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Rapid Bacterial Testing Method by Size Distribution Measurement with Laser Light Scattering

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SUMMARY A rapid testing is required in clinical laboratory. A new method for rapid bacterial testing is proposed by size distribution measurement with laser light scattering. Bacterial sample is suspended in a saline solution and the scattering pattern is measured from 5 to 90 degrees with 5-degree intervals. The size distribution function is calculated from the light scattering pattern by the inversion technique based on the modified Rayleigh-Debye approximation. The difference in bacterial species and the influences of drug are investigated. The following results are obtained; (1) size distribution curve reflects the morphological characteristics of bacteria, and bacterial species are discriminated roughly by two characteristic parameters (mean size and dispersion width) obtained from the curve; (2) size distribution pattern changes in a short time (within one hour) indicating the effects of drug. It is useful for antibiotics susceptibility testing.

1. Introduction

Accurate identification of bacterial species and antibiotic susceptibility testing require many steps and long inspection time in clinical bacterial laboratory. Many authors proposed rapid and automated testing techniques by the measurement of impedance[1],[2], turbidity[3],[4], light scattering[5],[6], and simultaneous measurement of turbidity and impedance[7]. However, neither of them is satisfactory in practical use.

The particle size measurement method by light scattering has been developed by many workers[8]-[12]. These methods are applicable to particles of a few microns and hence applicable to bacterial size measurement. The size of bacteria differs for different bacterial species or for different growth conditions, and the size distribution measurement method seems a powerful tool for bacterial tests.

The present paper proposes a new method for rapid bacterial testing by the size distribution measurement with laser light scattering. The method uses the inversion technique developed by Shimizu and Ishimaru [12]-[14] to obtaine the size distribution from the scattering pattern. This technique is based on the modified Rayleigh-Debye approximation and has several advantages: 1. the inversion calculation is simple compared with other inversion techniques, 2. numerical calculation does not diverge, 3. modified Rayleigh-Debye approximation is valid even in the range of bacterial refractive index. Item 1 and 2 fit to the microcomputer analysis, and item 3 is useful for application to bacteria. In this paper, the possibilities of discriminating bacterial species and of detecting the influence of drugs on bacteria are demonstrated by size distribution measurement.

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2. Experimentals

2.1 Measurement System

The apparatus and optical arrangements are shown in Fig. 1. A He-Ne laser (6328Å, 5 mW) is used as a light source. The sample cell is made of glass tube with 7 mm in diameter and 40 mm in length. The laser light illuminates the sample through a chopper, a pinhole and a lens. The lens is put at the appropriate position in order to obtain a parallel light beam in the cylindrical sample cell. The scattering light is detected by a PIN photo-diode through two pinholes and transformed to an electrical signal. The signal is amplified by a lock-in amplifier (NF Circuit LI-575) and converted to an 8-bits digital signal and led to a microcomputer system (Commodore CBM 4032 micro computer, floppy disk, printer and plotter). The angular resolution of this system is about 0.5 degree.

2.2 Data Analysis

The inversion theory is valid when the following restrictions are satisfied: (1) the scattering pattern from a single particle is approximated well by the modified Rayleigh-Debye approximation, (2) the particle position is random in scattering volume, (3) multiple scattering is negligibly small. The size distribution $N(r/2)$ is calculated from scattering pattern $I(k)$ by[12]

$$N(r/2) \propto \frac{\partial}{\partial r}\frac{1}{r}\frac{\partial^2}{\partial r^2}\left[\int_0^\infty I(k)\, J_0(kr)\, k^2\, dk\right] \quad (1)$$

$$k = (4\pi/\lambda)\, n \sin(\theta/2) \quad (2)$$

where, k is the scattering wave number and the function of scattering angle θ, r is the radius of a particle, λ is the wavelength of light, n is the refractive index of scatterer, and J_0 is the 0-th order spherical Bessel function.

The scattering intensity $I(k)$ is measured from 5 to 90 degrees with 5-degree intervals. Measured points of original scattering pattern are 18 and too few for detailed calculations. Then, points are interpolated between the measured points by the least-square interpolation. The scattering

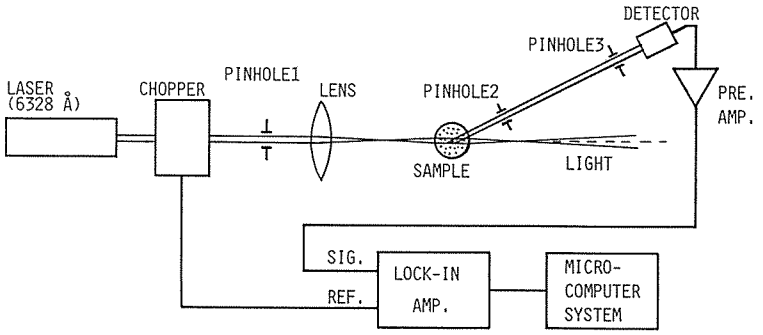

Fig. 1 Blockdiagram of the measuring system.

pattern reconstructed with 50 points is used in the analysis. The size distribution function is calculated by Eq.(1) from 0 to 2 μm with a 0.02 μm step. The refractive index of 1.4 is assumed for all bacteria[15],[16]. In the integral calculation, k should be extended up to infinity, but the scattering pattern is limited to a finite value of k corresponding to scattering angle of 90 degrees. In order to suppress the integration error, the scattering pattern is multiplied by the Hanning function. These calculations are carried out on micro-computer.

2.3 Sample Preparation

Bacterial species tested are mainly usual Enterobacteriaceae. The culture medium used in all experiments is Brain Heart Infusion (Difco Lab.). The samples are prepared by the following processes: Bacterial culture medium grown overnight at 37°C is centrifugalized with 1500 rpm for 15 minutes. The clear medium is gently removed and bacteria left in the tube bottom are diluted with 0.7 wt% saline solution. The solution is poured into the measuring cell and subjected to the scattering-pattern measurement. The concentration of bacteria in the suspension medium is adjusted to about 10^5 — 10^6 organisms/ml. Multiple scattering in this concentration is checked and found to be negligibly small.

The samples used for testing effects of drugs are prepared as follows: 0.5 ml of bacterial culture medium grown overnight is mixed with 4.5 ml of fresh medium containing drug. The concentration of drug is adjusted to 100 μg/ml. This mixture is incubated at 37°C for 0 to 180 minutes, and then the culture medium is centrifugalized. 15 minutes for centrifuge is added to the incubation time. The subsequent steps are the same as described above. The drugs used are the antibiotics of Chloramphenicol and Ampicillin (Fujisawa Pharmaceutical Co., Ltd.).

3. Results

3.1 Test for the Discrimination of Bacterial Species

Figure 2 shows typical size distribution curves of Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus). Photographs observed by scanning electron microscope (SEM) are also shown in the inset (×10000). The size distribution curve has an error at a small radius, and a false peak (not shown in Fig. 2) would appear at around 0.3 μm radius. The origine of this error will be discussed later. It is known that the size of bacteria is larger than 0.4 μm in radius. Thus, the curves in Fig. 2 are cut off at 0.4 μm and normalized at the significant peak.

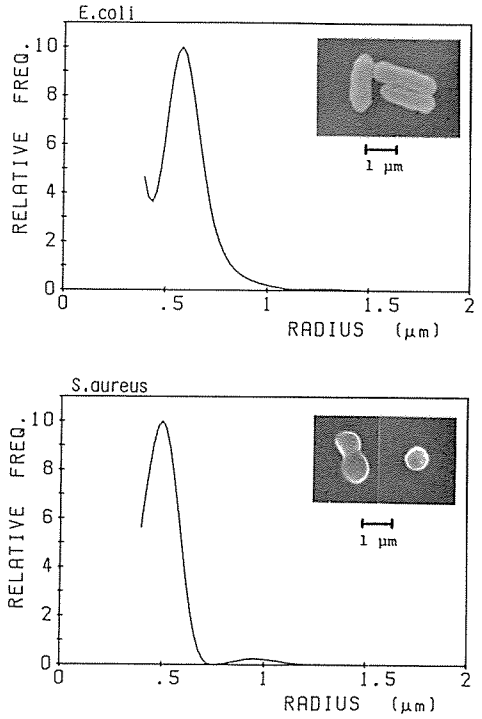

Fig. 2 Size distribution of E. coli and S. aureus. E. coli is a bacillus and S. aureus is a coccus as shown in the photograph. (×10000)

The radius corresponding to the peak of curve represents the mean size of scatterer. Mean size of bacteria estimated from Fig. 2 is about 0.6 μm for E. coli and 0.5 μm for S. aureus. Typical size read from SEM photograph is 0.44 μm for S. aureus, 0.65 μm in width and 2.0 μm in length for E. coli. The discrepancy for E. coli comes from their shape. E. coli of bacillus are regarded as prolate spheroids. Prolate spheroids can be approximated by spheres with the same volume. The equivalent radius calculated for E. coli from the size in photograph is 0.54 μm, which is fairly close to 0.6 μm. Remaining errors may be attributed to errors in the assumed value of the refractive index of bacteria or to changes in bacterial size during sampling processes for SEM.

Size distribution curves also show the morphorogical characteristics of bacteria. E. coli has wider dispersion compared with S. aureus and has a single peak. When E. coli is oriented randomly, they look like particles with sizes dispersed from minor axis to major axis of bacteria. On the contrary, S. aureus is a coccus and tends to form a chain. The sharp main peak may reflect single bactera, and the second peak at around 1 μm radius is indicating a duplex

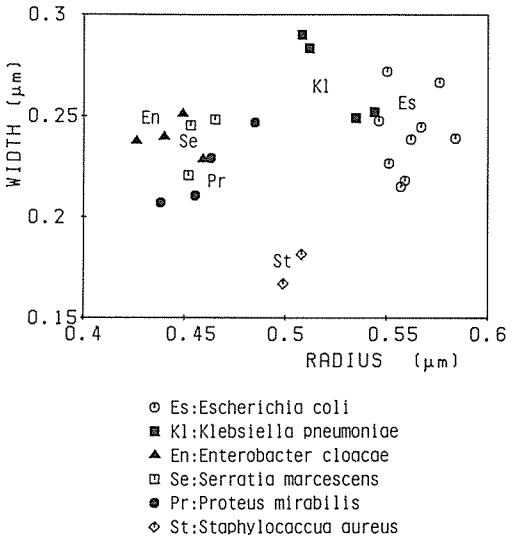

Fig. 3 Mean size and dispersion width obtained from size distribution curve. Six bacterial species are tested.

duplex chain of bacteria. We chose the mean radius and the dispersion width as characteristic parameters of bacteria. The dispersion width is difined by the difference between two radii corresponding to the peak and its 1/10 in the dominant peak.

The size distribution curves are measured for some other species of bacteria and the two characteristic parameters are obtained. The results are summarized in Fig. 3. Bacteria species tested may be roughly divided into three groups. Klebsiella pneumoniae (K. pnoumoniae) is a large bacillus and resembles E. coli. They site themselves in the upper right part of Fig. 3. Enterobacter cloacae (E. cloacae), Serratia marcescens (S. marcescens) and Proteus mirabilis (P. mirabilis) are small bacillus, and site themselves in the upper left part of the figure. S. aureus of coccus put itself quite apart from other bacillus bacteria.

3.2 Effect of Drugs

Figure 4 shows typical changes of size distribution curves for E. coli and S. marcescens, where bacteria are cultured in a medium containing 100 μg/ml of Chloramphenicol. In the figure, control refers to drug free condition and T_t indicates the incubation time in minutes at 37°C. In a drug free medium, bacteria swell and divide every about 30 minutes. The size of E. coli reaches maximum at about 30 minutes, as indicated by dashed curve in Fig. 4 (a), and returnes to the intial size with no futher change. The curves under antibiotic shift to the right as the time passes. Evidently bacterial size becomes large due to the effects of drug. Photographs in Fig. 5 indicate clearly that bacteria are fat and long under antibiotic condition. Since Chloramphenicol prevents a synthesis of protain [17] bacteria must grow large but can not divide. From these results, we conclude that both bacteria tested are sensitive to Chloramphenicol. In fact, MIC (Minimal Inhibitory Concentration) values for

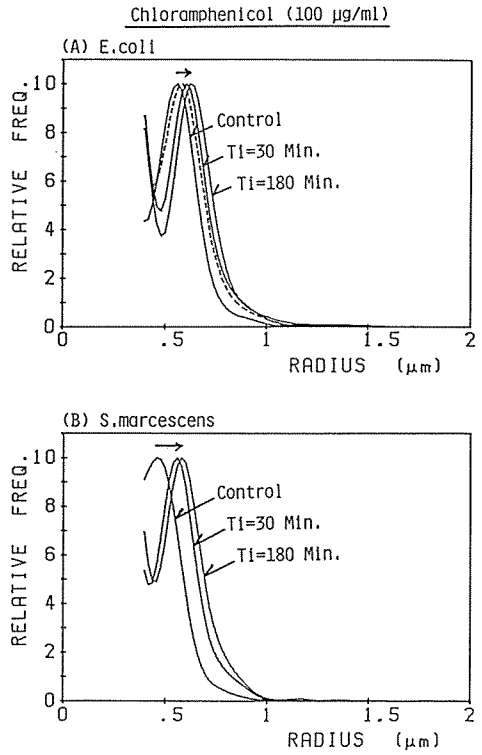

Fig. 4 Size distribution changes of E. coli and S. marcescens. Bacteria are cultured at 37°C in a medium containing 100 μg/ml of Chloramphenicol. Control refers to drug free condition and T_t indicates the incubation time in minutes. Dashed curve of E. coli indicates the maximum change in a drug free medium (T_t = 30 minutes).

E. coli and S. marcescens by conventional tube method [18] are 3.1 μg/ml and 25 μg/ml, respectively, indicating that both bacterial species are sensitive to Chloramphenicol. The results of size distribution measurement are consistent with the MIC test.

Figure 6 shows other examples of drug effect tests, where 100 μg/ml of Amplicillin is added to the medium. The changes of size distribution curve for E. coli indicate that the mean radius becomes large until at about half an hour and becomes small subsequently, and the size of bacteria becomes more dispersive with the time. After one hour, the scattered light intensity becomes too weak to make reliable measurement. On the contrary, the size of S. marcescens becomes slightly large with the time, but the pattern of the distribution does not change. MIC values of E. coli and S. marcescens are 12.5 μg/ml and greater than 200 μg/ml, respectively. Comparing the results of MIC test with the size distribution measurement, one finds that effects of drug are reflected in the change of distribution pattern. Since Amplicillin affects bacterial cell wall [17], E. coli may be broken into small pieces and dissolved in a medium. The drop off of the scattered light intensity at one hour can be understood from the above. Contrary to this, S. marcescens is resistive in MIC test, and the distribution pattern does not change.

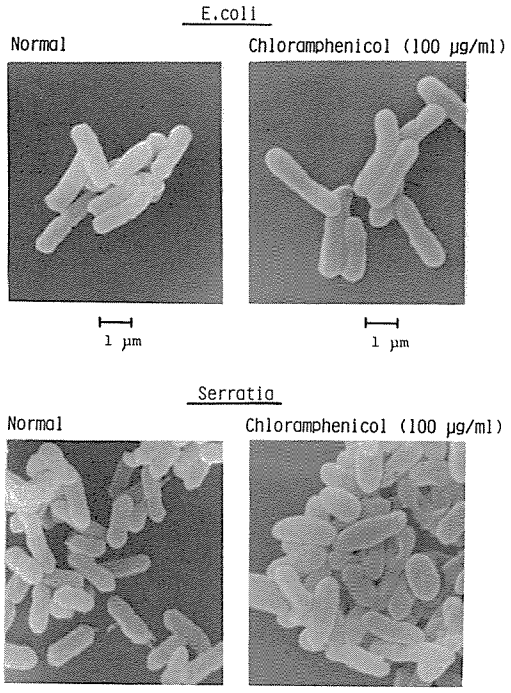

Fig. 5 Photographs of E. coli and S. marcescens observed by Scanning Electron Microscope. (×10000) Left side are the normal type bacteria and right side are the bacteria grown for three hours in a medium containing 100 μg/ml of Chloramphenicol.

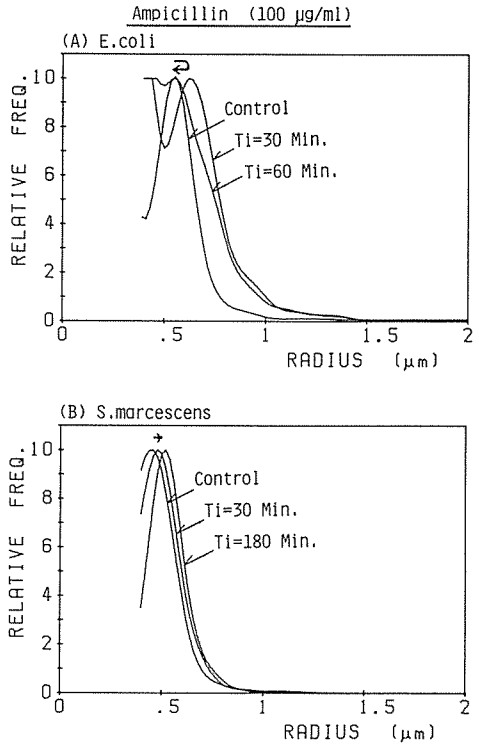

Fig. 6 Size distribution changes of E. coli and S. marcescens. Bacteria are cultured at 37°C in a medium containing 100 μg/ml of Amplicillin. Control refers to drug free condition and T_i indicates the incubation time in minutes.

4. Problem in the Analysis

As noted in Fig. 2, the size distribution couves have a false peak in small radius region. Therefore, we used the courves in the range of radius greater than 0.4 μm in bacterial tests. In this section, the origin of the false peak is discussed.

The measurement is carried out using latex sample with a known size. Figure 7 shows the measured scattering pattern (solid curve) and the analyzed size distribution curve of styrene-biniltoruen latex spheres whose mean size is 1.09 μm in diameter and refractive index is 1.6 (Duke Scientific). Size distribution curve has a peak at about 0.5 μm radius corresponding to the latex size. Another peak appears at around 0.3 μm radius, which is regarded as an error on the basis of data supplied by the manufacture. The size distribution curve can be divided into two parts at 0.4 μm (vartical line in Fig. 7). Dashed curve in Fig. 7 is a calculated scattering pattern from the part of size distribution curve for radii greater than 0.4 μm. The measured and calculated scattering patterns are similar, except that discrepancies become larger at larger angles, as to be expected. Shimizu suggested that the error of the scattering pattern in the large angle region is attributed to the refraction and reflection effects at the boundary of scatterer, and both are neglected in the modified Rayleigh-Debye approximation[13].

We tried to exclude the error, but have been unsuccessful so far because the approximation error in scattering pattern is not constant and varies with the size or the re-

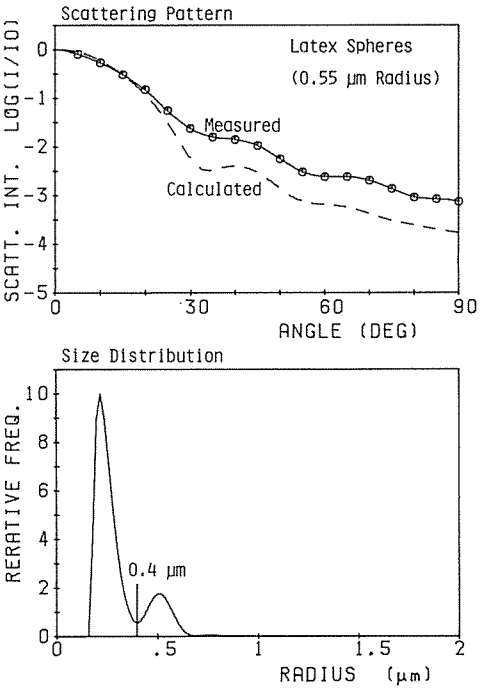

Fig. 7 Scattering pattern (solid curve) and size distribution curve of latex spheres with 0.55 μm radius. Dashed curve in scattering pattern is calculated from the part of size distribution curve for radii greater than 0.4 μm.

fractive index of scatterer. Therefore, at the present stage, we choose to use the portion of distribution curve for the radii greater than 0.4 μm.

5. Conclusion

The measurement of size distribution of coliform bacteria is carried out by analyzing the scattering pattern with laser light. The following results are obtained.

(1) The size distribution curve reflects the morphological characteristics of bacteria. Bacterial species can be grouped using two parameters obtained from the size distribution curve.

(2) The change of size distribution curve is found under antibiotic condition in a short time and is shown useful to indicate effects of drug.

The proposed method is applicable to rapid bacterial tests. We emphasize that the sensitivity of bacteria to antibiotics can be estimated within one hour using this method. This test requires a few hours at least with other methods[3],[6]. For practical use in clinical bacterial laboratories, however, it is necessary to compile more data for clinical samples.

The inversion technique used in the present method suffers from the error originating from the Rayleigh-Debye approximation error of scattering pattern in a large scattering angle. This problem must be improved in future to improve the accuracy of the method.

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