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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 sinuses that drain a serosanguinous exudate containing grains characteristic of the causative agents.[1] 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.[2]

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*.[3] *A. madurae* was the only species that was reported from all continents.[3] Its name comes from the first cases of mycetoma in the Madurai region of southern India.[4] 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 pseudofilaments.[5]

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.[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 *A. madurae* than by *N. brasiliensis*.[7,9] Furthermore, 13% of the 42 *A. madurae* strains tested in 1990 were found to be resistant to trimethoprim/sulfamethoxazole.[11] This necessitates the implementation of *in vitro* susceptibility testing in the clinic. Therefore, a simple standardized susceptibility assay for *A. madurae* is needed. CLSI developed the M24 guideline for susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes.[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.[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.[14] Therefore the MIC can be determined visually as being the first blue/purple well, or spectrophotometrically at 600 nm.[13]

Here, we aimed to develop an *in vitro* resazurin-based microdilution assay for *A. madurae*, based on the same principle as our recently published *in vitro* susceptibility assay for *Madurella mycetomatis*.[13]

Materials and methods

Strains

In this study, eight *A. madurae* reference strains (DSM43122, DSM46007, DSM43121, DSM43123, 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, *Staphylococcus aureus* ATCC 29213 was included.[12] All strains were molecularly identified to the species level by 16S rRNA sequencing.

Antibacterial agents

Susceptibility to 12 antibacterial agents was determined. These agents were dissolved in sterile DMSO (Merck, Darmstadt, Germany) or sterile distilled water according to the CLSI guidelines.[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 B802B0, 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 Lotlikar, Ev0004916), penicillin G (Sigma–Aldrich, P3032) and rifampicin (Sigma–Aldrich, R8883).

In vitro susceptibility assay

The *in vitro* susceptibilities were determined according to the CLSI-M24-A3 guidelines.[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).[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.[14] 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.[13] Percentages of growth inhibition for resazurin were calculated using equation below:

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

To determine whether a strain was susceptible or resistant to the antimicrobials under investigation, the breakpoints as established for *Nocardia* species were used, as described in the CLSI guidelines.[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 *A. madurae* strains and the *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

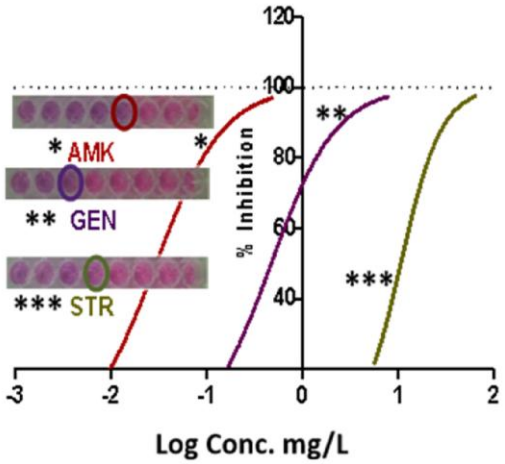

Figure 1. Percentage growth inhibition and visual MICs determined by resazurin assay for the susceptibility of *A. madurae* strain DSM 44005 to aminoglycosides: amikacin (AMK); gentamicin (GEN) and streptomycin (STR). Circles represent the visual MICs for amikacin (0.25 mg/L), gentamicin (2 mg/L) and streptomycin (8 mg/L). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

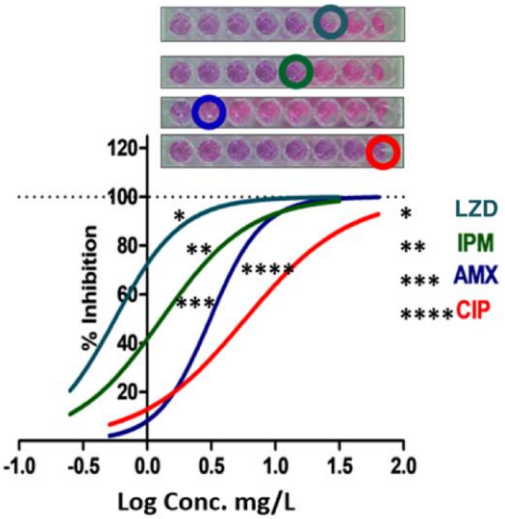

Figure 2. Percentage growth inhibition and visual MICs determined by resazurin assay for the susceptibility of *A. madurae* strain DSM 44005 to representative antibacterial agents: linezolid (LZD); imipenem (IPM); amoxicillin (AMX) and ciprofloxacin (CIP). Circles represent the visual MICs for each agent, with concentrations decreasing from left to right. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

from 66.7% to 100%. Not surprisingly, the lowest percentage agreement was obtained for ceftriaxone.

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 MIC_{50}s 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 MIC_{50}s were obtained for amoxicillin (MIC <0.5 to >64 mg/L), penicillin G (MIC <0.25 to >32 mg/L) and rifampicin

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

Antimicrobial agent	Reproducibility (%) of visual reading[a]	Reproducibility (%) of spectrophotometric reading at 600 nm[a]	% Agreement between visual and spectrophotometric reading[b]
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.

[a]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.

[b]The MICs obtained by visual endpoint reading were compared with those obtained by spectrophotometric reading at 600 nm.

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

Drugs/strains	Susceptible[a]	Intermediate[a]	Resistant[a]	DSM 43122	DSM 46007	DSM 43121	DSM 43123	DSM 43236	DSM 44005	DSM 43381	DSM 46181	SAK-A03	SAK-A05	ATCC 29213
Amikacin	≤8	—	≥16	0.0078	0.25	0.0625	0.0078	0.0078	0.25	0.25	0.25	0.0625	0.0312	2
Amoxicillin	≤8	16	>32	64	>64	>64	<0.5	64	8	>64	64	16	>64	32
Ceftriaxone	≤8	16–32	>64	64	8	<0.5	<0.5	<0.5	1	>64	<0.5	<0.5	<0.5	8
Ciprofloxacin	≤1	2	>4	<0.25	0.25	<0.25	<0.25	<0.25	<0.25	2	<0.25	<0.25	<0.25	0.5
Trimethoprim/ sulfamethoxazole	≤2/32	—	>4/76	0.5/9.5	1/19	0.5/9.5	0.03/0.59	0.25/4.75	1/19	0.5/9.5	0.25/4.75	0.25/ 4.75	0.25/ 4.75	0.5/9.5
Doxycycline	≤1	2–4	>8	<0.25	1	<0.25	<0.25	<0.25	<0.25	0.5	<0.25	<0.25	<0.25	<0.25
Gentamicin	—	—	—	0.25	1	0.5	1	1	2	1	1	0.5	0.5	0.25
Imipenem	≤4	8	>16	0.5	32	4	0.25	1	1	16	1	<0.25	<0.25	<0.25
Linezolid	≤8	—	—	1	2	1	<0.25	2	1	2	1	1	1	4
Penicillin G	—	—	—	0.5	>32	32	<0.25	32	0.5	>32	0.25	1	8	0.5
Rifampicin	≤1	2	>4	>32	>32	32	0.5	32	0.5	>32	>32	>32	32	<0.25
Streptomycin	—	—	—	2	64	8	16	ND	8	ND	2	64	8	ND

ND, not done.
[a]According to the CLSI M62 guideline for *Nocardia* spp.

(MIC 0.5 to >32 mg/L). *A. madurae* strains (DSM 43381 and DSM46007) showed some resistance to amoxicillin, penicillin, rifampicin and streptomycin (MIC >64 to >32 mg/L). DSM 43123 and DSM 44005 strains were susceptible to all tested antibacterial agents at variable MIC ranges (Table 2).

Discussion

Here we demonstrated that spectrophotometric endpoint reading using resazurin resulted in highly reproducible MICs for *A. madurae* for all 12 antibiotics tested. The lowest MICs were obtained for amikacin, ciprofloxacin, doxycycline, gentamicin and linezolid (MICs 0.0078–2 mg/L). The assay was easy to perform and the addition of resazurin allows easy visual MIC reading also in endemic settings.

The lack of standardized guidelines for drug susceptibility testing in *Actinomadura* 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.[12] 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.[14] 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*.[13]

The MICs obtained here were comparable with those reported for other aerobic actinomycetes[12,16] and indicate that breakpoints that apply to *Nocardia* spp. can tentatively be used for other aerobic actinomycetes.[16] 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.[17] However, already in 1990, resistance to trimethoprim/sulfamethoxazole was reported for *A. madurae*.[11] 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.[8] 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*.[7,10,18–20] The best clinical response to

streptomycin, a naturally derived aminoglycoside, combined with trimethoprim/sulfamethoxazole, was shown by *A. pelletieri*, *A. madurae* and *S. somaliensis*.[10] Gentamicin was employed in the modified two-step regimen for the management of invasive phase of actinomycetoma infection.[18]

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.[18] 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.[11] A previous study also revealed strong *in vitro* susceptibility of *N. brasiliensis* with MICs 0.5–4 mg/L.[21] 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.[22] 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). For 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.[7,9] 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.[11,22] Actinomycetoma infections have been reported to have very good clinical response to IV imipenem, a thienamycin derivative from *Streptomyces cattleya*.[20,23,24] The use of a combination of imipenem with amikacin is reported for resistant, severe cases of mycetoma, involving viscera or bones.[25]

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.[8] Rifampicin was added in the invasive and maintenance phases of management of actinomycetoma in the modified Welsh regimen.[18] However, it has been reported that rifampicin was the most effective antibiotic against *S. somaliensis* strains isolated from Sudanese patients.[20]

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.[9] Fibrosis is more pronounced in actinomycetoma caused by *A. madurae* than by *N. brasiliensis*.[9] In eumycetoma, it was already established that grains are less susceptible to antifungal agents than fungal hyphae are.[26] 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.

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