Development and validation of a resazurin assay for in vitro susceptibility testing of Actinomadura madurae: a common causative agent of actinomycetoma

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Objectives: Actinomycetoma is a chronic granulomatous disease affecting skin, subcutaneous tissue, fascia, muscle and bones. With increasing resistance against commonly used treatment regimens, susceptibility testing is urgently needed.

Methods: We developed an in vitro susceptibility assay for Actinomadura madurae, one of the common causative agents of actinomycetoma, employing resazurin for endpoint reading. Using this assay, reproducible MICs were determined for the most commonly used antibacterial agents for actinomycetoma treatment. The tested antibacterial agents included trimethoprim/sulfamethoxazole, amikacin, streptomycin, amoxicillin, ceftriaxone, gentamicin, ciprofloxacin, doxycycline, imipenem, linezolid, penicillin G and rifampicin.

Results: Following the clinical breakpoints as stated by CLSI, 100% of the tested strains were susceptible to trimethoprim/sulfamethoxazole (MIC 0.03/0.59–1/19 mg/L), amikacin (MIC 0.0078–0.25 mg/L), doxycycline (MIC <0.25–1 mg/L) and linezolid (MIC <0.25–2 mg/L), 90% to ciprofloxacin (MIC <0.25–2 mg/L), 80% to ceftriaxone (MIC <0.5 to >64 mg/L) and imipenem (MIC <0.25–32 mg/L) and only 20% to amoxicillin (MIC <0.5 to >64 mg/L) and rifampicin (MIC 0.5 to >32 mg/L).

Conclusions: Determinations of MICs by visual readings of colour changes versus spectrophotometric readings were comparable. This convenient visual reading has the advantage of feasible implementation in endemic settings.

Introduction

Mycetoma is a chronic granulomatous disease that affects the skin, subcutaneous tissue, fascia and muscle. Occasionally, the underlying bone and adjacent organs are affected as well. Mycetoma is characterized by firm tumefaction of the affected site, with abscesses, nodules and sinususes that drain a serosanguinous exudate containing grains characteristic of the causative agents. Mycetoma is endemic in Latin America, the Indian subcontinent and Africa, and a ‘mycetoma belt’ located between the latitudes of 15°S and 30°N around the Tropic of Cancer engulfs regions with high endemicity. Mycetoma can be caused by fungi (eumycetoma) or actinomycetes (actinomycetoma). Worldwide, approximately 60% of mycetoma is caused by actinomycetes, which are aerobic Gram-positive filamentous bacteria. Of the 4832 actinomycetes reported in 2013 in a meta-analysis study, 1946 cases were reported to be caused by Nocardia brasiliensis, 677 by Streptomyces somaliensis, 594 by Actinomadura madurae and 594 by Actinomadura pelletieri. A. madurae was the only species that was reported from all continents. Its name comes from the first cases of mycetoma in the Madurai region of southern India. Macroscopically, A. madurae is characterized by large, white/yellow granules that can be seen with the naked eye. On microscopic examination with haematoxylin and eosin stain, these grains are purple and exhibit peripheral pink pseudothreads. Despite the fact that no therapeutic guidelines are available, actinomycetoma is usually more responsive to combined antibiotic treatment, with cure rates ranging from 60% to 90%. To date, the Welsh regimen, consisting of trimethoprim/sulfamethoxazole and amikacin, forms an integral part of actinomycetoma management and is considered the gold standard treatment.
However, aminoglycosides, tetracyclines, rifampicin, ciprofloxacin and amoxicillin/clavulanic acid have also been successfully used.\textsuperscript{5–10} Currently, antimicrobial therapy for actinomycetoma is prescribed without prior antimicrobial susceptibility testing. However, recently it was demonstrated that the Welsh regimen was less successful in patients with actinomycetoma caused by \textit{A. madurae} than by \textit{N. brasiliensis}.\textsuperscript{7,9} Furthermore, 13\% of the 42 \textit{A. madurae} strains tested in 1990 were found to be resistant to trimethoprim/sulfamethoxazole.\textsuperscript{11} This necessitates the implementation of \textit{in vitro} susceptibility testing in the clinic. Therefore, a simple standardized susceptibility assay for \textit{A. madurae} is needed. CLSI developed the M24 guideline for susceptibility testing of mycobacteria, \textit{Nocardia} spp., and other aerobic actinomycetes.\textsuperscript{12} However, in this guideline, visual reading is recommended. The viability dye resazurin is an affordable, readily soluble, cell-permeable indicator that offers extra advantages in terms of its fast and exact visual endpoint determination. Due to its non-toxic nature and its half-life of 10 days it can be added to the cultured bacteria during inoculation.\textsuperscript{13,14} Upon adding, resazurin is non-fluorescent and deep blue-coloured. When bacteria start to grow, the blue-coloured resazurin is metabolically reduced by NADH to the fluorescent pink-coloured resorufin.\textsuperscript{14} Therefore the MIC can be determined visually as being the first blue/purple well, or spectrophotometrically at 600 nm.\textsuperscript{13}

Here, we aimed to develop an \textit{in vitro} resazurin-based microdilution assay for \textit{A. madurae}, based on the same principle as our recently published \textit{in vitro} susceptibility assay for \textit{Madurella mycetomatis}.\textsuperscript{13}

Materials and methods

Strains

In this study, eight \textit{A. madurae} reference strains (DSM43122, DSM46007, DSM43121, DSM43236, DSM44005, DSM43381 and DSM46181) and two clinical strains (SAK-A03 and SAK-A05) were used. All reference strains were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). These strains were originally isolated from patients in the first decades of the 1900s and had been deposited to the DSMZ collection before 1993. The clinical strains were obtained from the University of Science and Technology (UST) depository of strains during the period between 2018 and 2019. As a quality control, \textit{Staphylococcus aureus} ATCC 29213 was included.\textsuperscript{15} All strains were molecularly identified to the species level by 16S rRNA sequencing.

Antibacterial agents

Susceptibility to 12 antibacterial agents was determined. These agents dissolved in sterile DMSO (Merck, Dormstadt, Germany) or sterile distilled water according to the CLSI guidelines.\textsuperscript{12} Concentrations ranged between 0.03/0.59 and 4/76 mg/L for trimethoprim/sulfamethoxazole (Sigma-Aldrich, S7507, T7883), 0.0156 and 32 mg/L for amikacin hydrate (Sigma-Aldrich, A3650), 0.5 and 64 mg/L for streptomycin (Reyoung Pharmaceuticals Co. Ltd, China), amoxicillin (Centrafarm, Lot B80280, the Netherlands) and ceftriaxone (Sigma–Aldrich, C5793), 0.0625 and 8 mg/L for gentamicin (Centrafarm, Lot 2007211, Netherlands) and 0.25 and 32 mg/L for ciprofloxacin (Interchem, the Netherlands), doxycycline HCL (Sigma–Aldrich, D9891), imipenem monohydrate (Sigma–Aldrich, I0160), linezolid (Manisha Lottikar, Ev0004916), penicillin G (Sigma–Aldrich, P3032) and rifampicin (Sigma–Aldrich, R8883).

In vitro susceptibility assay

The \textit{in vitro} susceptibilities were determined according to the CLSI-M24-A3 guidelines.\textsuperscript{12} Resazurin was used to ease endpoint reading. In short, a bacterial suspension for each strain was prepared in CAMHB and adjusted to absorbance between 0.08 and 0.1 at 625 nm. A 100 µL suspension was added to each well of a round-bottom 96-well plate (Greiner Bio-One, The Netherlands) along with 1 µL of the antibacterial agent and 1 µL of resazurin solution (0.15 g/L).\textsuperscript{15} A growth control consisting of only the bacterial suspension, the solvent and resazurin solution, as well as a negative control consisting of only the culture medium and resazurin solution, were included. The plates were then sealed and incubated at 35°C ± 2°C for 5–7 days. The quantity of resorufin produced was proportional to the number of viable cells and was assessed both visually and spectrophotometrically.\textsuperscript{16} The MIC was determined visually as the first blue/purple well for each agent as from the third day of incubation. For spectrophotometric endpoints, on the seventh day of incubation, 100 µL of the supernatant was transferred to flat-bottom 96-well plates (Greiner Bio-One). Absorbance was measured at 600 nm using a microplate reader (Epoch 2, BioTek, USA); the MIC was defined as the lowest concentration of antibacterial agent resulting in 100\% reduction of viable organisms, or 80\%-90\% growth inhibition in the case of trimethoprim/sulfamethoxazole.\textsuperscript{13} Percentages of growth inhibition for resazurin were calculated using equation below:

\[
\text{Percentage growth inhibition} = 100 - \left( \frac{OD_{600nm, GC} - OD_{600nm, test}}{OD_{600nm, GC}} \right) \times 100
\]

To determine whether a strain was susceptible or resistant to the antimicrobials under investigation, the breakpoints as established for \textit{Nocardia} species were used, as described in the CLSI guidelines.\textsuperscript{16}

Calculation of reproducibility and agreement of the different methods of endpoint reading

To determine the reproducibility of the assay, the percentage agreement between replicates was determined. The assay was considered reproducible for a certain isolate when the MICs obtained by replicates differed by no more than a single dilution. The reproducibility was calculated for visual endpoint reading as well as for spectrophotometric endpoint reading. To determine the percentage agreement between the two methods for endpoint reading, the MIC data were compared. An MIC was considered to be in agreement when no more than a single-dilution difference between the visual MIC and the spectrophotometric MIC was found.

Results

Here we determined the antibacterial susceptibilities of the 10 \textit{A. madurae} strains and the \textit{S. aureus} control strain for 12 antimicrobial agents using both visual as well as spectrophotometric endpoint reading. As can be seen in Figures 1 and 2, in general, MICs obtained with spectrophotometric endpoint reading were comparable to those obtained with visual endpoint reading. The reproducibility of visual reading ranged between 60\% and 100\% for the 12 antibiotics tested (Table 1). Lowest reproducibility was obtained with ceftriaxone, while for gentamicin and amikacin 100\% reproducibility was obtained. Reproducibility of spectrophotometric reading was higher and ranged from 80\% to 100\%. For ceftriaxone, reproducibility of 90\% was obtained when spectrophotometric reading was used. The agreement between visual endpoint reading and spectrophotometric reading ranged
In vitro resazurin assay for testing Actinomadura madurae

Due to the higher reproducibility of spectrophotometric endpoint reading and the high agreement between visual and spectrophotometric reading, we depict the MICs obtained via spectrophotometric endpoint reading in Table 2. As can be seen in Table 2, all MICs for the S. aureus control strain were similar to those reported in the CLSI guidelines. The lowest visual and spectrophotometric MICs were obtained for amikacin, ciprofloxacin, doxycycline, gentamicin and linezolid (MICs: 0.0078–2 mg/L). All strains tested (100%) were inhibited by trimethoprim/sulfamethoxazole (MICs 0.03/0.59–1/19 mg/L). Higher MICs were obtained for amoxicillin (MIC <0.5 to >64 mg/L), penicillin G (MIC <0.25 to >32 mg/L) and rifampicin from 66.7% to 100%. Not surprisingly, the lowest percentage agreement was obtained for ceftriaxone.

Table 1. Reproducibility and accuracy of visual reading compared with spectrophotometric reading

| Antimicrobial agent                  | Reproducibility (%) of visual reading<sup>a</sup> | Reproducibility (%) of spectrophotometric reading at 600 nm<sup>b</sup> | % Agreement between visual and spectrophotometric reading<sup>b</sup> |
|-------------------------------------|-----------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Amikacin                            | 100                                           | 100                                             | 100                                             |
| Amoxicillin                         | 80                                            | 90                                              | 88.8                                            |
| Ceftriaxone                         | 60                                            | 90                                              | 66.7                                            |
| Ciprofloxacin                       | 80                                            | 100                                             | 80                                              |
| Trimethoprim/sulfamethoxazole       | 90                                            | 100                                             | 90                                              |
| Doxycycline                         | 90                                            | 90                                              | 90                                              |
| Gentamicin                          | 100                                           | 100                                             | 100                                             |
| Imipenem                            | 70                                            | 90                                              | 77.7                                            |
| Linezolid                           | 90                                            | 100                                             | 90                                              |
| Penicillin G                         | 80                                            | 80                                              | 80                                              |
| Rifampicin                          | 70                                            | 80                                              | 87.5                                            |
| Streptomycin                        | 75                                            | 87                                              | 86.2                                            |

MICs were considered to be in agreement when no more than a single-dilution difference between MICs was found for a single isolate between the two endpoint reading methods.

<sup>a</sup>The reproducibility of the visual endpoint reading or the spectrophotometric reading was determined by calculating the percentage of agreement between the replicates. The assay was considered reproducible for a certain isolate when the MICs obtained by triplicate tests differed by no more than a single dilution.

<sup>b</sup>The MICs obtained by visual endpoint reading were compared with those obtained by spectrophotometric reading at 600 nm.
In our study we tested the amino-

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Table 2.

| Drugs/strains | Susceptible | Intermediate | Resistant |
|---------------|-------------|--------------|-----------|
| Amikacin      | ≤0.0078     | 0.0625–0.25  | >0.25     |
| Amoxicillin   | 8           | >16          | >32       |
| Ceftriaxone   | ≤0.5        | >64          | >32       |
| Ciprofloxacin | 1           | >4           | >8        |
| Trimethoprim  | 0.5         | >16          | >32       |
| Streptomycin  | 64          | >64          | >16       |

Nocardia spp. prompted us to develop a reproducible in vitro susceptibility assay for A. madurae. A slight modification to the protocol specified by CLSI-M24-A3 for susceptibility testing of Nocardia and other aerobic actinomycetes was made to ease endpoint reading. The readily soluble, cell-permeable and non-
toxic redox indicator resazurin was employed as viability indicator in the present protocol. The addition of resazurin allowed both visual readings by the changes from blue/purple to pink in metabolically active A. madurae as well as quantitative readings by either measuring absorbance, as done in our study, or by fluorescence, as also performed by others. Furthermore, addition of resazurin enhanced the reproducibility of endpoint reading for 8 out of 12 antibiotics tested. This indicated that adding resazurin to ease endpoint reading was not only beneficial for the eumycetoma causative agent M. mycetomatis but also for the actinomycetoma causative agent A. madurae.

The MICs obtained here were comparable with those reported for other aerobic actinomycetes and indicate that breakpoints that apply to Nocardia spp. can tentatively be used for other aerobic actinomycetes. In general, A. madurae was most susceptible to amikacin, ciprofloxacin, doxycycline, gentamicin and linezolid. The two clinical strains used in this study (SAK-A03 and SAK-A05) were susceptible to all agents except amoxicillin and rifampicin, similar to the reference strains used in the study.

As mentioned before, the Welsh regimen consisting of trimethoprim/sulfamethoxazole and amikacin is currently the gold standard treatment for treating actinomycetoma. However, already in 1990, resistance to trimethoprim/sulfamethoxazole was reported for A. madurae. In our present in vitro results, all A. madurae strains tested were susceptible to trimethoprim/sulfamethoxazole at MICs ranging between 0.03/0.59 and 1/19 mg/L. Despite their potential nephrotoxicity and ototoxicity, as well as drug interactions, the addition of aminoglycosides to treatment regimens for actinomycetoma was shown to be beneficial and shorten the treatment period. In our study we tested the aminoglycosides amikacin, gentamicin and streptomycin (Figure 1). In vitro, the tested A. madurae strains were most susceptible to amikacin (MICs 0.0078–0.25 mg/L), followed by gentamicin (MICs 0.25–2 mg/L) and streptomycin (MIC 2–64 mg/L). Amikacin was very active in vitro in combination with trimethoprim/sulfamethoxazole against the other actinomycetoma causative agent Nocardia asteroides. The best clinical response to

Table 2. MIC distribution for the susceptibility of eight A. madurae reference (DSM) strains, two clinical (SAK) strains and the control strain S. aureus ATCC 29213 to 12 standard antibacterial agents in the resazurin assay using spectrophotometric endpoint reading.
Streptomycin, a naturally derived aminoglycoside, combined with trimethoprim/sulfamethoxazole, was shown by A. pelletieri, A. madurae and S. somaliensis. Gentamicin was employed in the modified two-step regimen for the management of invasive phase of actinomycetoma infection. With respect to the other classes of antibiotics, the A. madurae strains included in this study were all susceptible to doxycycline and linezolid. Doxycycline was combined with trimethoprim/sulfamethoxazole for the treatment of actinomycetoma infections according to a modified two-step regimen. Linezolid revealed 100% in vitro activity against A. madurae strains under the present investigation (Figure 2). The MICs reported in our study are in agreement with the in vitro activity reported for 24 strains of A. madurae with MICs between 0.031 and 0.25 mg/L. A previous study also revealed strong in vitro susceptibility of N. brasiliensis with MICs 0.5–4 mg/L. Linezolid displayed a statistically significant decrease in the formation of N. brasiliensis lesions in an experimental murine model of mycetoma compared with that for the animals treated with saline solution. However, the high cost of this drug represents a real problem for actinomycetoma patients in this part of the globe where this disease is very closely associated with poverty.

The majority of the A. madurae strains were also susceptible to the fluoroquinolone ciprofloxacin, the cephalosporin ceftriaxone and the carbapenem imipenem (Figure 2). Ciprofloxacin, only one strain was intermediate, the rest were susceptible. For ceftriaxone and imipenem, 80% of the tested strains were susceptible. In patients, ciprofloxacin in combination with trimethoprim/sulfamethoxazole showed good results against actinomycetoma. It has been reported that no A. madurae strain was resistant to ceftriaxone in vitro, whilst >50% of N. brasiliensis isolates were resistant to this agent. Actinomycetoma infections have been reported to have very good clinical response to IV imipenem, a thienamycin derivative from Streptomyces cattleya. The use of a combination of imipenem with amikacin is reported for resistant, severe cases of mycetoma, involving viscera or bones.

It is notable that the β-lactam antibacterial agents and rifampicin were poorly active in vitro in the present study. Only two strains were susceptible to amoxicillin, one was intermediate and the remaining seven were resistant (Table 2 and Figure 2). Penicillin G variably inhibited A. madurae strains under testing at MICs <0.25 to >32 mg/L. Amoxicillin and penicillin G were added to the two-step regimen (Ramam regimen) for actinomycetoma treatment. Rifampicin was added in the invasive and maintenance phases of management of actinomycetoma in the modified Welsh regimen. However, it has been reported that rifampicin was the most effective antibiotic against S. somaliensis strains isolated from Sudanese patients.

Since susceptibility testing is not routinely used for A. madurae, and resistance rates to commonly used antibiotics are increasing, this convenient assay would assist in the implementation of susceptibility testing and subsequent appropriate therapy in clinical settings in endemic regions. The resazurin assay offers prompt execution in terms of inoculum preparation and standardization, with no need for sophisticated equipment, coupled with providing flexibility in plate layouts besides being a less costly viability dye. Performing routine in vitro susceptibility testing could facilitate a correlation between in vitro susceptibility and clinical outcomes. A. madurae forms large grains with extensive fibrosis surrounding these grains. These are two barriers an antimicrobial agent needs to cross before reaching the causative agent. Fibrosis is more pronounced in actinomycetoma caused by A. madurae than by N. brasiliensis. In eumycetoma, it was already established that grains are less susceptible to antifungal agents than fungal hyphae are. For actinomycetoma grains, this correlation is still to be determined. Therefore before we can use the MIC as the sole indication for predicting the clinical outcome, clinical studies in which the MIC will be linked to clinical outcome are needed to establish if there is a correlation. The resazurin in vitro susceptibility assay developed here would be an excellent tool for that. Furthermore, the use of resazurin as a viability dye would allow better quantification of growth, which makes this assay suitable for drug discovery too.

In summary, we developed, a simple, reproducible in vitro microdilution assay, with a flexible readout system, compatible with the colorimetric viability dye resazurin for fast and efficient profiling of antibacterial susceptibility in Actinomadura spp. Determinations of MICs by visual readings of colour changes versus spectrophotometric readings were comparable, which makes this assay suitable for implementation in endemic settings.

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### Transparency declarations
The authors declare no conflicts of interest.

### References
1. Welsh O. Mycetoma: current concepts in treatment. Int J Dermatol 1991; 30: 387–98. https://doi.org/10.1111/j.1365-4632.1991.tb03892.x
2. Arneen M, Arenas R. Developments in the management of mycetomas. Clin Exp Dermatol 2009; 34: 1–7. https://doi.org/10.1111/j.1365-2330.2008.03028.x
3. Van de Sande WWJ. Global burden of human mycetoma: a systematic review and meta-analysis. PLoS Negl Trop Dis 2013; 7: e2550. https://doi.org/10.1371/journal.pntd.0002550
4. Nenoff P, van de Sande WW, Fahal AH et al. Eumycetoma and actinomycetoma—an update on causative agents, epidemiology, pathogenesis, diagnostics and therapy. J Eur Acad Dermatol Venereol 2015; 29: 1873–83. https://doi.org/10.1111/jdv.13008
5. Kar S, Prasad K, Madke B et al. Abdomino-pelvic actinomycetoma successfully treated with combination chemotherapy. Australas J Dermatol 2014; 55: 230–2. https://doi.org/10.1111/ajd.12211
6. Welsh O, Al-Abdely HM, Salinas-Carmona MC et al. Mycetoma medical therapy. PLoS Negl Trop Dis 2014; 8: e3218. https://doi.org/10.1371/journal.pntd.0003218
7. Bonifaz A, Tirado-Sanchez A, Vazquez-Gonzalez D et al. Actinomycetoma by Actinomadura madurae: clinical characteristics and treatment of 47 cases. Indian Dermatol Online J 2021; 12: 285–9. https://doi.org/10.4103/idoj.IDOJ_474_20
8. Agarwal P, Jagati A, Rathod SP et al. Clinical features of mycetoma and the appropriate treatment options. Res Rep Trop Med 2021; 12: 173–9. https://doi.org/10.2147/RRTM.S282266
9. Bonifaz A, Tirado-Sanchez A, Vazquez-Gonzalez D et al. Actinomycetoma by Actinomadura madurae. Clinical and therapeutic characteristics of 18
cases with two treatment modalities. J Dermatolog Treat 2020; 33: 1954–8. https://doi.org/10.1080/09546634.2020.1793887

10 Welsh O, Salinas MC, Rodriguez MA. Treatment of eumycetoma and actinomycetoma. Curr Top Med Mycol 1995; 6: 47–71.

11 McNeil MM, Brown JM, Jarvis WR et al. Comparison of species distribution and antimicrobial susceptibility of aerobic actinomycetes from clinical specimens. Rev Infect Dis 1990; 12: 778–83. https://doi.org/10.1093/clinids/12.5.778

12 CLSI. Susceptibility Testing of Mycobacteria, Nocardia spp., and Other Aerobic Actinomycetes—First Edition: M24. 2018.

13 Abd Algaffar SO, Verbon A, van de Sande WWJ et al. Development and validation of an in vitro resazurin-based susceptibility assay against Madurella mycetomatis. Antimicrob Agents Chemother 2021; 65: e01338-20. https://doi.org/10.1128/AAC.01338-20

14 O’Brien J, Wilson I, Orton T et al. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000; 267: 5421–6. https://doi.org/10.1046/j.1432-1327.2000.01606.x

15 Mahmoud AB, Abd Algaffar S, van de Sande W et al. Niclosamide is active in vitro against mycetoma pathogens. Molecules 2021; 26: 4005. https://doi.org/10.3390/molecules26134005

16 CLSI. Performance Standards for Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes—First Edition: M62. 2018.

17 Carroll GF, Brown JM, Haley LD. A method for determining in-vitro drug susceptibilities of some nocardiae and actinomadurae: results with 17 antimicrobial agents. Am J Clin Pathol 1977; 68: 279–83. https://doi.org/10.1093/ajcp/68.2.279

18 Relhan V, Mahajan K, Agarwal P et al. Mycetoma: an update. Indian J Dermatol 2017; 62: 332–40. https://doi.org/10.4103/0019-5154.198046

19 Welsh O, Vera-Cabrera L, Welsh E et al. Actinomycetoma and advances in its treatment. Clin Dermatol 2012; 30: 372–81. https://doi.org/10.1016/j.clderm.2011.06.027

20 Nasher MA, Hay RJ, Mahgoub ES et al. In vitro studies of antibiotic sensitivities of Streptomyces somaliensis—a cause of human actinomycetoma. Trans R Soc Trap Med Hyg 1989; 83: 265–8. https://doi.org/10.1016/0035-9203(89)90675-5

21 Vera-Cabrera L, Gomez-Flores A, Escalante-Fuentes WG et al. In vitro activity of PNU 100766 (linezolid), a new oxazolidinone antimicrobial, against Nocardia brasiliensis. Antimicrob Agents Chemother 2001; 45: 3629–30. https://doi.org/10.1128/AAC.45.12.3629-3630.2001

22 Gomez-Flores A, Welsh O, Said-Fernandez S et al. In vitro and in vivo activities of antimicrobials against Nocardia brasiliensis. Antimicrob Agents Chemother 2004; 48: 832–7. https://doi.org/10.1128/AAC.48.3.832-837.2004

23 Hamid ME. Variable antibiotic susceptibility patterns among Streptomyces species causing actinomycetoma in man and animals. Ann Clin Microbiol Antimicrob 2011; 10: 24. https://doi.org/10.1186/1476-0711-10-24

24 Wortman PD. Treatment of a Nocardia brasiliensis mycetoma with sulfamethoxazole and trimethoprim, amikacin, and amoxicillin and clavulanate. Arch Dermatol 1993; 129: 564–7. https://doi.org/10.1001/archderm.1993.01680260032002

25 Gombert ME, Aulicino TM, duBouchet L et al. Therapy of experimental cerebral nocardiosis with imipenem, amikacin, trimethoprim-sulfamethoxazole, and minocycline. Antimicrob Agents Chemother 1986; 30: 270–3. https://doi.org/10.1128/AAC.30.2.270

26 Van de Sande WWJ. In vitro susceptibility testing for black grain eumycetoma causative agents. Trans R Soc Trap Med Hyg 2021; 115: 343–54. https://doi.org/10.1093/trstmh/traa184