However, there are a few studies in which different series of patients were treated with either ketoconazole,68,69 itraconazole,6,70 or terbinafine.6,71 Of the 571 black-grain patients with...
a complete data set and treated with 200–400 mg ketoconazole twice daily and surgery, only 321 (56.2%) were cured and 35 (6.1%) underwent an amputation. Of the 23 black-grain mycetoma patients treated with a combination of itraconazole with the antibiotics trimethoprim–sulfamethoxazole and surgery, all were cured (100% cure rate). Of the 22 black-grain mycetoma patients treated with terbinafine for which data were available until the end of treatment, 20 (90.9%) patients were cured and 2 (9.1%) had a recurrence, indicating that despite an MIC above the attainable serum level, cure was possible. A comprehensive clinical trial is needed to determine if the MIC is indicative of clinical success or failure.

Way forward
As reviewed here, in vitro susceptibility assays have been developed for the most common causative agents of black-grain eumycetoma. These assays have been used to establish if causative agents are inhibited in growth by various antifungal agents but not for clinical decision making. For drug discovery, the assay proved useful in determining which compounds were able to prevent hyphal growth and had a direct effect on the fungal cell. A clear correlation between in vitro inhibition in terms of IC50 or MIC50 and therapeutic efficacy in terms of G. mellonella larval survival was not found. This indicates that for drug discovery, other in vitro models are needed to predict the in vivo efficacy of a drug. These models should mimic certain properties of the mycetoma grain. As already seen, adding M. mycetomatis melanin to culture medium influenced the MICs obtained for ketoconazole and itraconazole and the formation of biofilms in E. dermatitidis also influenced the MICs for the azoles. Therefore, models mimicking various features of the mycetoma grain might overcome these shortcomings.

Before the in vitro susceptibility assays can be used for clinical decision making, they need to be assessed to determine if a correlation between MIC and clinical outcome exists. For that, large data sets are needed. In the current ongoing proof-of-concept superiority trial of fosravuconazole versus itraconazole in eumycetoma caused by M. mycetomatis in Sudan (Clinical-Trials.gov identifier: NCT03086226), in vitro susceptibility will be linked to clinical outcome and epidemiological cut-off values and break points will be determined for the early lesions included in the trial. However, more extensive data are needed to verify if these break points might also be valid in extensive lesions or when other body sites are infected. Furthermore, the current in vitro susceptibility tests use sonication to prepare a hyphal inoculum and viability dyes to assess fungal growth. These might be too labour intensive and expensive in clinical settings. When a clinical correlation is established, the next step would be to assess in which ways in vitro susceptibility assays could be made more user friendly for clinical use.

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