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A New Method for Antimicrobial Susceptibility Testing of *Vitro*-cultured Bacteria by Means of Resonance Light Scattering Technique

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A new method for antimicrobial susceptibility testing of *vitro*-cultured bacteria on an ordinary fluorescence spectrometer was developed. The viable bacteria reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to produce insoluble particles that displayed intense resonance scattering light. The assay showed a linear relationship between the number of viable bacteria and the intensity of resonance scattering light. Dead bacteria were unable to reduce MTT. Methicillin-resistant *Staphylococcus aureus* exposed to flavonoids from *Marchantia convoluta* showed a flavonoids concentration-dependent inhibition of the ability to reduce MTT. In the assay, less than 12 h was required to attain susceptibility results and fewer bacteria were utilized than in traditional methods. The RLS technique could, in combination with the MTT assay, be a rapid and sensitive measuring method to determine the *in vitro* activity of new antimicrobials.

Keywords: MTT, antimicrobial susceptibility testing, resonance light scattering

Conventional methods for antimicrobial susceptibility testing such as the dry weight method, viable cell counting methods, turbidity method, etc., are time-consuming and cumbersome. Since the 1980s, several analytical methods for antimicrobial susceptibility testing of bacteria have been developed [8], such as the Kirby-Bauer disk diffusion antibiotic susceptibility testing [14, 33], Wadsworth method [28], ROSCO tablets method [30], broth dilution method [10], and PCR-based genotype MTBDR assay [31]. Great improvements have been made compared with conventional methods, as these new methods are more rapid or simpler. The standard Kirby-Bauer method issued by the National Committee on Clinical Laboratory Standards has been especially used for susceptibility testing in many countries. The accuracy and reproducibility of the Kirby-Bauer method are dependent on maintaining a standard set of procedures. However, commonly used methods of antimicrobial susceptibility testing are much too time-consuming (18–24 h), particularly with microorganisms such as tuberculosis bacteria, which grow very slowly in the culture dish. Moreover, these methods require a large number of bacteria (1.5×10^8 CFU/ml).

Resonance light scattering (RLS), an elastic scattering, occurs when an incident beam in energy is close to an absorption band. RLS is an appropriate technique for detecting and characterizing the extended aggregates of chromophores. Pasternack *et al.* [7, 26, 27] first established the RLS technique to study the biological macromolecules by means of an ordinary fluorescence spectrometer. Because of its high sensitivity, selectivity, and convenience, RLS studies have attracted great interest among researchers [2, 15, 18, 21]. RLS has emerged as a very attractive technique used to monitor molecular assemblies and to characterize the extended aggregates of chromophores [7, 18, 21, 26, 27]. In recent years, the RLS technique has been used to determine pharmaceutical [12, 37] and various biological macromolecules, such as nucleic acids [5, 20, 31], proteins [6, 11, 41], glucose [1], and ions [36]. However, the application of RLS technique for antimicrobial susceptibility testing has not been reported.

The yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by mitochondrial dehydrogenase in living cells to produce insoluble purple MTT formazan crystals (Fig. 1), which, after solubilization, can be measured spectrophotometrically [9, 13, 19, 35, 38, 39]. This property has long been used to assess the viability of cells. Herein, we assessed the possibility of using the RLS technique combined with MTT assay to establish a rapid and sensitive method for antimicrobial susceptibility testing of *vitro*-cultured bacteria.

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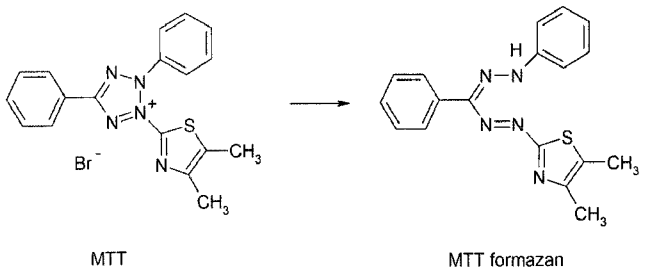

Fig. 1. Reaction scheme.

MATERIALS AND METHODS

Apparatus

RLS spectra were obtained by synchronous scanning in the wavelength region from 250 to 750 nm on a JASCO FP-6500 spectrofluorometer (Tokyo, Japan) using quartz cuvettes (1.0 cm). The width of the excitation and emission slits was set at 3.0 nm. The ultraviolet-visible (UV-vis) absorption spectra were carried out on a UV-2450 spectrophotometer (Shimadzu, Japan) with 1-cm quartz cells.

Bacterial Strain

Bacterial strain used in this study was methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 25923, which had been isolated by Dr. M. Xu in Hunan Want-want Hospital (Changsha, China). The bacterial strains were cultured overnight at 37°C in Mueller Hinton broth medium. Before the determination of the number of CFU, the bacteria were washed three times with phosphate-buffered saline (PBS) and plated for microcolony counting.

Inactivation of Bacteria

MRSA were inactivated by heat treatment at 91°C for 15 min in a boiling water bath or by treatment with a 5% hydrogen peroxide. Bacteria treated with hydrogen peroxide were washed three times with phosphate-buffered saline (PBS).

Standard Procedure

Freshly grown MRSA were standardized to a cell density of 1.5×10^8 CFU/ml (McFarland No. 0.5), and diluted to 1.5×10^6, 3.0×10^6, 4.5×10^6, 6.0×10^6, 7.5×10^6, 9.0×10^6, 1.05×10^7, 1.20×10^7, 1.35×10^7, 1.50×10^7, and 1.65×10^7 CFU/ml with PBS. The MTT assay was carried out as described by Mosmann [23], with some modification. Briefly, each well of a flat-bottom microtiter plate received 100 μl of bacterial suspension. Triplicate wells were used for each experimental condition. MTT (Sigma, U.S.A.) was dissolved with PBS (pH 7.2) to obtain a concentration of 5.0 mg/ml. Twenty μl of the MTT solution was then added to each culture well, and the plates were incubated for 10 h at 37°C. Then, the bacterial suspension was diluted to 5 ml with PBS (pH 7.2). The RLS spectrum was obtained by scanning simultaneously the excitation and emission monochromators with the wavelength range of 250–750 nm with $\Delta\lambda$=0 nm. The spectral bandwidths of the excitation and emission monochromators were both kept at 3.0 nm. RLS intensity was measured at 560 nm. The enhanced RLS intensity was represented as $\Delta I=I-I_0$ (I and I_0 are the RLS intensities of the system with and without MRSA).

Extracting Flavonoids from *Marchantia convoluta*

The flavonoids from *Marchantia convoluta* (MCF) were extracted according to the reference [20]. The powdered plant material (500 g) was extracted three times with 3,000 ml ethanol-water (1:1, v/v). The accumulated alcoholic extract was concentrated to dryness under reduced pressure and extracted three times with petroleum ether (100 ml). The residue was extracted three times with ethyl acetate (100 ml). Then, it was extracted three times with *n*-butanol (100 ml). The *n*-butanol extract was then filtered and concentrated to dryness under reduced pressure. The yield of MCF amounted to 4.83% (w/w). The content of total flavonoids in the *n*-butanol extract was determined through a visible spectrophotometer. The content of total flavonoids in MCF was 95.02%. MCF was dissolved in PBS to form stock solutions, and sterilized by filtration (0.2 μm) before testing.

MCF Susceptibility Testing

The MRSA (1.20×10^7 CFU/ml) were treated with various concentration of MCF for 24 h. After the incubation period, MCF susceptibility was determined by the RLS method as described above. The methicillin was used as a positive control group.

RESULTS

MTT-Bacteria System

Fifteen MTT-bacteria systems with different MRSA concentrations (1.5×10^6–6.0×10^7 CFU/ml) were investigated. The pictures of these systems are shown in Fig. 2. It was found that the solution color almost kept the color of the MTT dye solution with the MRSA concentration from 1.50×10^6 to 1.20×10^7 CFU/ml. However, when the MRSA concentration arrived at 1.35×10^7 CFU/ml, the obvious purple was seen and increased with the addition of bacteria. For the RLS assay, only the low concentrations of MRSA (<1.20×10^7 CFU/ml) without color change were considered. However, large numbers of MRSA (1.5×10^8 CFU/ml) were used in a traditional MTT assay by means of spectrophotometry.

Characteristics of the RLS Spectra

The RLS spectra of the MTT-MRSA system are shown in Fig. 3. It can be seen that the RLS intensity of MTT was quite weak in the whole scanning wavelength region

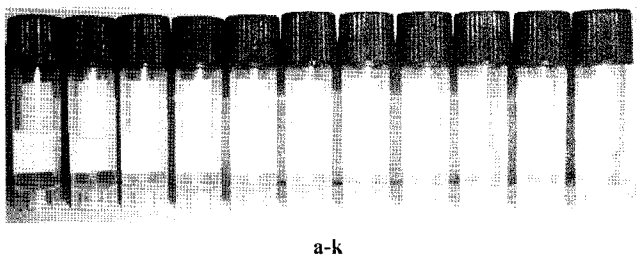

Fig. 2. The pictures of MTT-bacteria systems (a–k: 1.65×10^7, 1.50×10^7, 1.35×10^7, 1.20×10^7, 1.05×10^7, 9.0×10^6, 7.5×10^6, 6.0×10^6, 4.5×10^6, 3.0×10^6, and 1.5×10^6 CFU/ml).

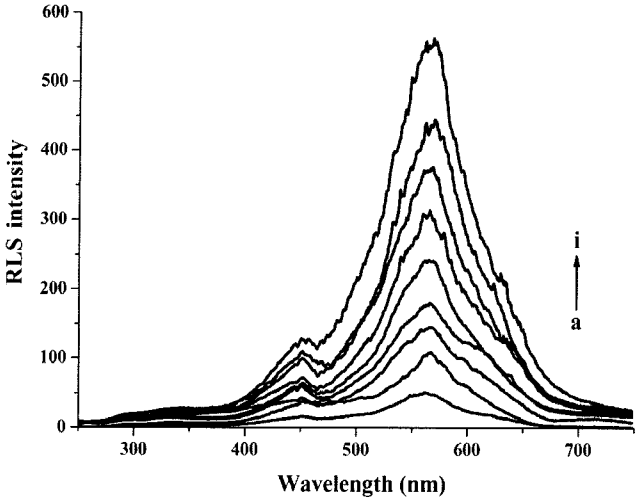

Fig. 3. RLS spectra of the MTT-bacteria system: 20 μg/ml of MTT (a); 1.20×10^7 CFU/ml (b); (c)–(i), 20 μg/ml of MTT; 1.5×10^6, 3.0×10^6, 4.5×10^6, 6.0×10^6, 7.5×10^6, 9.0×10^6, and 1.20×10^7 CFU/ml.

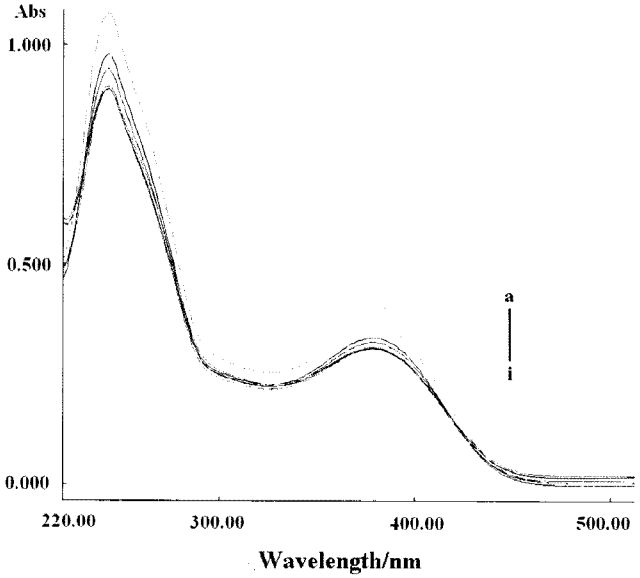

Fig. 4. UV spectra of the MTT-bacteria system: 20 μg/ml of MTT. a–i, 0, 1.5×10^6, 3.0×10^6, 4.5×10^6, 6.0×10^6, 7.5×10^6, 9.0×10^6, 1.05×10^7, and 1.20×10^7 CFU/ml.

(Fig. 3, spectrum a). In contrast, with the addition of trace amount of MRSA to MTT solution, a remarkably enhanced RLS with a maximum peak at 560 nm was observed under the same circumstances (Fig. 3, spectra b–i). It can be clearly observed that there were two peaks located at 452 and 560 nm in the RLS spectrum of the MTT-MRSA system. The addition of increasing MRSA to the MTT solution led to the gradual enhancement in RLS intensity, exhibiting a concentration-dependent relationship. The production of RLS and its intensity were correlative with the formation of the aggregate and its particle dimension in solution [3].

As shown in Fig. 3, spectra a and b, when the RLS intensities of MRSA and MTT were considered alone, they were quite weak. It thus can be concluded that the MRSA reacted with MTT and produced a new-formed compound (formazan) whose RLS intensity was much higher than that of MRSA or MTT when they existed separately. Moreover, the dimension of formazan particles may be much less than the incident wavelength, and thus the enhanced light-scattering signal occurs in the given conditions. In this way, the resonance light scattering formula [22, 39] could be applicable to the MTT-MRSA system.

Characteristics of the UV Spectra

The UV spectra of the MTT-bacteria system are shown in Fig. 4. It can be clearly observed that there were two peaks located at 243 and 378 nm in the UV spectrum of the MTT-MRSA system. It was found that the UV absorption decreased with the addition of increasing MRSA to the MTT solution. However, at the MRSA concentrations beyond 1.2×10^5 CFU/ml, the UV absorption hardly changed. MTT was reduced to insoluble particles by living MRSA, so the concentration of MTT decreased. It thus resulted in weakening UV absorption of MTT.

Establishment of the MTT Assay

To establish the range of bacterial concentrations in which the reduction of MTT was reproducibly proportional to the number of viable bacteria, various concentrations of MRSA were plated and the MTT assay was carried out as described in Materials and Methods. Fig. 5 shows that there was a linear relationship between the intensity of the MTT-MRSA system and the MRSA concentration when

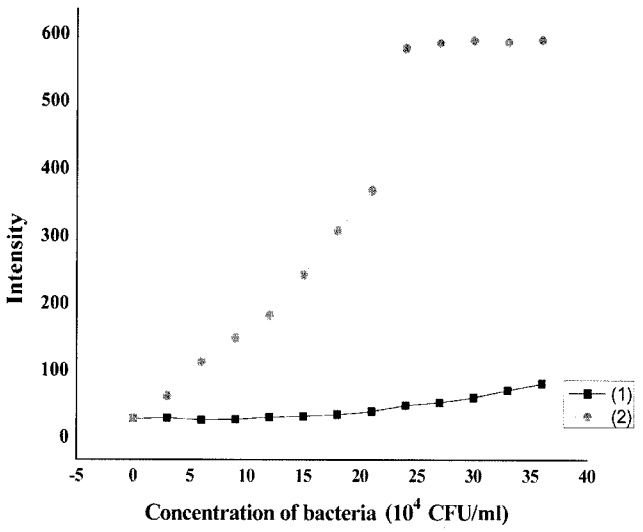

Fig. 5. Reduction of MTT by MRSA *in vitro* is dependent on the concentration of the bacteria (1). Bacteria killed by heating (immersion in a boiling water bath at 91°C) cannot reduce MTT (2).

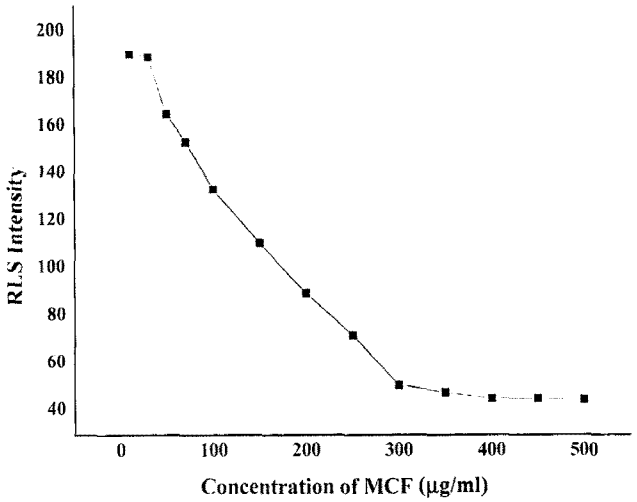

Fig. 6. Effect of MCF on the ability of MRSA to reduce MTT.
MRSA treated with various concentrations of MCF for 48 h showed a drug dose-dependent inhibition of the ability to reduce MTT.

the MRSA concentration ranged from 3.00×10^4 to 2.40×10^5 CFU/ml. The linear regression equation with the least-square method was $\Delta I=13.35+22.79C_{bacteria}$ (r=0.9976, $P<$ 0.001) [Fig. 5(1)]. MRSA that had been placed in a boiling water bath (91°C) for 15 min was unable to reduce the MTT dye [Fig. 5(2)].

MCF Susceptibility Testing

MRSA sensitive to MCF showed a significant and MCF dose-dependent inhibition of MTT reduction (Fig. 6). This effect was seen after incubation with MCF for 24 h. Fig. 6 also shows that the RLS intensity was very weak when MRSA were treated with MCF at concentrations equal to or greater than 300 μg/ml, suggesting that no viable MRSA was found. When MCF was used at concentrations ranging from 30–300 μg/ml, there was a significant linear relationship between the drug concentration and the inhibition of MTT reduction. From this point, the MTT-bacteria assay could be used to detect MRSA susceptibility to pharmaceuticals. To verify that this relationship was caused by killing MRSA by pharmaceuticals, similarly treated MRSA was plated for microcolony counting. Fig. 7 shows that there was a strong linear relationship between the number of CFU and the concentration of MCF. Fig. 7 also shows that MRSA treated with MCF at 500 μg/ml did not produce any colonies, which supported our previous observation.

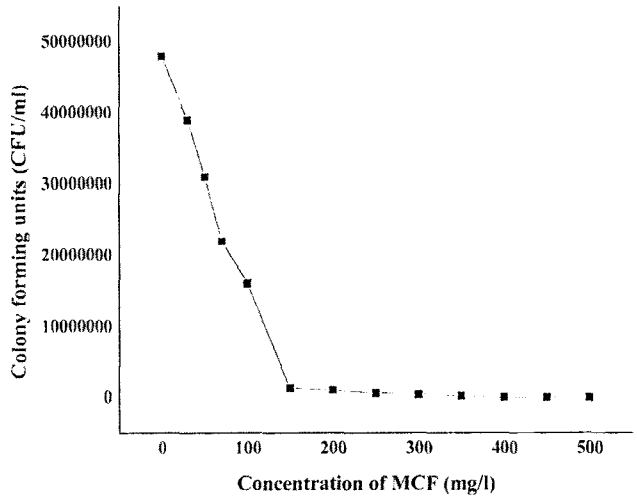

Fig. 7. Inhibition effect of MCF on MRSA.
MRSA treated with various concentrations of MCF for 48 h showed a drug dose-dependent inhibition of the MRSA.

Comparison of Effect of MCF on MRSA, Determined by the RLS and Microcolony Counting Methods

The effects of MCF on MRSA determined by different methods are shown in Fig. 8. For the RLS method with MTT reduction assay, the effect of MCF on MRSA viability was evaluated by determining the percentage of RLS intensity upon incubation of MRSA with increasing extract concentrations in the range 30–500 μg/ml (Fig. 8A). For the microcolony counting method, the effect of MCF on MRSA viability was evaluated by determining the percentage of living MRSA (Fig. 8B). As shown in Figs. 8A and 8B, the RLS results were in agreement with the microcolony counting method, which proved that study of *in vitro* activity of antimicrobial agents against MRSA by the RLS was practical. The MIC values obtained by RLS and microcolony counting method were 30 μg/ml. The RLS technique could, in combination with the MTT assay, be a rapid and sensitive measuring method to determine the *in vitro* activity of new antimicrobials.

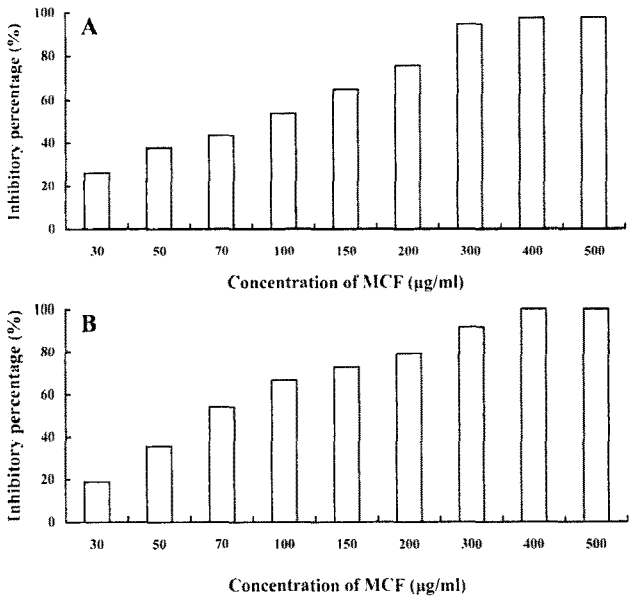

Fig. 8. Inhibitory effect of MCF on MRSA by RLS (**A**) and microcolony counting method (**B**).

Discussion

Today, methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial problem for the failure of antimicrobial treatments and an increasing problem in community-acquired infections [16, 17, 25].

Recently, the application of MTT reduction has received great interest. de Nooijer *et al.* [24] discussed a new viability assay based on the reduction of MTT by living foraminifera, confirming that living individuals of *Ammonia beccarii* and *Globobulimina turgida* converted MTT and became stained within 24 h. Some dead foraminifers may continue enzymatic activity for several days, but produce a different coloration compared with that of stained living foraminifers. Sanchez and Kanigsberg [29] adapted the MTT assay to corroborating mitochondrial respiration in yeast. It provided a good idea to apply the MTT assay. Aziz [3, 4] examined and validated a simple and less costly MTT test to determine bovine sperm and equine sperm viability, and compare the efficiency of this test with a flow cytometer. The results revealed a strong correlation between the results of MTT reduction rate and the results simultaneously determined by a flow cytometer.

Like MTT, 3-(4,5-dimethylthiazol-2-yl-5)-3-carboxy-methylphenyl)-2-(4-sulfonyl)-2-tetrazolium (MTS) is also reduced by mitochondrial dehydrogenases, but unlike the MTT reduction product, that of MTS is water soluble and thus it cannot be used to perform the RLS assay.

Another tetrazolium compound, sodium 3,3'-[1[(phenyl-amino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), was compared with MTT for use in a colorimetric assay for quantitating bovine neutrophil bactericidal activity [32]. Dead bacteria and lyzed neutrophils did not react with MTT or XTT plus coenzyme Q. Live bacteria converted XTT to water-soluble orange formazan in the presence of coenzyme Q. Absorption of formazan produced by bacteria from XTT was measured at 450 nm. Formazan produced by bacteria from MTT was solubilized by adding isopropanol and measured by absorption at 560 nm. The RLS technique with MTT reduction assay was quicker and easier to perform, because bacteria converted MTT to a formazan that did not need to be solubilized before measuring the RLS intensity. The obtained results were almost in agreement with those by the currently used dilution method (results not reported). It could be a rapid and sensitive screening method for MRSA.

The need to know whether an organism is likely to respond to antimicrobial therapy is as old as chemotherapy itself. The classical methods of antimicrobial susceptibility testing of *vitro*-cultured bacteria are expensive and time-consuming. Herein, we describe a simple and cheap RLS method that can be used to screen drug resistance in MRSA. The RLS needs only one culture cycle to produce enough bacteria for drug resistance testing. Compared with the traditional MTT assay, the RLS test itself takes a maximum of 12 h. The RLS technique could, in combination with the MTT assay, be a rapid and sensitive measuring method to test the antimicrobial susceptibility of *vitro*-cultured bacteria.

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