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ARTICLE

Rapid susceptibility testing for slowly growing nontuberculous mycobacteria using a colorimetric microbial viability assay based on the reduction of water-soluble tetrazolium WST-1

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Abstract Rapid susceptibility testing for slowly growing nontuberculous mycobacteria (NTM) using a colorimetric microbial viability assay based on the reduction of the water-soluble tetrazolium salt {2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1)} using 2,3,5,6-tetramethyl-1,4-benzoquinone as an electron mediator was developed. Using the Clinical and Laboratory Standards Institute (CLSI) method, a long-term incubation time (7–14 days) was required to determine the minimum inhibitory concentrations (MICs) of the slowly growing NTM. The MICs for a variety of different antibiotics against the slowly growing NTM were determined by the WST-1 colorimetric method and compared with those obtained using the broth microdilution methods approved by the CLSI. Good agreement was found between the MICs determined after 3–4 days using the WST-1 colorimetric method and those obtained after 10–14 days using the broth microdilution method. The results suggest that the WST-1 colorimetric assay is a useful method for the rapid determination of the MICs for the slowly growing NTM.

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Introduction

Nontuberculous mycobacteria (NTM) are ubiquitous environmental microorganisms that can be found in soil and water [1–4]. NTM are considered to be opportunistic pathogens, and several species are associated with human disease. Slowly growing NTM, including *M. avium-M. intracellulare* complex (MAC), *Mycobacterium kansasii*, and *M. marinum*, have been implicated in a variety of different human diseases, including pulmonary disease [5–8], as well as disseminated disease in immunocompetent and immunocompromised patients, including those with acquired immunodeficiency syndrome (AIDS) [9–12]. In recent years, the prevalence of clinical infections caused by these NTM has continued to increase throughout the world [13–15].

With this in mind, the development of a rapid and accurate susceptibility testing system for slowly growing NTM is, therefore, particularly important to allow for effective patient management and clinical surveillance. For the susceptibility testing of mycobacteria, the standard method approved by the Clinical and Laboratory Standards Institute (CLSI) is frequently used [16]. According to this method, the incubation time allowed for determining the antimicrobial susceptibility for slowly growing NTM should be set at 7 days. In addition, the M24-A2 document provided by the CLSI specifies that the incubation time should be extended to 10–14 days when the growth is poor. In the CLSI method, a long incubation time is required to allow for the visual detection of the growth of slowly growing NTM. With this in mind, the development of a rapid susceptibility testing system for slowly growing NTM would add significant value to the existing methods of analysis.

Tetrazolium salts have been widely used as a tool for measuring the metabolic activities of mammalian cells and microorganisms [17]. One of the most commonly used tetrazolium

salts in colorimetric assays for testing microorganisms is 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT), which undergoes a reduction reaction to yield a water-soluble formazan derivative that can be easily quantified colorimetrically. The application of XTT in conjunction with an electron mediator such as 2-methyl-1,4-napthoquinone (NQ) or phenazine methosulfate (PMS) has been used in rapid colorimetric assays for the antimicrobial susceptibility testing of both bacteria and fungi [18–20]. The XTT colorimetric method has also been applied to the susceptibility testing of mycobacteria [21–24]. In the previously reported method, however, XTT was only added to the test solution after the mycobacteria and antibiotics had been incubated for a suitable period of time. The XTT was added in this way to avoid the non-cellular reduction of XTT that can occur in the medium during the incubation period. We have previously reported that XTT can be readily reduced by a variety of the different components present in the culture media, such as peptones, glycated proteins, and some antibiotics [25, 26]. The underestimation of the activities of the antimicrobial substances can be caused by the non-cellular reduction of XTT. The time required for susceptibility testing by the XTT colorimetric method is slightly shorter than or almost equal to the time period required by the CLSI method. Therefore, if the indicator reagents could be added to the test solution together with the mycobacteria and the antibiotics in the initial step, the new method could add significant value to the overall process in terms of enhancing the rapidity and convenience of the assay.

Recently, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) has been developed [27]. WST-1 has been successfully used in assays for cell viability and cytotoxicity [28, 29]. WST-1, however, has not yet been applied to the susceptibility testing of slowly growing NTM. With this in mind, we propose the development of a colorimetric method based on the reduction of WST-1 with 2,3,5,6-tetramethyl-1,4-benzoquinone (BQ) for use as a microbial viability assay. In this method, 2,3,5,6-tetramethyl-1,4-BQ used as an electron mediator is reduced by mycobacteria, and WST-1 is then reduced by the produced hydroquinone to its formazan, which exhibits a maximum absorbance at 440 nm. Based on our current results, the combination of WST-1 and 2,3,5,6-tetramethyl-1,4-BQ can be superior to the combination of XTT with 2-methyl-1,4-NQ or PMS in terms of the metabolic efficiency of the mycobacteria and the stability of the materials in the medium.

The purpose of this study was to apply the WST-1 colorimetric method to the susceptibility testing of a variety of different slowly growing NTM and to demonstrate the advantages of the current method over the broth microdilution methods approved by the CLSI. In addition, we have evaluated combinations of two tetrazolium salts, namely WST-1 and XTT, with electron mediators for the assays of slowly growing NTM in terms of the metabolic efficiency of the mycobacteria and the influence of the different components within the medium on the outcome of the process. The WST-1 colorimetric method could provide a useful means for the rapid determination of the minimum inhibitory concentration (MIC) values for slowly growing NTM.

Materials and methods

Chemicals and media

2,3,5,6-Tetramethyl-1,4-BQ was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Methyl-1,4-NQ, PMS, and XTT were obtained from Sigma Chemicals (St. Louis, MO, USA). WST-1 and 1-methoxy-PMS were provided by Dojindo (Kumamoto, Japan). Middlebrook 7H9 broth and Middlebrook ADC enrichment were purchased from Becton Dickinson and Company (Parsippany, NJ, USA). All of the other chemicals used were of analytical reagent grade and were used without further purification.

Detection reagents

The tetrazolium salts (WST-1 and XTT) were dissolved in distilled water at a concentration of 11.1 mM and then filter sterilized with a membrane filter (0.2 μm). 2,3,5,6-Tetramethyl-1,4-BQ and 2-methyl-1,4-NQ were used as electron mediators and were dissolved in DMSO at a concentration of 8.0 mM. PMS and 1-methoxy-PMS as electron mediators were prepared with distilled water to a concentration of 8.0 mM. The tetrazolium salt solution was then mixed with the electron mediator solution in a 9:1 (*v*/*v*) ratio. The resulting detection reagent contained 10 mM of the tetrazolium salts and 0.8 mM of the electron mediators.

Antibiotics

The representative antibiotics employed in the current study were as follows: amikacin (AMK), azithromycin (AZM), ciprofloxacin (CPFX), clarithromycin (CAM), ethambutol (EB), isoniazid (INH), levofloxacin (LVFX), linezolid (LZD), moxifloxacin (MFLX), rifabutin (RBT), rifampicin (RFP), and streptomycin (SM).

Slowly growing NTM strains and growth conditions

M. kansasii ATCC12478, *M. intracellulare* ATCC13950, and *M. avium* GTC603 were purchased as the type strains from the American Type Culture Collection (ATCC, Rockville, MD, USA) and Gifu University (Gifu, Japan). Clinical isolates (*M. kansasii* and *M. avium*) were obtained from Gifu University. CAM-resistant *M. kansasii* ATCC12478cr and RFP-

resistant *M. kansasii* ATCC12478rr were produced from *M. kansasii* ATCC12478 according to a repeated 3-weekly passage in Middlebrook 7H10 agar containing increasing levels of CAM or RFP up to 128 μg/ml.

Slowly growing NTM strains were incubated in Middlebrook 7H9 broth (pH 6.8) containing 10 % ADC enrichment and 0.2 % glycerol for 14 days at 37 °C.

Susceptibility testing

Reference MIC values were determined by the broth microdilution method currently recommended by the CLSI [16]. Serial two-fold dilutions of each antibiotic were prepared in Middlebrook 7H9 broth containing 10 % ADC enrichment and 0.2 % glycerol. The pH of the medium was adjusted to 6.8 for *M. kansasii*. For *M. avium* and *M. intracellulare*, a pH of 7.4 was employed because macrolides display a greater level of in vitro activity under mildly alkaline conditions than under mildly acidic conditions [16]. The slowly growing NTM strains were adjusted with phosphate-buffered saline (PBS) to give a turbidity value equal to that of the 0.5 McFarland standard, and then diluted 10-fold with PBS. The prepared mycobacteria suspension was further added to an antibiotic solution to provide a final inoculum density of approximately 10^4–10^5 colony-forming units (CFU)/ml in each well of a 96-well microtiter plate. Each of the wells was then inoculated with 200 μl of inoculum, and the plate was sealed with a transparent film (FG-DM100PCR, Nippon Genetics Co., Tokyo, Japan) before being incubated for 7–14 days at 37 °C. Following the incubation, the MIC was read as the lowest concentration of antibiotic at which there was no visible growth.

For the susceptibility testing using the WST-1 colorimetric method, 10 μl of the detection reagent was added to each inoculum (190 μl), which had been prepared as described above. The 96-well microtiter plate was then sealed with a transparent film and incubated at 37 °C. A blank test, which contained antibiotics and the detection reagent in the absence of mycobacteria, was also set up in a similar manner. The formazan produced was measured at 440 nm using a microplate reader (VersaMax, Molecular Devices Co., Sunnyvale, CA, USA) at 0, 1, 2, 3, 4, 7, 10, and 14 days. The measured absorbance values were calculated by the subtraction of the blank value from the test values to eliminate the influence of the background with antibiotics. When the control absorbance value, which was obtained from the mycobacteria in the absence of antibiotics, provided a value greater than 0.5, the MIC was read as the lowest concentration of antibiotic at which the measured value was less than one-fifth of the control value.

Results

Comparison of WST-1 and XTT

The WST-1 and XTT compounds contain sulfonate groups that give them a net negative charge and reduce their ability to move across microbial cell membranes [17]. It is, therefore, necessary to use an electron mediator to facilitate the cellular reduction of the tetrazolium salts. In previous reports, XTT has been used in combination with an electron mediator such as 2-methyl-1,4-NQ or PMS for the susceptibility testing of mycobacteria [21–24]. The WST-1 and XTT colorimetric methods were, therefore, compared in terms of their reactivity with *M. kansasii* and *M. avium* (Fig. 1a, b). In the proposed method, the combination of WST-1 and 2,3,5,6-tetramethyl-1,4-BQ was used. On the other hand, XTT was combined with 2-methyl-1,4-NQ, PMS, and 1-methoxy-PMS, with each combination being subsequently evaluated. Figure 1a, b reveal that the combination of WST-1 and 2,3,5,6-tetramethyl-1,4-BQ was superior to the other XTT combinations in terms of the reactivity for slowly growing NTM.

In addition, the influences of the Middlebrook 7H9 broth, which is usually used for the susceptibility testing of mycobacteria, on the reductions of WST-1 and XTT in the presence of an electron mediator were compared (Fig. 1c). When XTT was used in combination with an electron mediator, the non-cellular reductions in the Middlebrook 7H9 broth in the absence of mycobacteria were pronounced. In contrast, when WST-1 was used in combination with 2,3,5,6-tetramethyl-1,4-BQ, the level of non-cellular reduction in the Middlebrook 7H9 broth was negligible.

Taken together, these results indicate that the WST-1 colorimetric method is superior to the conventional XTT colorimetric methods with regard to the reactivity for slowly growing NTM and the influences of the components in the medium.

Cell proliferation assay

The cell proliferation assay was investigated to evaluate the applicability of the WST-1 colorimetric method for the susceptibility testing of slowly growing NTM. Figure 2 shows the relationship between the incubation time and the absorbance in *M. kansasii* and *M. avium*.

When the detection time is defined as the time required to give an absorbance change of 0.5, the detection time (y) can be expressed as follows:

$$M.kansasii : y = -16.47 \log [x] + 243.9 \quad (1)$$

$$M.avium : y = -14.10 \log [x] + 222.9 \quad (2)$$

where $[x]$ is the initial cell density (CFU/ml). With good correlation coefficients ($r > 0.991$), linear relationships

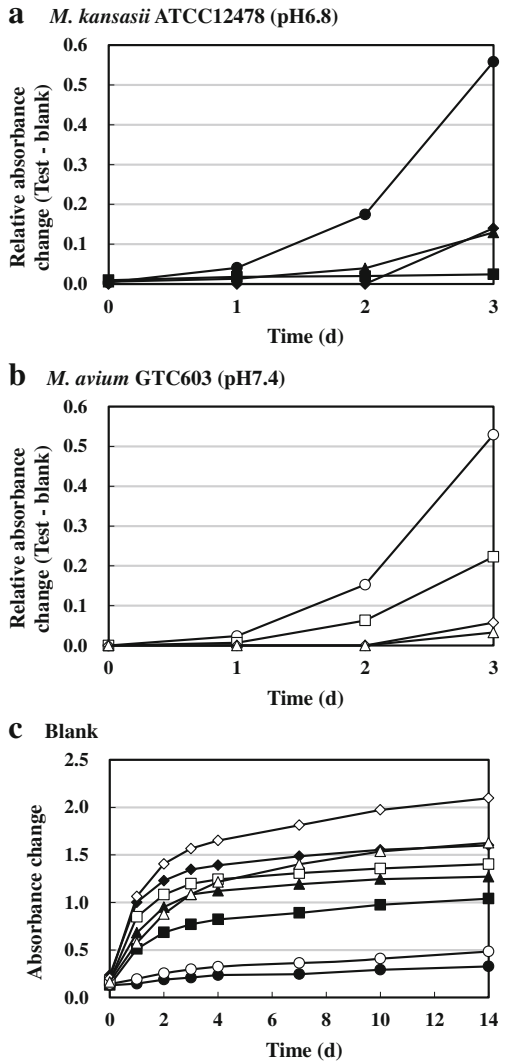

Fig. 1 Comparison of the WST-1 colorimetric method and the conventional XTT method on the basis of the reactivity of slowly growing NTM and the influence of the media components. *black circles*, *white circles*, WST-1 and 2,3,5,6-tetramethyl-1,4-BQ; *black squares*, *white squares*, XTT and 2-methyl-1,4-NQ; *black diamonds*, *white diamonds*, XTT and PMS; *black triangles*, *white triangles*, XTT and 1-methoxy-PMS; *black symbols*, pH 6.8; *white symbols*, pH 7.4. The data represent the means for four identical wells of a microtiter plate from each experiment

between the detection time (y) and the initial cell density (x) were subsequently obtained. Equation (1) shows that it takes about 54 h to produce an absorbance change of 0.5 with *M. kansasii* at 10^5 CFU/ml. Similarly, the detection time for *M. avium* at 10^5 CFU/ml was estimated to be about 60 h using Eq. (2). These results indicate that a cell density of approximately 10^5 CFU/ml could be detected in 3 days by the WST-1 colorimetric method.

Based on these results, it is possible that the susceptibility testing of slowly growing NTM could be completed by the WST-1 colorimetric method in 3 days, because the initial

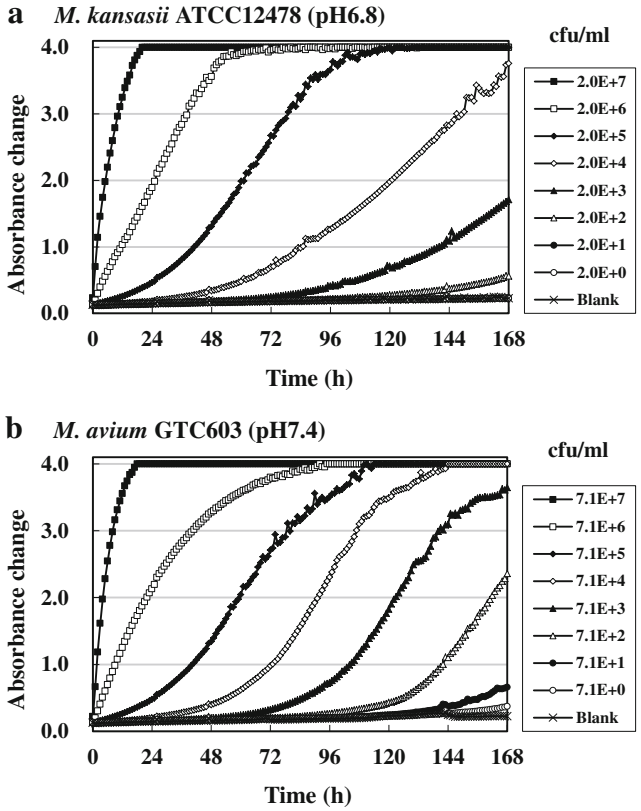

Fig. 2 Cell proliferation assays for *M. kansasii* and *M. avium* using the WST-1 colorimetric method. The data represent the means for four identical wells of a microtiter plate from each experiment

inoculum density was approximately 10^4–10^5 CFU/ml in the susceptibility testing.

Susceptibility curves and rapid MIC determination

To confirm the incubation time required to obtain MICs for slowly growing NTM using the WST-1 colorimetric method, the effects of incubation time on the susceptibility curves were studied using *M. kansasii* and CAM or RFP (Fig. 3). CAM- and RFP-susceptible *M. kansasii* ATCC12478, CAM-resistant *M. kansasii* ATCC12478cr, and RFP-resistant *M. kansasii* ATCC12478rr were employed for the susceptibility testing. For the CAM-susceptible *M. kansasii*, the MIC towards CAM was estimated to be 0.5 μg/ml at an incubation time of 7 days (Fig. 3a). Furthermore, to shorten the detection time required for the MICs, we defined that the MIC was read as the lowest concentration of antibiotic at which the measured absorbance value was less than one-fifth of the control absorbance value when the absorbance provides a value greater than 0.5. As shown in Fig. 3a, the control absorbance value was greater than 0.5 at 3 days, and the MIC value was consequently estimated to be 0.5 μg/ml. On the other hand, the MIC for CAM against CAM-resistant *M. kansasii* was estimated to be >32 μg/ml at 3 days (Fig. 3b). These MIC values agreed

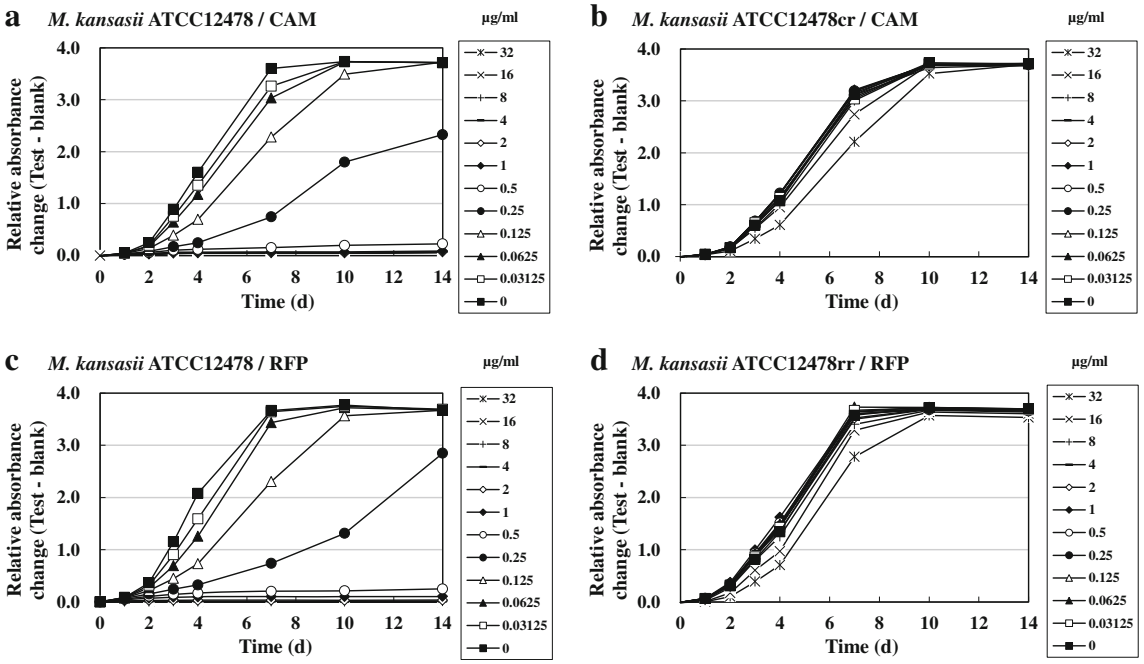

Fig. 3 Effect of incubation time on the susceptibility curves using drug-susceptible and drug-resistant *M. kansasii*. The data represent the means for four identical wells of a microtiter plate from each experiment

with those obtained using the CLSI method at 10–14 days (data not shown). RFP-susceptible *M. kansasii* ATCC12478 and RFP-resistant *M. kansasii* ATCC12478rr were similarly employed for the susceptibility testing using the proposed method and the broth microdilution method. On the basis of the definition provided above for the rapid determination of the MIC, the MIC for RFP against *M. kansasii* ATCC12478 was also measured by the WST-1 colorimetric method at 3 days and estimated to be 0.5 μg/ml (Fig. 3c). On the other hand, the MIC for RFP against RFP-resistant *M. kansasii* was estimated to be >32 μg/ml at 3 days (Fig. 3d). These MIC values were in good agreement with those determined using the broth microdilution method at 10–14 days. Taken together, these results suggested that the MICs for slowly growing NTM could be determined by the WST-1 colorimetric method at 3 days on the basis of the definition provided above for the rapid determination of the MIC. Furthermore, the results suggested that the proposed method would allow for the drug-susceptible and drug-resistant NTM to be effectively distinguished at 3 days.

Determination of the MIC values for the slowly growing NTM

The broth microdilution method approved by the CLSI recommends an incubation time of 7–14 days to obtain the final antimicrobial susceptibility results for slowly growing NTM [16]. The development of a method capable of providing rapid susceptibility testing for slowly growing NTM would, therefore, be particularly useful. To evaluate the applicability of the WST-1 colorimetric method to rapid susceptibility testing, MIC values determined by the proposed method were compared with those obtained by the broth microdilution method at a variety of different incubation times.

The type strains of slowly growing NTM, *M. kansasii* ATCC12478, *M. avium* GTC603, and *M. intracellulare* ATCC13950, were employed for this comparison. Table 1 shows a comparison of the MIC values determined for a variety of different antibiotics using the WST-1 colorimetric method and the broth microdilution method in *M. kansasii* ATCC12478. In the broth microdilution method, the MIC values tended to increase with increasing incubation time. It is noteworthy that more than 7 days was required to obtain consistent MIC values. There was excellent agreement between the MIC values obtained at 3 days using the WST-1 colorimetric method and those obtained after 10 or 14 days using the broth microdilution method, within ±1 $\log_2$ difference. Similarly, the MIC values of a variety of different antibiotics against *M. avium* GTC603 and *M. intracellulare* ATCC13950 using the broth microdilution method tended to increase with increasing incubation time (Table 2). More than 7 days was required in the broth microdilution method to obtain consistent MIC values against *M. avium* and *M. intracellulare*. The MIC values obtained at 3 days using the proposed method were in good agreement with those obtained after 10–14 days using the broth microdilution method, within ±1 $\log_2$ difference. In the type strains of slowly growing NTM, the percentage of MIC values located at ±1 $\log_2$ difference was 100 %.

Table 1 Comparison of the MIC values (in μg/ml) of a variety of different antibiotics determined by the WST-1 colorimetric method and the broth microdilution method in *M. kansasii* ATCC12478

Antibiotics	WST-1 colorimetric method (3 days)	Broth microdilution method		
		7 days	10 days	14 days
Primary agents				
CAM	0.5	0.25	0.5	0.5
RFP	0.5	0.25	0.25	0.5
Secondary agents				
AMK	8	4	4	4
CPFX	1	1	2	2
LVFX	2	2	4	4–8
EB	8	4	4	4–8
LZD	2	2	2	2–4
MFLX	0.125	0.0625	0.125	0.25
RBT	0.0078	0.0078	0.0078	0.0165
Others				
INH	4	4	4	8
SM	>256	>256	>256	>256
AZM	8	4	8	16

To evaluate the utility of the WST-1 colorimetric method for the rapid determination of antimicrobial susceptibility, this approach was also used to assess the susceptibility of clinical isolates of slowly growing NTM in the presence of a variety of different antibiotics. Eleven strains of *M. kansasii* and six strains of *M. avium* were employed as clinical isolates. Table 3 shows a comparison of the MIC values determined by the WST-1 colorimetric method and by the broth microdilution method at 10–14 days. The time required for the measurement of the MIC values in the WST-1 colorimetric method was found to be 3–4 days on the basis of the definition provided for the rapid determination of the MIC. This difference likely occurred because the growth of a few clinical isolates may have been very slow. The incubation period was, therefore, extended to 4 days and the MIC values were then determined by the proposed method. The percentages of the MIC values

Table 2 Comparison of the MIC values (in μg/ml) of a variety of different antibiotics determined by the WST-1 colorimetric method and the broth microdilution method in *M. avium* GTC603 and *M. intracellulare* ATCC13950

Antibiotics	WST-1 colorimetric method (3 days)	Broth microdilution method		
		7 days	10 days	14 days
M. avium GTC603				
Primary agents				
CAM	0.0625	0.125	0.125	0.125
Secondary agents				
LZD	1	1	2	2
MFLX	2	2	4	4
Others				
AZM	1	2	2	2
LVFX	4	8	8	8
M. intracellulare ATCC13950				
Primary agents				
CAM	0.0313	0.0313	0.0313	0.0313
Secondary agents				
LZD	1	0.25	0.5	1
MFLX	0.125	0.0313	0.125	0.25
Others				
AZM	0.5	1	1	1
LVFX	0.5	0.5	1	1

Table 3 Comparison of MIC values of a variety of different antibiotics determined by the WST-1 colorimetric method and the broth microdilution method in clinical isolates of *M. kansasii* and *M. avium*

Isolates	Antibiotics	Number of isolates with the following differences in MICs[a]					Agreement[b] (%)
		−2	−1	0	+1	+2	
M. kansasii (*n*=11)	CAM, RFP, AMK, CPFX, EB, LZD, MFLX, RBT	3	26	35	22	2	94.3
M. avium (*n*=6)	CAM, LZD, MFLX, AZM, EB, LVFX	1	10	10	8	2	90.3
Total		4	36	45	30	4	93.3

[a] Zero indicates the number of isolates for which the MICs are identical; −2, −1, +1, and +2 indicate −2, −1, +1, and +2 $\log_2$ differences, respectively

[b] Percentage of the isolates within the accuracy limits of the test (±1 $\log_2$ dilution)

located at ±1 $\log_2$ difference between the proposed method and the broth microdilution method was 93.3 %. Furthermore, to better assess the degree of agreement between the MIC results obtained from the WST-1 colorimetric method and the broth microdilution method, the Wilcoxon signed-ranked test was performed. *p*-values derived from the Wilcoxon signed-rank test demonstrated no significant differences (p= 0.705) between the proposed method and the broth microdilution method. The result emphasized that there was good agreement between the MIC values determined by the WST-1 colorimetric method at 3–4 days and those obtained by the broth microdilution method at 10–14 days.

Taken together, these findings, therefore, suggest that the present method provides a useful tool for the rapid determination of the MIC values for slowly growing NTM.

Discussion

We have successfully developed a WST-1 colorimetric method based on the determination of microbial viability, and demonstrated the advantages of this method over the conventional XTT colorimetric method. The results of this study revealed that 2,3,5,6-tetramethyl-1,4-BQ was the electron mediator most effectively metabolized by the slowly growing NTM. Furthermore, it became clear that WST-1 was superior to the conventional tetrazolium salt XTT with regard to its reactive efficiency with the hydroquinones produced by the slowly growing NTM and the way in which it was influenced by the different components in the medium. The XTT colorimetric method has already been applied to the susceptibility testing of mycobacteria [21–24]. According to the previously reported method, however, XTT was added to the test solution following the incubation of the mycobacteria and the antibiotics, because of the non-cellular reduction of the XTT that can occur in the medium during the incubation period. The time required for susceptibility testing by the XTT colorimetric method was, therefore, slightly shorter than or almost equal to that required by the CLSI method. In addition, the procedure for the addition of the XTT following the incubation could potentially lead to the injury of the operators because the lid of the 96-well microtiter plate containing the pathogenic microorganisms must be opened. In contrast, the indicator reagent can be added to the test solution together with the mycobacteria and the antibiotics in the initial step of the WST-1 colorimetric method. The proposed method was, therefore, superior to the XTT colorimetric method with regard to the overall rapidity, convenience, and safety of the assay.

Furthermore, a method was successfully established for the rapid susceptibility testing of slowly growing NTM using the WST-1 colorimetric method, and its advantages over the broth microdilution method approved by the CLSI were demonstrated. The incubation time suggested by the CLSI method to determine the antimicrobial susceptibility of slowly growing NTM is between 7 and 14 days [16]. With this in mind, the WST-1 colorimetric method was applied to the susceptibility testing, and the MIC values determined by the proposed method were compared with those obtained by the broth microdilution method at a variety of different incubation times. For the type strains of slowly growing NTM, there was excellent agreement between the MIC values determined after 3 days using the WST-1 colorimetric method and those obtained after 10–14 days using the broth microdilution method. Furthermore, to evaluate the utility of the proposed method for the rapid determination of antimicrobial susceptibility for slowly growing NTM, we used this approach to assess the susceptibility of clinical isolates in the presence of a variety of different antibiotics. The concordance rate of the MIC values determined by the proposed method at 3 or 4 days to those obtained by the broth microdilution method at 10–14 days was 93.3 %, with no significant differences observed between the data obtained from the two different methods. This result suggested that susceptibility testing of clinical isolates could be achieved more rapidly by the WST-1 colorimetric method than the broth microdilution method.

In the WST-1 colorimetric method, an additional procedure is required for the addition of the detection reagent compared with the broth microdilution method. In spite of this minor difference, however, the proposed method has several advantages over the CLSI method, and the WST-1 colorimetric

method, therefore, provides a good alternative to the tedious and time-consuming features of the broth microdilution method.

Recently, the prevalence of clinical infections caused by NTM has continued to increase throughout the world [13–15]. Furthermore, the emergence of infectious diseases caused by multidrug-resistant *M. tuberculosis* continues to expand throughout the world [30–32]. With this in mind, we believe that the current WST-1 colorimetric method could be applied to the rapid susceptibility testing of *M. tuberculosis*, and hope that this method might be used in the future as an alternative to the CLSI method for the susceptibility testing of slowly growing mycobacteria.

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Conflict of interest The authors declare no conflict of interest.

References

1. Covert TC, Rodgers MR, Reyes AL, Stelma GN Jr (1999) Occurrence of nontuberculous mycobacteria in environmental samples. Appl Environ Microbiol 65:2492–2496
2. De Groote MA, Pace NR, Fulton K, Falkinham JO 3rd (2006) Relationships between *Mycobacterium* isolates from patients with pulmonary mycobacterial infection and potting soils. Appl Environ Microbiol 72:7602–7606. doi:10.1128/AEM.00930-06
3. Falkinham JO 3rd (2009) Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. J Appl Microbiol 107:356–367. doi:10.1111/j.1365-2672.2009.04161.x
4. Fernandez-Rendon E, Cerna-Cortes JF, Ramirez-Medina MA, Helguera-Repetto AC, Rivera-Gutierrez S, Estrada-Garcia T, Gonzalez-y-Merchand JA (2012) *Mycobacterium mucogenicum* and other non-tuberculous mycobacteria in potable water of a trauma hospital: a potential source for human infection. J Hosp Infect 80:74–76. doi:10.1016/j.jhin.2011.10.003
5. Shitrit D, Baum GL, Priess R, Lavy A, Shitrit ABG, Raz M, Shlomi D, Daniele B, Kramer MR (2006) Pulmonary *Mycobacterium kansasii* infection in Israel, 1999–2004: clinical features, drug susceptibility, and outcome. Chest 129:771–776. doi:10.1378/chest.129.3.771
6. Winthrop KL, McNelley E, Kendall B, Marshall-Olson A, Morris C, Cassidy M, Saulson A, Hedberg K (2010) Pulmonary nontuberculous mycobacterial disease prevalence and clinical features: an emerging public health disease. Am J Respir Crit Care Med 182:977–982. doi:10.1164/rccm.201003-0503OC
7. Maekawa K, Ito Y, Hirai T, Kubo T, Imai S, Tatsumi S, Fujita K, Takakura S, Niimi A, Iinuma Y, Ichiyama S, Togashi K, Mishima M (2011) Environmental risk factors for pulmonary *Mycobacterium avium-intracellulare* complex disease. Chest 140:723–729. doi:10.1378/chest.10-2315
8. Huang CT, Tsai YJ, Wu HD, Wang JY, Yu CJ, Lee LN, Yang PC (2012) Impact of non-tuberculous mycobacteria on pulmonary function decline in chronic obstructive pulmonary disease. Int J Tuberc Lung Dis 16:539–545. doi:10.5588/ijtld.11.0412
9. Horsburgh CR Jr, Gettings J, Alexander LN, Lennox JL (2001) Disseminated *Mycobacterium avium* complex disease among patients infected with human immunodeficiency virus, 1985–2000. Clin Infect Dis 33:1938–1943. doi:10.1086/324508
10. Taillard C, Greub G, Weber R, Pfyffer GE, Bodmer T, Zimmerli S, Frei R, Bassetti S, Rohner P, Piffaretti JC, Bernasconi E, Bille J, Telenti A, Prod'hom G (2003) Clinical implications of *Mycobacterium kansasii* species heterogeneity: Swiss national survey. J Clin Microbiol 41:1240–1244. doi:10.1128/JCM.41.3.1240-1244.2003
11. Henry MT, Inamdar L, O'Riordain D, Schweiger M, Watson JP (2004) Nontuberculous mycobacteria in non-HIV patients: epidemiology, treatment and response. Eur Respir J 23:741–746. doi:10.1183/09031936.04.00114004
12. Lai CC, Lee LN, Ding LW, Yu CJ, Hsueh PR, Yang PC (2006) Emergence of disseminated infections due to nontuberculous mycobacteria in non-HIV-infected patients, including immunocompetent and immunocompromised patients in a university hospital in Taiwan. J Infect 53:77–84. doi:10.1016/j.jinf.2005.10.009
13. Moore JE, Kruijshaar ME, Ormerod LP, Drobniewski F, Abubakar I (2010) Increasing reports of non-tuberculous mycobacteria in England, Wales and Northern Ireland, 1995–2006. BMC Public Health 10:612. doi:10.1186/1471-2458-10-612
14. Lai CC, Tan CK, Chou CH, Hsu HL, Liao CH, Huang YT, Yang PC, Luh KT, Hsueh PR (2010) Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000–2008. Emerg Infect Dis 16:294–296. doi:10.3201/eid1602.090675
15. Park YS, Lee CH, Lee SM, Yang SC, Yoo CG, Kim YW, Han SK, Shim YS, Yim JJ (2010) Rapid increase of non-tuberculous mycobacterial lung diseases at a tertiary referral hospital in South Korea. Int J Tuberc Lung Dis 14:1069–1071
16. Clinical and Laboratory Standards Institute (CLSI) (2011) Susceptibility testing of Mycobacteria, Nocardiae, and other aerobic Actinomycetes; Approved standard—Second edition. CLSI document M24-A2. CLSI, Wayne, PA, USA
17. Berridge MV, Herst PM, Tan AS (2005) Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev 11:127–152. doi:10.1016/S1387-2656(05)11004-7
18. Meletiadis J, Mouton JW, Meis JF, Bouman BA, Donnelly JP, Verweij PE; EUROFUNG Network (2001) Colorimetric assay for antifungal susceptibility testing of *Aspergillus* species. J Clin Microbiol 39:3402–3408. doi:10.1128/JCM.39.9.3402-3408.2001
19. Tunney MM, Ramage G, Field TR, Moriarty TF, Storey DG (2004) Rapid colorimetric assay for antimicrobial susceptibility testing of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 48:1879–1881. doi:10.1128/AAC.48.5.1879-1881.2004
20. Moriarty F, Elborn S, Tunney M (2005) Development of a rapid colorimetric time–kill assay for determining the in vitro activity of ceftazidime and tobramycin in combination against *Pseudomonas aeruginosa*. J Microbiol Methods 61:171–179. doi:10.1016/j.mimet.2004.11.010
21. De Logu A, Uda P, Pellerano ML, Pusceddu MC, Saddi B, Schivo ML (2001) Comparison of two rapid colorimetric methods for determining resistance of *Mycobacterium tuberculosis* to rifampin, isoniazid, and streptomycin in liquid medium. Eur J Clin Microbiol Infect Dis 20:33–39. doi:10.1007/PL00011234
22. De Logu A, Pellerano ML, Sanna A, Pusceddu MC, Uda P, Saddi B (2003) Comparison of the susceptibility testing of clinical isolates of *Mycobacterium tuberculosis* by the XTT colorimetric method and the NCCLS standards method. Int J Antimicrob Agents 21:244–250. doi:10.1016/S0924-8579(02)00350-3

23. De Logu A, Borgna R, Uda P, Sanna A, Pellerano ML, Saddi B (2003) The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay as rapid colorimetric method for determination of antibiotic susceptibility of clinical *Mycobacterium tuberculosis* isolates in liquid medium. Clin Lab 49:357–365
24. Singh U, Akhtar S, Mishra A, Sarkar D (2011) A novel screening method based on menadione mediated rapid reduction of tetrazolium salt for testing of anti-mycobacterial agents. J Microbiol Methods 84:202–207. doi:10.1016/j.mimet.2010.11.013
25. Tsukatani T, Suenaga H, Higuchi T, Akao T, Ishiyama M, Ezoe K, Matsumoto K (2008) Colorimetric cell proliferation assay for microorganisms in microtiter plate using water-soluble tetrazolium salts. J Microbiol Methods 75:109–116. doi:10.1016/j.mimet.2008.05.016
26. Tsukatani T, Higuchi T, Suenaga H, Akao T, Ishiyama M, Ezoe T, Matsumoto K (2009) Colorimetric microbial viability assay based on reduction of water-soluble tetrazolium salts for antimicrobial susceptibility testing and screening of antimicrobial substances. Anal Biochem 393:117–125. doi:10.1016/j.ab.2009.06.026
27. Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He P (1993) A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull 41:1118–1122
28. Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K (1996) A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 19:1518–1520
29. Johnsen AR, Bendixen K, Karlson U (2002) Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates by using the respiration indicator WST-1. Appl Environ Microbiol 68:2683–2689. doi:10.1128/AEM.68.6.2683-2689.2002
30. Mäkinen J, Marjamäki M, Haanperä-Heikkinen M, Marttila H, Endourova LB, Presnova SE, Mathys V, Bifani P, Ruohonen R, Viljanen MK, Soini H (2011) Extremely high prevalence of multidrug resistant tuberculosis in Murmansk, Russia: a population-based study. Eur J Clin Microbiol Infect Dis 30:1119–1126. doi:10.1007/s10096-011-1200-7
31. Agrawal D, Udwadia ZF, Rodriguez C, Mehta A (2009) Increasing incidence of fluoroquinolone-resistant *Mycobacterium tuberculosis* in Mumbai, India. Int J Tuberc Lung Dis 13:79–83
32. Sanchez-Padilla E, Dlamini T, Ascorra A, Rüsch-Gerdes S, Tefera ZD, Calain P, de la Tour R, Jochims F, Richter E, Bonnet M (2012) High prevalence of multidrug-resistant tuberculosis, Swaziland, 2009–2010. Emerg Infect Dis 18:29–37. doi:10.3201/eid1801.110850