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Measurement of Bacterial (*Escherichia coli*) Concentration by Flow Cytometry

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Periodontitis is a multi-microbial disease and the comparison of a series of periodontopathogenic and non-periodontopathogenic bacteria in terms of microbe-host interaction may provide clues to understand the microbial etiology of the disease better. When we deal with twenty different bacterial species in a study, the first technical issue is how to measure the accurate concentration and use the same number of bacterial cells. We measured bacterial concentration by enumerating bacteria stained with SYTOX green for constant time using a flow cytometer and compared the results with those obtained by plate counting. Concentrations calculated by two different methods were very close. Therefore, flow cytometric counting allowed the rapid analysis of live/dead bacteria, offering the advantage of turbidity measurement and that of colony counting together.

Keywords: bacteria, concentration, measurement, flow cytometryAbstract

Introduction

Periodontitis is the inflammation of periodontal tissues initiated by subgingival plaque-associated bacteria. Unlike most infectious diseases, periodontitis is a multi-microbial disease and ten to twenty out of about 500 bacterial species constituting the subgingival plaque are known to be periodontopathogenic based on the prevalence of bacteria in disease and health (Socransky and Haffajee, 2002). However, the line between periodontopathogenic bacteria and normal flora is not clear because periodontopathogenic bacteria are often found in healthy individuals or healthy sites, too. Although many studies have been done to understand the pathogenesis of the periodontopathogenic bacteria so far, each study was focused on one or two bacteria (Nishihara and Koseki, 2004). The comparison of a series of perio-dontopathogenic and non-periodontopatho-genic bacteria in terms of microbe-host interaction may provide clues to understand the microbial etiology of perio-dontitis better.

We wanted to test our hypothesis that periodontopathogenic bacteria are more resistant to phagocytosis than non-pathogenic bacteria. When we deal with twenty different bacterial species in a study, the first technical issue is how to measure the accurate concentration and use the same number of bacterial cells for each species. Conventionally, the measure of bacterial concentration represents a viable cell count determined by counting colonies formed on agar plates. Although the turbidity of liquid culture measured by a spectrophotometer is often used in most cases, a standard curve between the viable counts and the turbidity has to be established first (Brooks *et al.*, 2001). Recently, a new method to measure the bacterial concentration by flow cytometry has been introduced, which is fast and handy but has not been used widely, yet (Caron *et al.*, 1998; Shapiro, 2000).

In this study, we verified the usefulness of flow cytometry in the measurement of bacterial concentration in comparison with plate counting, and reported technical considerations in detail to obtain consistent data and avoid potential pitfalls.

Materials and Methods

Bacterial culture

Escherichia coli DH5α were cultured in Luria-Bertani (LB) broth (Difco, Detroit, MI, USA) at 37°C in an aerobic condition with orbital shake. *Streptococcus gordonii* and

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Actinobacillus actinomycetemcomitans ATCC 43719 was cultured in brain heart infusion (BHI) broth (Difco). *Eikenella corrodens* ATCC 23834 was cultured in BHI supplemented with 10 μg/ml of vitamin K. *Porphyromonas gingivalis* ATCC 49417 and was cultured in BHI supplemented with 5 μg/ml of hemin and 10 μg/ml of vitamin K. The four oral bacteria were cultured at 37°C in an anaerobic atmosphere (5% H_2, 10% CO_2 and 85% N_2).

Bacterial detection and live/dead discrimination by flow cytometry

Fresh cultured *E. coli* were mixed with *E. coli* that were killed with 70% alcohol in various ratios, stained with SYTOX green (Molecular Probe, Eugene, Oregon, USA) by incubating with 5 μM SYTOX green in PBS for 5 min at room temperature, and then analyzed with FACSCalibur (BD Bioscience, San Diego, CA, USA). Bacteria (3 mL) were harvested in log phase, washed with PBS once, fixed with 4% paraformaldehyde for 20 min at room temperature, and then washed with PBS once again. Bacteria resuspended in 1 mL PBS were stained with 5 [and -6]-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probe) at a final concentration of 1-10 μM at room temperature for 20 min. Staining was checked under a fluorescence microscope and then bacteria were washed with PBS twice and analyzed by FACSCalibur (BD Bioscience). Instrument settings are as follow: FSC-E02 for *E. coli* and FSC-E03 for oral bacteria, logarithmic amplification; SSC-460 V, logarithmic amplification; FL1-600 V, logarithmic amplification. Threshold was set with either SSC or FL1. After checking events per second in the initial analysis, bacterial samples were diluted to obtain less than 1000 events per second.

Measurement of bacterial concentration

E. coli (2 mL) cultured for 3 hrs or overnight were diluted serially in PBS. A half of each dilution was taken to measure the turbidity using a spectrophotometer, Gene Spec III (Naka Instruments, Japan). For flow cytometric analysis, 200 μL of each dilution was transferred to FACS tubes, to which SYTOX green was added to make 5 μM, and enumerated for 15 s at low mode. Each sample was acquired three times. In addition, BD Liquid Counting Beads (BD Biosciences) diluted with PBS at 1/100 were enumerated separately, and then counted with a hemocytometer under a microscope to confirm the concentration of beads. From the bead concentration, the volume analyzed by FCM for 15 s was determined. For plate counting, 10 μL of each dilution was plated on a LB agar plate in triplicates. LB broth (140 μL) was added on the plates to help spreading.

Results

Detection of labeled or unlabeled bacteria by flow cytometry

Small bacterial cells have to be discriminated from dusts and debris very carefully upon flow cytometric analysis. We found that the best way is either to run a couple of different dilutions or to label bacterial cells with fluorescent dyes. Fig. 1A shows plots of fresh cultured *E. coli* that were analyzed at the dilutions of 1/500 and 1/2500 after staining with SYTOX green dye. It is often difficult to tell bacterial cells apart from debris clearly on the FSC vs. SSC plot, but the FL1 vs. SSC plot separates them into distinct populations. In diluted sample, while the bacterial population (R2, M2) is diluted out, debris (R4, M1) is not. SYTOX green penetrates only dead cells (R3, M3) and increases fluorescence by thousand-fold upon binding to DNA, providing a good tool to examine the viability of bacteria. When entire bacterial cells are labeled with fluorescence, debris can be excluded easily by setting threshold with FL-1 because debris is not labeled (Fig. 1B).

The usefulness of flow cytometry in the measurement of bacterial concentration

To verify the usefulness of flow cytometry in the evaluation of bacterial viability, live and killed *E. coli* were mixed in various proportions, stained with SYTOX green, and analyzed by flow cytometry. The results reflected the real viabilities of the prepared samples fairly well (Fig. 2A). Next, we enumerated bacterial cells using a flow cytometer for constant time (15 s) and calculated bacterial concentration. By comparing the number of standard beads counted by the flow cytometer and that by a hemocytometer, we reasoned that the flow cytometer analyzes 10.6 μL for 15 s at low mode. To avoid co-incidence upon flow cytometric analysis, events/second has to be below 1000. Three counts by flow cytometry were pretty consistent, suggesting constant flow during analysis (Table 1). In general, counts by flow cytometry were slightly greater than those by plate counting (Table 1 and 2) but two were correlated to each other well (Fig. 2B, upper panel). Bacterial concentration was calculated by extrapolating observed counts and dilution factors (Table 2). We extrapolated bacterial concentrations and turbidities of original and 1/10 diluted culture from two different experiments (Fig. 2B, lower panel). Bacterial cell concentration/optical density was calculated as 5.0×10^8 *E. coli* and 2.8×10^8 *E. coli* cells by flow cytometric counts and plate counting, respectively. Therefore, concentrations calculated by two different methods were close but those by flow cytometry fit turbidity better.

Discussion

Flow cytometry has not been applied to bacteria widely although the method was introduced already in 1996 (Davey and Kell). The distinction between small bacteria and

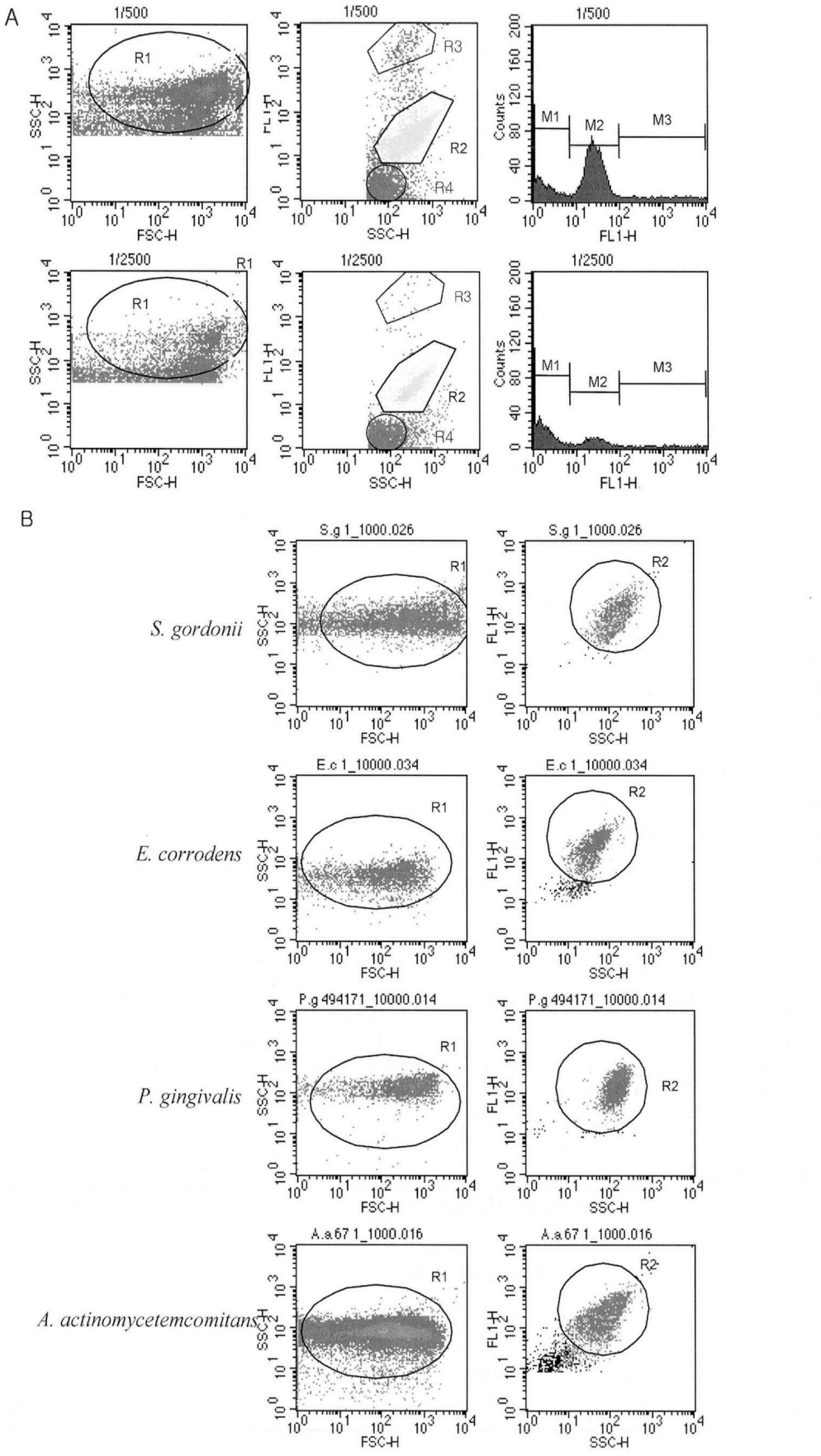

Fig. 1. Detection of bacteria on flow cytometric analysis. A. Fresh cultured *E. coli* were diluted with PBS at 1/500 and 1/2500, and stained with SYTOX Green. Bacterial population was roughly gated as R1 on the density plot of FSC vs. SSC. R1 gated events were analyzed on the dot plot of SSC vs. FL1 and a histogram for FL1. R2, R3, and R4 represent live bacteria, dead bacteria, and debris, respectively. B. Fresh cultured oral bacteria were washed, fixed, and stained with CFSE. Following proper dilution to obtain < 1000 events/second, bacteria were analyzed for 15 s at low mode.

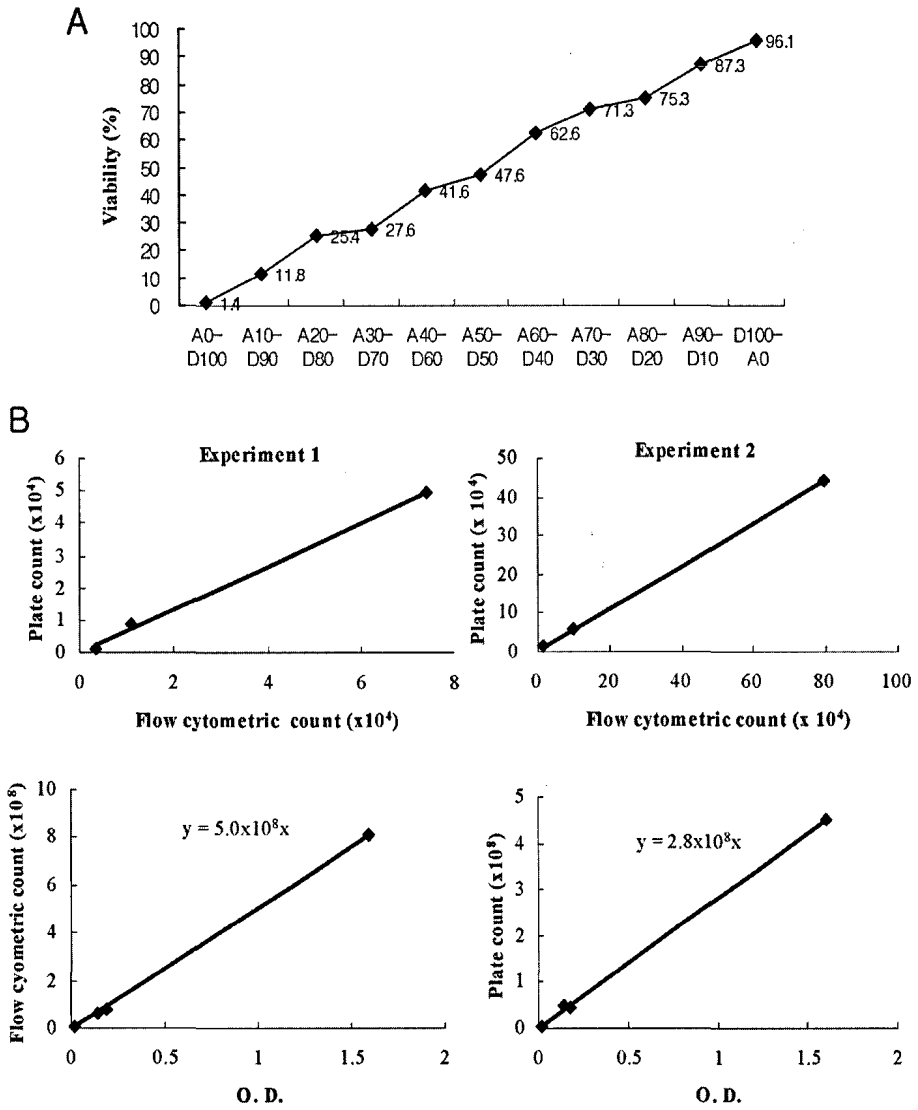

Fig. 2. Usefulness of flow cytometry in the measurement of bacterial concentration. A. Determination of live/dead bacteria by flow cytometry. Live and killed *E. coli* were mixed in various proportions, stained with SYTOX Green, and analyzed by flow cytometry. Live and dead bacteria populations were determined as in Fig. 1A and viability was calculated from the numbers of live and dead bacteria. B. Measurement of bacterial concentration. Fresh cultured *E. coli* were serially diluted and the turbidity was measured with a spectrophotometer. Subsequently, a portion was subjected to flow cytometric enumeration and the other portion was plated on LB agar plates. The correlation between flow cytometric and plate counts is shown on top panel. The correlation of turbidity to bacterial concentrations determined by flow cytometry or plate counting is shown on bottom panel.

Table 1. Example of viable count

dilution	Flow Cytometric Count (10.6 μl)			Plate Count (10 μl)		
1						
1/10	Events/second > 1000			Too crowded to count		
1/100						
1/1000	8443	8521	8311	4033	5047	4103
1/10000	968	1023	1066	267	866	654
1/100000	151	169	168	95	256	99

background debris on flow cytometric analysis is tricky at first, but it can be accomplished easily by running different dilutions of labeled bacteria (Fig. 1). After verifying the bacterial population, setting a threshold with either SSC or FL1 (in case bacteria are labeled with a dye detected in FL1) is recommended to improve the accuracy of analysis. Several different dyes can be used to label bacteria depending on the purposes. CFSE labeled both live and

Table 2. Comparison of bacterial concentrations measured by flow cytometry (FCM) and plate counting

dilution	Exp. 1			Exp. 2		
	O. D.	FCM count (cells/ml)	Plate count (cells/ml)	O. D.	FCM count (cells/ml)	Plate count (cells/ml)
1	1.599			0.138		
1/10	0.184			0.015		
1/100	0.022			0	614000	
1/1000		790000	440000		74000	49000
1/10000		100000	60000		11000	8600
1/100000		20000	15000			1000
Calculated concentration		8.1×10^8/ml	4.5×10^8/ml		6.2×10^7/ml	5.0×10^7/ml

fixed bacteria uniformly with a small deviation in the fluorescence intensities and was well contained even in fixed bacteria. Due to the far greater fluorescence enhancement (~1000 fold by SYTOX green vs. 20 to 30 fold by PI) upon binding DNA, SYTOX green dye provided the better discrimination of dead bacteria than PI. Commercial bacterial viability kits provide PI to label dead bacteria and SYTO 9 or thiazole orange to stain both live and dead bacteria. Similarly, SYTOX green can be used together with SYTO red fluorescent dyes such as SYTO 59-64 to stain both live and dead bacteria for two-color analysis. However, the use of SYTOX green alone was enough to determine the viability of *E. coli* (Fig. 2A). Since flow cytometric analysis is performed in aerobic condition, the accuracy of viable cell detection is decreased slightly for anaerobic bacteria.

Recently, a bacterium counting kit using flow cytometer was introduced by a couple of commercial companies. The kit provides dye to stain bacteria and standard beads of a known concentration. By running bacteria mixed with the beads of known concentration, the concentration of bacteria is determined based on that of beads. When we tested the kit, however, beads (6 μm) were too large than many oral bacteria to acquire within one plot using a same instrument setting. Therefore, we modified the method. Instead analyzing bacteria and standard beads at the same time, we enumerated them separately for a constant time utilizing the constant flow of the flow cytometer. From the concentration of beads determined by hemocytometer counting, we estimated the volume analyzed by flow cytometry, and the concentration of bacteria. The obtained results were very close to those obtained by plate counting and correlated with the turbidity of culture very well (Table 2, Fig. 2B). In conclusion, the greatest advantage of flow cytometric counting is that it can analyze live/dead bacteria rapidly, offering the advantage of turbidity measurement and that of colony counting together.

Acknowledgement

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