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Determination of bactericidal activity against 3HC-2-Tre-labelled *Mycobacterium abscessus* (*Mycobacteroides abscessus*) by automated fluorescence microscopy

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ABSTRACT

The minimum bactericidal concentration (MBC) of antibiotics is an important parameter for the potency of a drug in eradicating a bacterium as well as an important measure of the potential of a drug candidate in research and development. We have established a fluorescence-based microscopy method for the determination of MBCs against the non-tuberculous mycobacterium *Mycobacterium abscessus* (*Mycobacteroides abscessus*) to simplify and accelerate the performance of MBC determination compared to counting colony forming units on agar. Bacteria are labelled with the trehalose-coupled dye 3HC-2-Tre and analysed in a 96-well plate. The results of the new method are consistent with MBC determination by plating on agar. The method was used to evaluate the bactericidality of the antibiotics rifabutin, moxifloxacin, amikacin, clarithromycin and bedaquiline. Bactericidal effects against *M. abscessus* were observed, which are consistent with literature data.

1. Introduction

Mycobacterium abscessus (*Mycobacteroides abscessus*) is a fast-growing, multidrug-resistant mycobacterium classified as a non-tuberculous mycobacterium (NTM). It is an opportunistic pathogen responsible for severe respiratory infections in patients with pre-existing medical conditions such as immunosuppression or cystic fibrosis (Baldwin et al., 2019). In addition, *M. abscessus* can cause skin and soft tissue infections. It is difficult to treat infections caused by *M. abscessus* because the pathogen is resistant to a large number of antibiotics (Boudehen and Kremer, 2021). For example, the cornerstones of tuberculosis therapy, rifampicin and isoniazid, are not effective against *M. abscessus* (Wu et al., 2018) and are not used clinically.

The therapeutic regimen for *M. abscessus* infection depends highly on the subspecies and individual strain (Daley et al., 2020). It usually consists of at least three antibiotics. The combination is based on a macrolide combined with a parenteral aminoglycoside and either cefoxitin, imipenem or tigecycline. The duration of therapy is usually 12 months and the defined end point of sputum conversion is rarely achieved (Wu et al., 2018). This uncertain success in therapy, together with several antibiotics causing side effects negatively affects the patient's adherence and compliance (Wu et al., 2018; Holt and Baird, 2023).

A major challenge in the treatment of *M. abscessus* is the lack of antibiotics with bactericidal activity against the pathogen, *e.g.* clarithromycin and tigecycline, which show no or only weak bactericidal activity (Maurer et al., 2014) against *M. abscessus*. To improve *M. abscessus* therapy and to shorten the duration of treatment, it is necessary to include antibiotics with sterilising properties in the treatment regimen. The established method - determination of bactericidal activity by colony counting on agar - is time-consuming and labour-intensive, which limits its applicability in clinical microbiology, but also during early drug development. The transfer of bacterial suspensions to agar plates restricts the number of replicates and usability. For this reason, we have developed a robust and efficient method to analyse whether substances have bactericidal properties against *M. abscessus*.

The goal of the study is to establish a straightforward experimental procedure that allows efficient analysis of compounds in 96-well plate format without the need for manual plating or colony counting. In order to accomplish this, we use a fluorescent trehalose probe 2-(6-(diethylamino)benzofuran-2-yl)-3-hydroxy-4*H*-chromen-4-one Trehalose (3HC-2-Tre), which is capable of selectively staining mycobacteria, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Mycobacterium aurum* or *M. abscessus* (Kamariza et al., *2018*; Richter et al., *2024*; Kamariza et al., *2021*). Trehalose conjugates are a recent development

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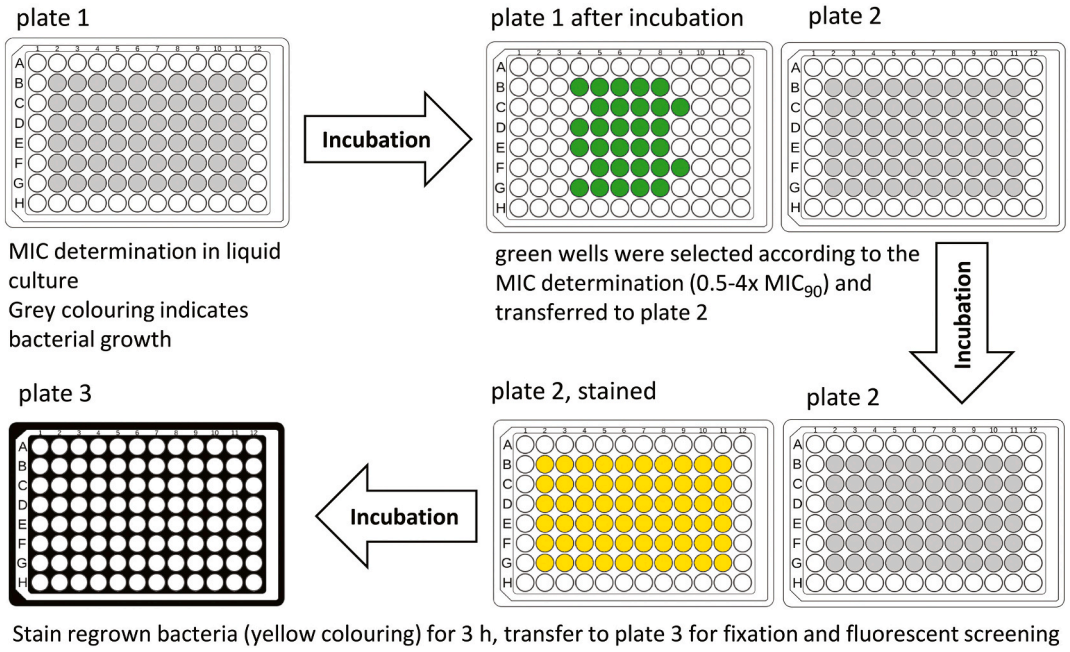


Fig. 1. Visualisation of the MBC determination by 3HC-2-Tre staining.

that are of great use for fluorescence microscopic detection of mycobacteria: Kamariza et al. (2018) first published 4-*N*,*N*-Dimethylamino-1,8-naphthalimide conjugate of trehalose (DMN-Tre) as a dye that is selectively incorporated into the mycobacterial cell wall by the enzyme Ag85 (Kamariza et al., 2018). Additionally, DMN-Tre labelling was reported to show comparable results to the commonly used auramine staining. In 2020, Sahile et al. published that DMN-Tre is capable of labelling intracellular *M. tuberculosis* (Sahile et al., *2020*). In 2021, Kamariza et al. published two closely related dyes, 3HC-3-Tre and 3HC-2-Tre, for which the fluorescence intensity was further enhanced. Importantly, a straightforward synthetic procedure to make the dyes easily accessible was published (Kamariza et al., 2021). Here we present a method we have developed to simplify the determination of MBC against *M. abscessus*. Results will also be presented to demonstrate that the method provides results comparable to MBC determination by CFU (colony forming units) counting.

2. Material and methods

2.1. Bacteria and culture medium

Stocks of *M. abscessus* subsp. *abscessus* type strain ATCC 19977 were cultivated in complete 7H9 broth (Sigma Aldrich, St. Louis, MO, USA) and stored as cryo stocks with approximately 15% glycerol at −80 °C. The cryo stocks were used to inoculate complete 7H9 broth supplemented with 10% ADS (ADS supplement is a filter-sterilized solution of 0.8% sodium chloride, 5.0% bovine serum albumin, and 2.0% dextrose in purified water) and 0.05% polysorbate 80. The culture volume was 10 mL in a 50 mL Falcon tube, which was shaken in an incubator at 37 °C. Solid cultures were grown on 7H10 medium supplemented with 0.5% glycerol and 10% ADS (Mann et al., 2022).

2.2. MIC_{90} determination in 7H9 by OD_{600} measurement

MICs were determined against *M. abscessus* ATCC 19977 utilizing a broth microdilution method in 7H9 containing 10% ADS and 0.05% polysorbate 80. A nine-point 2-fold serial dilution of each compound was prepared in 96-well flat-bottom plates (Sarstedt, 3924500, Nümbrecht, Germany) with a final volume of 100 µL. Column 2 of the 96-well plate included eight negative controls, column 3 contained eight positive controls. Column 1 contained only medium as a sterile control. The concentration of the inoculum was $5*10^7$ CFU/mL (an OD_{600} (optical density) of 0.1 is equal to $1*10^8$ CFU/mL). The starting inoculum was diluted from a preculture at the mid-log phase (OD_{600}, 0.2 to 0.8). The plates were sealed with Parafilm and incubated for 3 days at 37 °C. After incubation, the plates were analysed by OD measurement at 550 nm (BMG labtech Fluostar Optima, Offenburg, Germany). The assay was performed in duplicate.

2.3. Calculation of the MIC_{90}

Each assay plate contained eight wells with DMSO (1%) as a negative control, which correspond to 100% bacterial growth, and eight wells with amikacin (100 µM) as a positive control, in which 100% inhibition of bacterial growth was reached. The controls were used to monitor assay quality through the determination of the Z score (>0.6) and for normalizing the data. The Z factor was determined using the following formula (Richter et al., 2018):

$$Z^{'} = 1 - \frac{3\left(SD_{\text{positive control}} + SD_{\text{DMSO}}\right)}{M_{\text{positive control}} - M_{\text{DMSO}}}$$

where SD is the standard deviation and M is the mean.

Percent inhibition was calculated as follows:

$$\%\text{inhibition} = -100\% \times \frac{\text{signal(sample)} - \text{signal(DMSO)}}{\text{signal(DMSO)} - \text{signal(positive control)}}$$

The lowest concentration exceeding 90% inhibition was considered as the MIC_{90} value.

2.4. MBC determination on agar

For MBC (minimal bactericidal concentration) determination, *M. abscessus* ATCC 19977 was incubated in a microplate assay as described above. Subsequently, the MBC was determined by CFU counting. Petri dishes filled with 7H10 agar supplemented with 0.5% glycerol, 10% ADS were used for this purpose. Serial 10-fold dilutions were prepared in phosphate-buffered saline (PBS) containing 0.025% polysorbate 80. 10 µL of each concentration were pipetted onto the agar

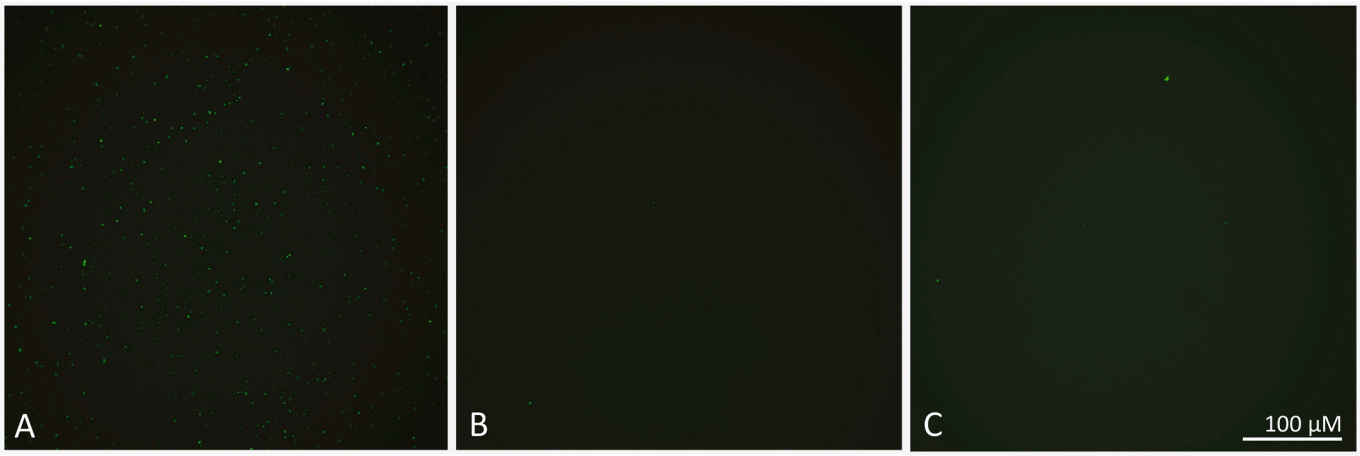


Fig. 2. Microscopic images (10× magnification) of fluorescent *M. abscessus* bacteria (green) taken with CX5 (Thermo Fisher Scientific). A: Inoculum, B: 100 µM rifabutin, C: 50 µM MMV688845. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dish. The colonies were counted after 3 days of incubation at 37 °C and the experiment was performed in triplicate. The concentration in CFU per mL was calculated based on the number of CFU in the inoculum after the three-day incubation from day 0.

2.5. Labelling of *M. abscessus* cells with trehalose conjugate 3HC-2-Tre

The synthesis of the compound 3HC-2-Tre was described by Richter et al. 2023 (Richter et al., 2023), following the method previously reported by Kamariza et al. 2021 (Kamariza et al., 2021). After MIC determination (as described above) 1 µL of each well with >90% growth inhibition was transferred in a second 96-well plate containing 100 µL of 7H9 medium supplemented with 10% ADS and 0.05% polysorbate 80 per well. The bacteria were incubated at 37 °C for two days.

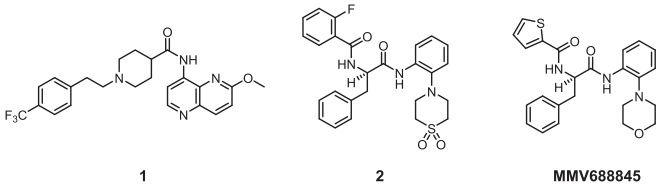
Afterwards 1 µL of 3HC-2-Tre (10 mM in DMSO) was added to each well resulting in a final dye concentration of 100 µM followed by incubation at 37 °C for 3 h. After incubation, 1 µL of each well was transferred to a black, clear, flat-bottom 96-well plate (Greiner bio one, 6550909, Frickenhausen, Germany) containing 199 µL of filtered 4% paraformaldehyde solution (PFA) in PBS for fixation. Fluorescence microscopy was then performed using a Thermo Fisher Scientific CellInsight CX5 instrument (Waltham, MA, USA). Samples were analysed at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 510–531$ nm. Images were acquired for 21 fields in one well. The valid object count of the GFP filter was matched to the number of bacteria counted. The valid field count has been used as a check that all fields are in focus of the microscope.

3. Results

3.1. Preparation of 96-well plates and microscopic readout

The MBC method developed in this study is based on a microplate dilution MIC assay for *M. abscessus*, as shown in Fig. 1. In contrast to the CFU assay, no plating on agar is performed and the bacteria that are still viable after incubation with a drug are cultivated in liquid medium.

After an initial 3-day incubation with a serial drug dilution on plate 1 and determination of MIC_{90} values, the bacteria are resuspended and wells with drug concentrations of 0.5×, at or above the MIC_{90} are transferred to plate 2 containing drug-free medium. This step is followed by a second incubation period of 48 h at 37 °C for growth of any still viable *M. abscessus* cells. During the transfer from plate 1 to plate 2, there is a 100-fold dilution, which reduces the drug concentration below the MIC_{90} to subinhibitory concentrations. In this way, the influence of residual antibiotics can be excluded as an error for drug concentrations close to the MIC.



Scheme 1. Structures of experimental compounds 1 (Beuchel et al., 2022), 2 (Lang et al., 2023) and MMV688845 (Mann et al., 2022; Ballell et al., 2013).

After the 48 h incubation period of plate 2, 3HC-2-Tre is added at a concentration of 100 µM to stain the bacteria. After 3 h incubation for the sufficient staining the wells are homogenized by pipetting, and an aliquot is transferred to plate 3 containing 4% PFA in PBS for fixation. This second dilution step is necessary to reduce the background fluorescence that is otherwise caused by the dye. Plate 3 (a clear flat-bottom 96-well plate) can be used for analysis by automated fluorescence microscopy.

Automated fluorescence microscopy was performed with a CellInsight CX5 under use of the GFP channel, for determination of the object count (Sahile et al., 2020). However, the method described here is not dependent on a particular dye, and is rather intended to allow a free choice of dye to suit equipment and experience.

To determine the number of bacteria in the inoculum, the inoculum

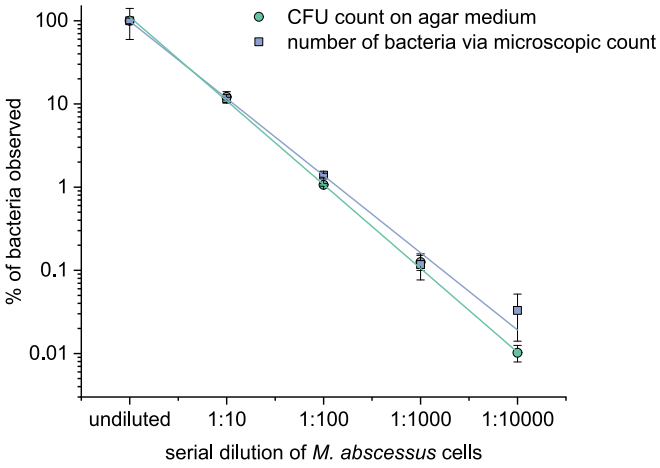


Fig. 3. Comparison of CFU with microscopic count of *M abscessus* in a tenfold dilution series.

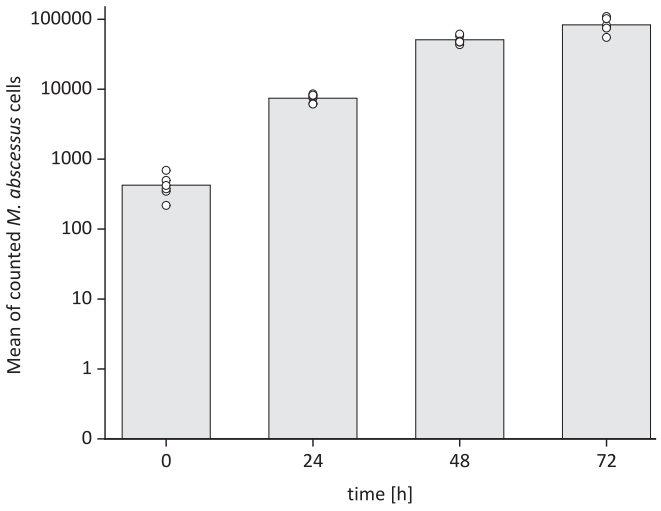


Fig. 4. Mean of 3HC-2-Tre labelled *M. abscessus* cells after the respective time of bacterial growth.

is transferred directly to plate 2 without the initial 3-day incubation on plate 1. After two days of incubation, the bacteria are stained and examined microscopically as described. The object count obtained in this way represents 100% viable bacteria and the object counts obtained from the MBC assay are normalised to this value, similar to the evaluation of the CFU assay.

Fig. 2 shows the microscopic images of the bacteria stained with 3HC-2-Tre after incubation with 100 μM rifabutin or 50 μM MMV688845 (Scheme 1) compared to the inoculum. The image data after incubation with the active substances already show a clear decrease in the number of objects, which indicates a bactericidal effect in this assay.

3.2. Correlation of CFU on agar and microscopic count

Fig. 3 shows the results of a comparison between the CFU count on agar and the microscopic count of fluorescent labelled bacteria. The aim is to show that, in an identical dilution series, both methods can reliably detect the number of bacteria over the entire dilution range, from undiluted culture to 1:10,000 dilution. The bacterial count in the undiluted culture (determined using CFU count on agar or fluorescence microscopy) is defined as 100%. For the experiment the starting culture was diluted by a factor of 10 and the bacterial count was determined with the respective method. The comparison of the two methods shows that the fluorescence microscopy method can determine the number of bacteria in a similar concentration range as the CFU count after plating, even at high dilutions and low numbers of bacteria.

3.3. Determination of regrowth-period

Because mycobacteria grow faster in liquid medium than on agar, the incubation time for regrowth of bacteria on plate 2 is shorter than for bacteria grown on solid agar. Therefore, we adjusted the incubation time for *M. abscessus* based on the measured number of regrown bacteria after 24, 48 and 72 h, as shown in Fig. 4. The inoculum was treated as described above. After fixation, the number of bacterial cells was determined by automated fluorescence microscopy. We chose to incubate for 48 h because the number of bacteria (about 50,000 cells) is 1000 times higher than the number on day 0, which was about 500 cells. Furthermore, with 50,000 cells, individual bacteria can still be detected by microscopic counting, but there are enough cells to detect the low numbers of bacteria within wells containing bactericidal test substances.

3.4. Antibiotics analysed for bactericidal activity against M. abscessus

The described assay was used to analyse the bactericidal efficacy of eight different antibiotics against *M. abscessus*. To substantiate the correlation shown in Fig. 2, we wanted to demonstrate the comparability of MBCs determined by CFU count and by fluorescence labelling.

We selected compound 2 and MMV688845 shown in Scheme 1 for which bactericidal activity has already been confirmed in a previous study by CFU counting on agar (Lang et al., 2023). The results for both RNA polymerase inhibitors MMV688845 and compound 2 are shown in Fig. 5. The data obtained by CFU counting (orange bars), showed comparable bactericidal activity to the automated assay based on 3HC-2-Tre labelling (green bars). Both compounds are bactericidal, but compound 2 is a derivative with improved activity, reducing the number of viable bacteria by two log units at 6.3 μM, whereas a considerably higher concentration (25 μM) of MMV688845 is required for a comparable effect.

As the results of the new method described in this study were consistent with the CFU count on agar, we selected additional

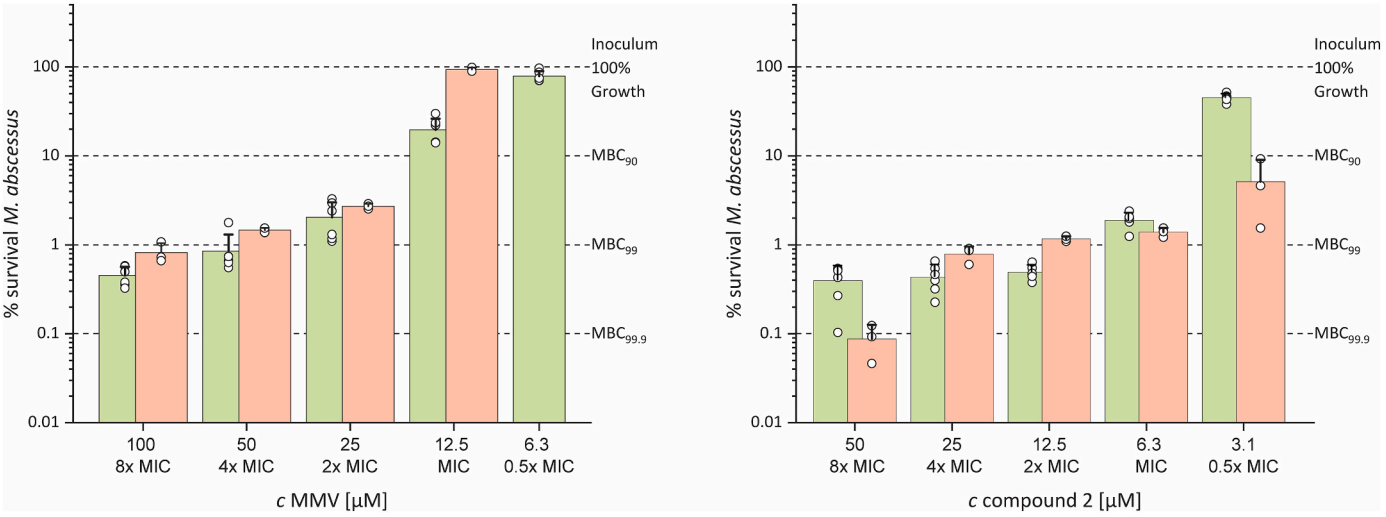


Fig. 5. Percentage of viable *M. abscessus*, after incubation with MMV688845 and compound 2. Green bars are determined by fluorescence microscopy, red bars by manual plating on agar and colony counting. (red bars: the experiment was carried put in triplicate, green bars: the experiment was carried out in biological duplicate and technical triplicate). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

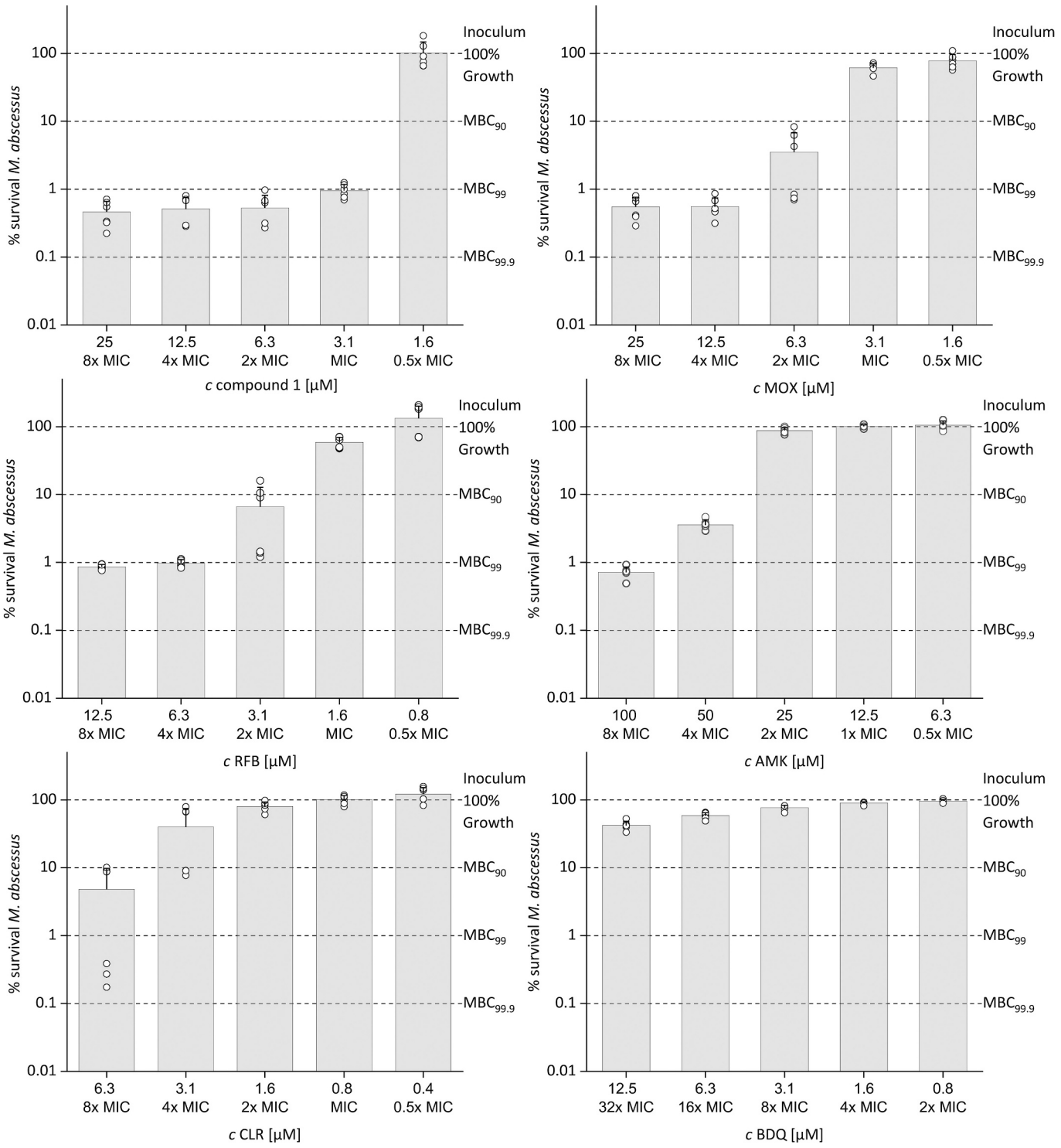


Fig. 6. Percentage of viable *M. abscessus*, normalised to the object count of the inoculum. (AMK = amikacin, BDQ = bedaquiline, CLR = clarithromycin, MOX = moxifloxacin, RFB = rifabutin) Experiments were performed in biological duplicates and technical replicate. All six data points and the standard deviation are shown.

antimycobacterial substances and performed further experiments. Six antimycobacterial compounds with different mechanisms of action and known bactericidal or bacteriostatic properties were analysed in the fluorescence based assay. The results are summarized in Fig. 6.

We expected bactericidal activity for the DNA gyrase inhibitors moxifloxacin and the novel bacterial topoisomerase inhibitor compound 1 (Beuchel et al., 2022) (see Scheme 1). As shown by Negatu et al. (Negatu et al., 2021) compound 1 leads to a 90% reduction in CFU of *M. abscessus* ATCC 19977 at 4× MIC and a comparable effect is observed for moxifloxacin at 8× MIC. This corresponds to a compound concentration of 4.5 µM (compound 1) or 9.0 µM (moxifloxacin). We obtained an MBC_{90} for compound 1 at 3.1 µM (1× MIC) and for moxifloxacin at 6.3 µM (2× MIC), reflecting a very similar dose range and also a weaker effect of moxifloxacin compared to compound 1.

For the RNA polymerase inhibitor rifabutin we determined an MBC_{90} at 3.1 µM (2× MIC) and an MBC_{99} from 6.3 µM (4× MIC). In a previous study, we determined an MBC_{90} of 2.4 µM (2× MIC) (Mann et al., 2022) by CFU count on agar. In a publication using *M. abscessus* Bamboo, an

MBC_{90} of 5 μM is reported for rifabutin (Xie et al., 2023). In another study using different strains of *M. abscessus*, including *M. abscessus* subsp. *abscessus* ATCC 19977, rifabutin showed an MBC_{90} of 6 μM (2× MIC) (Dick et al., 2020). The MBC_{90} and MBC_{99} values determined by 3HC-2-Tre labelling are within a similar dose range.

As shown in Fig. 6, amikacin showed a reduction of viable bacteria (MBC_{90}) for a rather high concentration of >50 μM, which is consistent with literature reporting an MBC_{90} of 40 μM (Xie et al., 2023) against *M. abscessus* Bamboo. Clarithromycin reduced the bacterial count by 90% at 6.3 μM (8× MIC), which is consistent with literature data reporting a 90% reduction at 12.5 μM (8× MIC) (Aziz et al., 2017). However, clarithromycin shows weak bactericidal activity overall, as it does not achieve a 99% reduction of viable bacteria at 8× MIC.

For bedaquiline, we did not observe a reduction in bacterial counts up to a concentration of 12.5 μM. A publication investigating the activity of bedaquiline also showed no colony-reducing effect against two different *M. abscessus* strains: $MBC_{99.9} > 2$ μg/mL (3.6 μM) (Aguilar-Ayala et al., 2017). However, it is possible to achieve bactericidal activity against *M. abscessus* with bedaquiline, by extending the incubation period. Xie et al. published an MBC_{90} of 2 μM against *M. abscessus* bamboo with an incubation period of 10 days (Xie et al., 2023).

4. Discussion

We developed a method to determine the bactericidal activity of antibiotics against *M. abscessus* using automated fluorescence microscopy with the trehalose conjugate 3HC-2-Tre. Automated evaluation based on bacterial staining provides a straightforward method for analysing the effect of antibiotics on the ability of bacterial cells to divide. Compared to plating on agar, this method saves time and material, which leads to a higher throughput during substance characterisation. The method also allows a greater number of objects to be analysed than with colony counting and requires fewer dilution steps. We are confident that the method described here represents an improvement over CFU assays performed by plating on agar, particularly in terms of sample throughput, accurate quantification of residual antibiotic concentration and robustness of results.

A study based on fluorescence to facilitate the determination of the MBC against *Escherichia coli* and *Pseudomonas aeruginosa* was carried out by Bär et al. in 2009 (Bär et al., 2009) using propidium iodide and SYTO™ 9 for staining. Here, however, two dyes are used to determine a ratio of viable to non-viable bacteria and an algorithmic evaluation is proposed. The staining with propidium iodide and SYTO™ 9 is based on the membrane integrity of the cell. The method described in our study is based on the ability of bacteria to grow in a liquid medium after exposure to antibiotics and determines an absolute number of bacteria.

In our experiments, we observed that propidium iodide stains viable and non-viable bacteria non-selectively, as described in Bärs study. For this reason, we decided to quantify the growth capacity of the cells after exposure to an antibiotic in liquid medium, rather than to differentiate between viable and non-viable bacteria by staining.

Another advantage of our method is that it does not require genetic modification of the bacteria. There are a number of methods for labelling mycobacteria, including episomal labelling or integration into the bacterial genome (Takaki et al., 2013; Biegas and Swarts, 2021; Sorrentino et al., 2016). However, this method is limited to genetically modified strains of bacteria, *e.g.* clinical isolates from patients cannot be analysed in this way without prior manipulation. When bacteria are modified with a plasmid, there is always a risk that the cells will lose the plasmid during the assay procedure and become undetectable by fluorescence microscopy (Wein et al., 2020). Another advantage of the method described in this study is that the unmodified wild-type bacteria can be used for substance testing, as the bacteria are not stained until the final step of the assay. Therefore, interference between the staining method or genetic modification and substance activity is rather unlikely.

The possibility of staining different types of mycobacteria, such as *M. tuberculosis* (Sahile et al., *2020*) or *M. aurum* (Richter et al., *2023*), with trehalose dyes has already been published. We therefore assume, even if this was not shown in the present study, that this assay can also be realised with the other subspecies of *M. abscessus*.

To summarise, our results show that the described method is suitable for determining the bactericidal properties of substances against the pathogenic mycobacterium *M. abscessus*.The method can discriminate between bacteriostatic and bactericidal effects of drugs and delivers equivalent results in comparison to the conventional method of plating on agar, while It is less time and material consuming. Since 3HC-2-Tre stains various mycobacterial species, the protocol described here can be adapted to other mycobacterial species, *e.g. M. tuberculosis*, by adjusting the incubation times.

CRediT authorship contribution statement

Lea Mann: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation. **Fabienne Siersleben:** Methodology, Investigation, Data curation. **Markus Lang:** Writing – review & editing, Investigation. **Adrian Richter:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Adrian Richter reports financial support was provided by German Research Foundation. Adrian Richter reports financial support was provided by Deutsche Mukoviszidose Stiftung. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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