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The performance and costs of XTT, resazurin, MTS and luciferin as viability dyes in *in vitro* susceptibility testing of *Madurella mycetomatis*

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Background: *in vitro* susceptibility testing for the non-sporulating fungus *Madurella mycetomatis* is performed with a hyphal suspension as starting inoculum and a viability dye for endpoint reading. Here we compared the performance of four different viability dyes for their use in *in vitro* susceptibility testing of *M. mycetomatis*.

Methods: To compare the reproducibility and the agreement between the viability dyes 2,3-bis-(2-methoxy-4-nitro-5-sulfphenyl)-2H-tetrazolium-5-carboxanilide salt (XTT), resazurin, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) and luciferin, the *in vitro* susceptibilities of 14 genetically diverse *M. mycetomatis* isolates were determined for itraconazole and amphotericin B. The reproducibility, agreement, price and ease of use were compared.

Results: Each of the four dyes gave highly reproducible results with >85.7% reproducibility. Percentage agreement ranged between 78.9% and 92.9%. Resazurin was the most economical to use (0.0009 €/minimal inhibitory concentration [MIC]) and could be followed in real time. Luciferin omitted the need to transfer the supernatant to a new 96-well plate, but cost 6.07 €/MIC.

Conclusion: All four viability dyes were suitable to determine the *in vitro* susceptibility of *M. mycetomatis* against itraconazole and amphotericin B. Based on the high reproducibility, high percentage agreement, price and possibility to monitor in real time, resazurin was the most suited for routine *in vitro* susceptibility testing in the diagnostic laboratory in mycetoma-endemic countries. Because luminescence could be measured directly without the need to transfer the supernatant to a new 96-well plate, luciferin is suitable for drug-screening campaigns.

Lay summary: To determine the *in vitro* susceptibility testing in the non-sporulating fungus *Madurella mycetomatis*, a viability dye is needed for endpoint reading. In this study we tested the viability dyes XTT, resazurin, MTS and luciferin for their use in *in vitro* susceptibility testing. It appeared that they all could be used but there were differences in time to result and costs associated with them.

Keywords: amphotericin B, *in vitro* susceptibility, itraconazole, luciferin, *Madurella mycetomatis*, MIC, MTS, mycetoma, resazurin, XTT

Introduction

Eumycetoma is a neglected tropical disease characterized by large subcutaneous swellings. It can be caused by >40 different fungi, but the fungus *Madurella mycetomatis* is by far the most common causative agent.[1] Currently, eumycetoma is treated with a combination of antifungal therapy and surgery.[2] Most often, the antifungal agent itraconazole is used. The outcome of this treatment is often disappointing and ranges from complete cure (8%) to partial response (69%), stable disease (23%) and massive recurrence (8%).[2]

Whether the initial susceptibility of the causative agent plays a role in treatment response cannot be predicted because *in vitro* susceptibility testing for *M. mycetomatis* is not routinely performed.[3,4] In the past, an *in vitro* susceptibility assay for *M. mycetomatis* was developed based on the M38 reference method for broth dilution antifungal susceptibility testing of filamentous fungi from the clinical and laboratory standards institute (CLSI).[3] This method differed only in the initial inoculation preparation. Because *M. mycetomatis* in general does not sporulate, a sonication step was introduced to generate a standardized hyphal inoculum.[3,4] Because this inoculum

was already turbid, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) or resazurin was used to assess the viability of the fungus after incubation.[3,5–7] Although these molecules are different, they are both reduced by NADH in viable cells. In the case of XTT, a water-soluble orange formazan product is produced, which can be measured spectrophotometrically.[3] In the case of resazurin, the deep blue-colored resazurin is transferred into pink-colored resorufin, and this change can be measured spectrophotometrically.[7] The downside of these two assays is that the supernatant needs to be transferred to another plate to measure the color because the hyphae inside the original culture plate will interfere with the spectrometric measurements. Furthermore, for XTT, a 5-h incubation is needed before the color reaction can be measured.[3]

Next to XTT and resazurin, different viability dyes can be used to measure the viability of other eukaryotic cells. Two dyes often used are the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), which is the dye present in the CellTiter 96 AQueous One Solution Cell Proliferation Assay and luciferin. MTS, like XTT and resazurin, is reduced by NADH in viable cells into a colored product. However, the time needed for this conversion is usually shorter.[8] It is not yet known if this viability dye could also be used to assess the viability of a filamentous fungus.

A viability dye not based on the reduction of NADH is luciferin present in the CellTiter-Glo 3D Cell Viability Assay. In this assay the enzyme luciferase will cleave luciferin in the presence of ATP to produce oxyluciferin. In this reaction, energy in the form of luminescence is released.[8] The luminescence can be measured in the reaction mixture inside the well, so there is no need to remove the supernatant to new plates to assess the viability.[8] This means that it is no longer necessary to transfer the supernatant to a clean 96-well plate before measuring. Furthermore, within 25 min of incubation at room temperature, the luminescence can be measured.[8] Here, we determined the percentage agreement between the four different viability dyes in *in vitro* susceptibility assays performed for *M. mycetomatis*. We also determined if all four dyes were equal in terms of reproducibility and cost.

Materials and Methods

Madurella mycetomatis strains

In total, 14 clinical isolates of *M. mycetomatis* were included in this study. Of these 14 isolates, seven isolates (mm13, mm14, mm30, mm41, mm49, mm54 and mm55) originated from the Mycetoma Research Centre in Khartoum, Sudan. Two isolates originated from India (CBS 132419 and CBS 132589), one from Mali (p1), one from Algeria (AL1), one from Somalia (SO1), one from Peru (Peru 72012) and one for which the country of origin was unknown (CBS 247.48). All isolates were identified to the species level by sequencing the internally transcribed spacer region and proved to be genetically different by MmySTR typing.[9] They were maintained in the laboratory on Sabouraud Dextrose agar plates (Tritium Microbiologie BV, Eindhoven, The Netherlands).

in vitro susceptibility assay

Minimal inhibitory concentrations (MICs) were tested independently in triplicate using our previously published protocol.[3,5] In short, *M. mycetomatis* was inoculated in colorless RPMI 1640 (cat. no. 11564456; Thermo Fisher scientific, Breda, The Netherlands) working medium containing 0.35 g/L L-glutamine (cat. no 25030024; Capricorn-scientific, Ebsdorfergrund, Germany) and 1.98 mM 4-morpholinepropane sulfonic acid (cat. no. M1254, MOPS; Sigma Aldrich, Zwijndrecht, The Netherlands). Then the mixture was sonicated for 10 s at an amplitude of 10 µm (Soniprep, Beun de Ronde, Abcoude, The Netherlands) to obtain hyphal fragments. The resulting suspension was cultured for 7 d at 37°C in RPMI 1640 working medium containing 0.35 g/L L-glutamine and 1.98 mM 4-morpholinepropane sulfonic acid. The mycelia were harvested by 5 min of centrifugation at 3400 rpm and again sonicated for 10 s at 10 µm. The resulting hyphae were further diluted to obtain a working inoculum consisting of a hyphal suspension of 68%–72% transmission at 660 nm (Novaspec II; Pharmacia Biotech, Sweden) in RPMI. When XTT, MTS or resazurin were used as viability dyes, we transferred 100 µl of this inoculum into each well of a round-bottom 96-well plate (cat. no. 3799; Thermo Fisher Scientific, Breda, The Netherlands). When luciferin was used as a viability dye, 100 µl of inoculum was pipetted into white flat-bottom 96-well plates (cat no. 655180; Greiner Bio-One, Alphen aan de Rijn, The Netherlands). A 100-times concentrated stock solution was made in DMSO as per previously developed guidelines.[3] In each well, 1 µl of the diluted compounds were added to reach final drug concentrations of 0.03 to 16 µg/ml for itraconazole (Janssen pharmaceutica, Beerse, Belgium) and amphotericin B (Bristol-Myers Squib, Princeton, USA). The assay was incubated for 7 d at 37°C before the viability of the fungi in each well was assessed.

The use of the four different viability dyes

For endpoint visualization, four different viability dyes were used, namely, XTT (cat no. ab146310; Abcam, Amsterdam, the Netherlands), MTS from the CellTiter 96 AQueous One Solution Cell Proliferation Assay (cat. no. G3581; Promega, Leiden, The Netherlands), resazurin (cat no. R7017; Sigma Aldrich, Zwijndrecht, The Netherlands) and luciferin from the CellTiter-Glo 3D Cell Viability Assay (cat no. G9683; Promega, Leiden, The Netherlands). XTT, MTS and luciferin were added after 7 d of incubation. Resazurin was added at the start of the incubation. For XXT, MTS and resazurin, the same spectrophometer (Epoch 2, BioTek, Santa Clara, USA) was used.

XTT: 100 µl of XTT working solution, consisting of 250 µg of XTT/ml and 58 µM of menadione (cat no. M5750; Sigma Aldrich, Zwijndrecht, The Netherlands), was added to every well. The wells were incubated with XTT for 2 h at 37°C and for another 3 h at room temperature. Thereafter, 100 µl of supernatant was transferred to a flat-bottom 96-well plate and measured spectrophotometrically at 450 nm.

MTS: 10 µl of MTS ready-to-use reagent was added into incubation plates. The plates were incubated for 2 h at 37°C, after which 100 µl of supernatant was moved into a flat-bottom plate and measured at 490 nm.

Resazurin: 20 µl of a resazurin working solution (0.15mg/ml) was added to the wells before incubation. At that time, a deep

blue color was noted in all wells. Then the plate was incubated for 7 d at 37°C. After incubation, 100 µl of supernatant was moved into a flat-bottom 96-well plate and measured at 600 nm.

Luciferin: 100 µl of ready-to-use reagent from CellTiter-Glo 3D Cell Viability Assay was added to the white incubation plates. The plates were incubated for 25 min at room temperature. Luminescence was measured with a fluorescence plate reader (CytoFluor Series 4000, PerSeptive Biosystems, Framingham, USA) directly. There was no need to transfer the supernatant to another plate.

The metabolic activity compared with the growth control (100% metabolic activity) and the negative control consisting of culture media only (0% metabolic activity) was determined using the following formulas[4]:

XTT:

$$\textit{Percentage metabolic activity} = \left(\frac{\textit{Absorbance test}_{450nm} - \textit{Absorbance NC}_{450nm}}{\textit{Absorbance GC}_{450nm} - \textit{Absorbance NC}_{450nm}}\right) * 100$$

MTS:

$$\textit{Percentage metabolic activity} = \left(\frac{\textit{Absorbance test}_{490nm} - \textit{Absorbance NC}_{490nm}}{\textit{Absorbance GC}_{490nm} - \textit{Absorbance NC}_{490nm}}\right) * 100$$

Resazurin:

$$\textit{Percentage metabolic activity} = \left(\frac{\textit{Absorbance NC}_{600nm} - \textit{Absorbance test}_{600nm}}{\textit{Absorbance NC}_{600nm} - \textit{Absorbance GC}_{600nm}}\right) * 100$$

Luciferin:

$$\textit{Percentage metabolic activity} = \left(\frac{\textit{Luminescence test} - \textit{Luminescence NC}}{\textit{Luminescence GC} - \textit{Luminescence NC}}\right) * 100$$

In these formulas, the formation of the end product is determined for XTT, MTS and luciferin, while for resazurin the reduction of resazurin into resorufin is measured at the wavelength of resazurin. Therefore, in the formula for resazurin, we calculate the amount of resazurin that has been used during *M. mycetomatis* growth, instead of the production of resorufin. The first concentration that resulted in a reduction of the metabolic activity of >80% was considered as the MIC.

Calculation percentage of agreement and reproducibility for four viability dyes

To evaluate the reproducibility and agreement of the four different viability dyes, the percentage between replicates was determined in both one dilution step and two dilution steps, according to the former calculations performed elsewhere.[5,10]

Costs of the viability assay

To calculate the costs of each viability dye per *in vitro* susceptibility assay, the price of each dye (as indicated in March 2023) was obtained. In this calculation we only included the costs of the dye and the plates, not the costs of the culture media, as they were the same. Also, the cost of the labor was not included, as the salaries differ per country. However, the reduction in time is measured and mentioned in the text. XTT was obtained from Abcam and cost €470 per 250 mg.[11] Menadione originated from Sigma and cost €42.20 per 25 g.[12] Resazurin was obtained from Sigma and cost €126 for 5 g.[13] The CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) and CellTiter-Glo 3D Cell Viability Assay were obtained from Promega and cost €972 and €506 per 100 ml, respectively.[14,15] Next, the amount of dye per well was calculated. When XTT, MTS and resazurin are used, the fungus is first cultured in Corning 96-well Clear Round Bottom plates (cat. no. 3799; Sigma-Aldrich, Zwijndrecht, The Netherlands), which cost €172.19 per case of 50 plates.[16] The supernatant is then transferred to flat bottom plates (cat. no. 655180; Greiner, Alphen aan den Rijn, The Netherlands) costing €70 per case of 100 plates.[17] When luciferin is used as the viability dye, the fungus is cultured in white 96-well plates (cat. no. 3917; Sigma-Aldrich, Zwijndrecht, The Netherlands) costing €544.81 per case of 100 plates.[18] When using XTT as the viability dye, in a single well, 25 µg of XTT and 1.6 µg of menadione are present, which cost €0.043 per well and €0.516 for a single MIC test consisting of 12 wells. When using MTS, 10 µl of CellTiter 96 AQueous One Solution Cell Proliferation Assay is present in each well. This costs €0.097 per well and €1.16 for a single MIC test. When using resazurin, 3 µg of resazurin is present in each well, costing €0.0000756 per well and €0.0009072 per MIC. When using luciferin, 100 µl of CellTiter-Glo 3D Cell Viability Assay is added to a single well, costing €0.506 per well and €6.072/MIC (Table 1). Furthermore, for XTT, MTS and resazurin, the fungus is cultured in round-bottom plates and the supernatant is transferred to new flat-bottom plates. The cost for a single MIC test is €0.52. For luciferin, white plates need to be used to measure the luminescence. These plates cost €0.68 for a single MIC test.

Results and Discussion

In this study, *in vitro* susceptibility assays were performed against itraconazole and amphotericin B for 14 genetically diverse *M. mycetomatis* isolates. The MICs were determined by different viability dyes, and the concentration in which a reduction of >80% in metabolic activity was noted was considered the MIC.[3] This differs from the criteria set by both the CLSI and EUCAST, where growth inhibition is used to measure the MIC. For *M. mycetomatis*, measuring growth inhibition visually is difficult as the fungal inoculum for *in vitro* susceptibility testing is prepared differently.[3] Under normal culture conditions, *M. mycetomatis* does not sporulate, which makes it impossible to use conidia or spores as a starting inoculum.[4] Instead, a hyphal inoculum is prepared by sonication.[4] Unfortunately, this inoculum is already turbid from the start, which complicates visual endpoint reading.[4] Previously, it was noted that the percentage agreement between visual endpoint reading of the modified CLSI method

Table 1. Reproducibility in *in vitro* susceptibility testing for itraconazole and amphotericin B against 14 *Madurella mycetomatis* strains comparing XTT, MTS, resazurin and luciferin

	Itraconazole				Amphotericin B			
Strain	**MIC (μg/ml) with XTT**	**MIC (μg/ml) with MTS**	**MIC (μg/ml) with resazurin**	**MIC (μg/ml) with luciferin**	**MIC (μg/ml) with XTT**	**MIC (μg/ml) with MTS**	**MIC (μg/ml) with resazurin**	**MIC (μg/ml) with luciferin**
mm13	0.06	0.125	0.125	0.125	0.5	0.5	0.25	0.5
mm14	0.06	0.06	0.06	0.125	0.5	0.5	1	0.25
mm30	0.06	0.06	0.06	0.125	0.06	0.25	0.25	0.06
mm41	0.125	0.125	0.06	0.25	0.5	1	0.5	0.5
mm49	0.5	0.06	0.125	0.03	0.5	0.25	0.25	0.5
mm54	0.06	0.03	0.03	0.03	0.5	0.5	0.25	0.5
mm55	0.125	0.125	0.125	0.25	0.5	0.5	0.25	0.5
CBS 132419	0.03	0.125	0.03	0.03	4	2	1	2
CBS 132589	0.25	0.25	0.5	0.25	1	1	1	1
p1	0.03	0.03	0.125	0.06	0.125	0.5	0.25	0.5
PARIJS 15580 AL1	0.03	0.03	0.06	0.03	0.5	0.25	0.5	0.5
Peru 72012	0.03	0.03	0.03	0.03	0.25	0.25	0.5	0.25
SO1 (LUMC1)	0.06	0.06	0.06	0.125	4	2	2	1
CBS 247.48	0.125	0.5	0.25	0.125	2	0.5	2	1
MIC50 (μg/ml)	0.06(0.03 to 0.5)	0.06(0.03 to 0.5)	0.06(0.03 to 0.5)	0.125(0.03 to 0.25)	0.5(0.06 to 4)	0.5(0.25 to 2)	0.5(0.25 to 2)	0.5(0.06 to 2)
Reproducibility within one-step dilution difference (%)	100	100	92.9	85.7	92.9	100	85.7	92.9
Reproducibility within two-step dilution difference (%)	100	100	100	100	100	100	100	100
Price of the viability dye per *in vitro* susceptibility assay	€0.564/MIC	€1.164/MIC	€0.0009/MIC	€6.072/MIC				

The MICs were obtained for 14 *M. mycetomatis* isolates against itraconazole and amphotericin B in this study. To compare the MICs, the percentage of reproducibility was calculated within one-step and two-step dilution.

Table 2. Percentage agreement for MICs obtained with XTT, MTS, resazurin and luciferin for 14 *M. mycetomatis* isolates against itraconazole and amphotericin B

	Itraconazole		Amphotericin B	
	One-dilution step difference (%)	Two-dilution step difference (%)	One-dilution step difference (%)	Two-dilution step difference (%)
XTT vs MTS	78.6	92.9	78.6	100
XTT vs resazurin	85.7	100	78.6	100
XTT vs luciferin	92.9	92.9	85.7	100
MTS vs resazurin	85.7	100	92.9	100
MTS vs luciferin	85.7	100	92.9	100
Resazurin vs luciferin	92.9	100	85.7	100

To compare the MICs, the percentage of agreement was calculated within one or two dilution steps, comparing with any two dyes in this study.

and the XTT method was 91.2% for itraconazole and 94.2% for amphotericin B.[5] This showed that, for *M. mycetomatis*, metabolic activity could be a replacement for visual endpoint reading. Furthermore, for both *Candida* spp. and *Aspergillus* spp., a direct relationship between colorimetric signal and the cell number of fungal biomass was obtained.[5,19,20]

The results in Table 1 show that, for itraconazole, an MIC50 of 0.06 µg/ml was obtained when XTT, resazurin and MTS were used as the viability dye. An MIC50 of 0.125 µg/ml for itraconazole was found when luciferin was used. These values were identical or within one dilution difference as the MIC50 values reported in previous studies, namely, 0.064 µg/ml when XTT was used and 0.125 µg/ml when resazurin was used as the viability dye.[3,5,21,22] For amphotericin B, an MIC50 of 0.5 µg/ml was obtained, regardless of the viability dye used. This was also comparable with the MIC50 values of 0.5 and 1 µg/ml reported previously using XTT.[23] Above all, MIC values in this study showed high comparability.

Of the four viability dyes tested, the highest reproducibility was obtained when the metabolic activity was determined with the formazan salts XTT and MTS. As shown in Table 2, for these two dyes, 100% agreement was obtained. For resazurin and luciferin, the reproducibility was lower, 92.9% and 85.7%, respectively. The high reproducibility obtained with each of the four dyes in this study indicated that all four dyes are suitable to be used to assess the MIC during *in vitro* susceptibility testing of *M. mycetomatis*.

To assess if the viability dyes gave comparable results with each other, the percentage agreement was calculated. As can be seen from Table 2, when the *in vitro* susceptibility was determined against itraconazole, the highest agreement was observed between luciferin and XTT (92.9% in agreement within one dilution difference) and luciferin and resazurin (92.9% agreement), and the lowest agreement was observed between XTT and MTS (78.6% agreement). When the *in vitro* susceptibility was determined against amphotericin B, the highest agreement was observed between MTS and resazurin (92.9% agreement within one dilution difference) and MTS and luciferin (92.9% agreement), and the lowest agreement was observed between XTT vs MTS (78.6% agreement) and MTS vs resazurin (78.6% agreement). The high agreement observed here between XTT and resazurin was in agreement with previous studies with *M. mycetomatis*,[5] but also for other fungal species, such as *Cryptococcus neorformans*.[24]

Because for all four dyes a high reproducibility and a high percentage of agreement were obtained, we wondered if there was a difference in ease of use and costs. Therefore, the price of each viability dye for a single MIC was determined. The most economical dye was resazurin, for which the costs of the viability dye were only €0.0009 per MIC (Table 1). This was followed by MTS (€0.516/MIC), XTT (€1.16/MIC) and luciferin (€6.072/MIC). In these costs, only the cost of the viability dye itself was included, not the costs of the different plates (€0.52 when using XTT, MTS and resazurin, or €0.68 when using luciferin), nor the costs of labor. The latter was omitted as the cost heavily depends on the country and the job description of the person performing the assay. However, there were differences in the time needed to perform the assay and these will be reflected in the costs as the number of man-hours will differ.

The time for each viability dye to develop ranged from 0 additional minutes (resazurin) to 5 additional hours (XTT). This indicated that although XTT is currently the gold standard as a viability dye for assessing the metabolic activity for many fungal species, including *M. mycetomatis*[3,5,22,23] and *A. fumigatus*,[25] it is not the most economical or rapid test. Resazurin seems to be more economical and more rapid. Furthermore, the growth can be observed in real time, which could reduce the incubation time.[7] Because of its high reproducibility, higher percentage agreement and its availability in endemic settings, it is currently recommended for use in *in vitro* susceptibility testing to guide therapeutic management.[7,21]

Although luciferin was the most expensive viability dye assessed, it had one benefit over the other dyes. To measure the luminescence it is not necessary to transfer the supernatant to a clean 96-well plate. It can be directly measured when the fungus is still present in the well in a luminometer. Furthermore, only 25 min was needed to assess the metabolic activity. Because of the high price and the requirement for a luminometer for endpoint reading, it is less feasible to be used as a viability dye in routine *in vitro* susceptibility assays for treatment guidance in mycetoma-endemic countries.[26] However, it might reduce the workload when screening large compound libraries for drug discovery

projects. For *M. mycetomatis*, currently, an Open Source Drug discovery project is ongoing, in which thousands of compounds are being screened for activity against *M. mycetomatis*.[27] Some of these libraries are screened in 384-well plates. To reduce the need to transfer the supernatant before measurement would be beneficial, and luciferin could have a place in these large drug-screening campaigns.

In conclusion, we tested four different viability dyes for their performance in endpoint reading in *M. mycetomatis in vitro* susceptibility assays. All four dyes seemed to be very reproducible and had high agreement in MIC performance. However, in terms of ease and price, resazurin seemed to be the cheapest and most time efficient and the most suitable for use in *in vitro* susceptibility testing in diagnostic laboratories in mycetoma-endemic countries. Luciferin, on the other hand, was the most expensive but also the most amendable for the screening of large compound libraries in drug discovery projects, as it omitted the need to transfer the supernatant before endpoint reading.

Authors' contributions: WvdS designed the study. JM and KE performed experiments. AF collected strains. JM calculated the data and wrote the draft of this manuscript. AV and WvdS supervised the research and revised the manuscript. All authors modified the draft and approved the final manuscript.

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