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Application of Flow Cytometry for Rapid Detection of *Lactococcus garvieae*

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ABSTRACT

Flow cytometry (FCM) technique was applied to rapid determination of cell number of *Lactococcus garvieae*. An antiserum against *L. garvieae* was prepared and its immunological property was examined. The present antibody would recognize some epitopes of *L. garvieae* with a high specificity. The optimum conditions for the FCM assay were as follows: discriminate value, 60; dilution ratio of the antiserum, 1.0×10^4. Calibration curve for *L. garvieae* cells was linear, in the range of 2.4×10^4–1.5×10^7 cells/mL. The detection of *L. garvieae* in cell suspensions contaminated with *Escherichia coli* was carried out. A good correlation was observed in the range of 20–90% for the mixing ratio of *L. garvieae*. One FCM assay could be completed within 2 min, and the total assay time, including the preparation of bacterial sample, was within 3 h.

Index Entries: Flow cytometry; *Lactococcus garvieae;* cell counts; enterococcal infection.

INTRODUCTION

Enterococcal infections have frequently been observed in marine and freshwater aquaculture. They present serious disease problems in fish such as yellowtail, sea bream, rainbow trout, and eel *(1–5)*. Yellowtails are known to be susceptible to infection by *Lactococcus garvieae* (*Enterococcus seriolicida*) *(6,7)*. In order to take preventive measures against infection, the detection of *L. garvieae* in aquaculture at an early stage is very important.

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For the determination of *L. garvieae*, a colony-counting method, using a commercial medium such as EF agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), has been commonly employed. Because this medium contains sodium azide, which selectively inhibits the growth of all Gram-negative bacteria, *L. garvieae* can grow in this medium *(8)*. However, this method requires an incubation period up to several days.

In recent years, the technique of flow cytometry (FCM) has made significant contributions to studies in several areas of biology, including medicine, cytology, immunology, and biotechnology. FCM combines the advantages of microscopy and biochemical analyses for the measurement of physical and biochemical characteristics of individual cells. In this method, cell particles in suspension flow in single file at a uniform speed through a laser light beam, with which they interact individually. This yields, for each cell, a light-scattering pattern that provides information about cell size, shape, density, and surface morphology. Furthermore, fluorophore labeling of cells, and subsequent measurement, can give quantitative data on specific target molecules or subcellular constituents and their distribution in the population *(9)*. Although FCM has been used primarily for studying eukaryotic cells, it has been recently applied to bacterial cells *(10–14)*. The authors have applied FCM to the rapid determination of cell number of viable bacteria *(15)*. A good correlation was observed between the values determine by the FCM method and the colony-counting method, in the range of 10^4–10^9 cells/mL. One FCM assay could be completed within 60 s. Therefore, the authors propose to apply the FCM method to detect *L. garvieae* selectively using an antigen (Ag)–antibody (Ab) reaction.

This article describes the following procedures relevant to the FCM technique: (1) preparation of anti-*L. garvieae* antiserum; (2) establishment of optimum condition for the Ag–Ab reaction, and (3) detection of *L. garvieae* among several species of bacteria commonly found in aquaculture.

MATERIALS AND METHODS

Materials

Freund's complete adjuvant and Freund's incomplete adjuvant were obtained from Rockland (Gilbertsville, PA). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Fluorescein isothiocynate (FITC)-labeled second Ab (antirabbit IgG [Goat]) and extract of bonito were purchased from Wako Pure Chemical Industries (Osaka, Japan). Peptone, tryptone, and yeast extract were obtained from Difco (Detroit, MI). Other chemicals were obtained commercially and were of analytical reagent grade.

Microorganisms and Their Cultivation

L. garvieae (H 9507), isolated from the yellowtail, *Seriola quinqueradiata,* which was obtained from a fish farm in Kagoshima, Japan, was used as model microorganism for the enterococcal infections. Several species of bacteria (*Escherichia coli* [JM 109], *Staphylococcus aureus* [FDA 209], *Pseudomonas fluorescens* [IAM 12022], *Shewanella putrefaciens* [IAM 12089], and *Vibrio* sp. [S8-A403]), commonly found in the coastal waters and in aquaculture, were chosen as reference microorganisms.

L. garvieae and *Vibrio* sp. were cultured in Bonito-Peptone-Glucose (BPG) medium consisting of (g/L): extract of bonito (5), peptone (5), glucose (1), NaCl (25), $MgSO_4$ (2.5), and KCl (1). *S. aureus, P. fluorescens, S. putrefaciens,* and *E. coli* were cultured in LBS medium consisting of (g/L): tryptone (10), yeast extract (5), and NaCl *(15).* The microorganisms were incubated at 25°C for 15–20 h, respectively.

Preparation of Anti-*L. garvieae* Rabbit Antiserum

L. garvieae was grown in BPG medium, and the cells were harvested by centrifugation, then suspended in 1% glutaraldehyde for 5 min at 25°C, and washed twice with 0.9% NaCl solution. A 200-mg wet cell mass was emulsified in Freund's complete adjuvant and subcutaneously injected into a female rabbit. After 2 wk, the same amount of Ag, emulsified in Freund's incomplete adjuvant, was administered. The same procedure was repeated every 2 wk thereafter. Three mo after the first injection, blood was collected from the ear vein using a catheter. The whole blood was incubated at 37°C for 60 min and the serum containing the Ab against *L. garvieae* was separated by centrifugation at 1700 *g* for 20 min, and stored at −80°C until used.

Analyses by Electrophoresis

Sodium dodecyl sulfate-polyacrylamlde gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli *(16)* using a 12.5% gel. Protein standards used for molecular mass determination were obtained from Sigma and consisted of rabbit skeletal muscle myosin heavy chain (205 kDa), β-galactosidase from *E. coli* (116 kDa), rabbit skeletal muscle phosphorylase b (97.4 kDa), bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase from bovine erythrocyte (29 kDa), and parvalbumin from carp skeletal muscle (11 kDa). Immunoblotting was carried out according to the method of Burnette *(17)* by using a transfer membrane (PVDF, Millipore, Bedford, MA).

FCM Measurement of *L. garvieae*

Preparation of Bacteria for Antigen–Antibody Reaction

Each bacterial species was grown to the exponential phase of growth in broth. Twenty-mL samples of culture broth were transferred to 50-mL

test tubes, and the cells were harvested by centrifugation at 1700*g* for 20 min. The growth medium was decanted and the cells were washed (1700*g*, 20 min, 4°C) with 0.9% NaCl solution. A 1-mL sample of the cell suspension was transferred to a 1.5-mL Eppendorf tube, pelleted by centrifugation (8500*g*, 2 min), and suspended in 1% glutaraldehyde. Following incubation for 5 min at 25°C, the cells were washed (8500*g* for 2 min), pelleted, and blocked by incubation with 1 mL 1% BSA in 0.1 *M* phosphate buffer saline (PBS), pH 7.8, at 25°C for 10 min. One mL of the antiserum against *L. garvieae*, diluted 5×10^2–1×10^4-fold with 1% BSA in 0.1 *M* PBS, pH 7.8, was added to the pelleted cells in the tube and incubated at 25°C for 60 min in the dark. The cells were then washed with 1% BSA in 0.1 *M* PBS, pH 7.8 and resuspended in 1 mL of the FITC-labeled second Ab solution, diluted 1×10^2-fold with 1% BSA in 0.1 *M* PBS, pH 7.0. After incubation for 60 min at 25°C in the dark, the cells were washed twice and resuspended in 1% BSA in 0.1 *M* PBS, pH 7.0. The cells were used for the FCM analyses.

FCM Assay

Flow cytometry was performed using EPICS XL (Coulter, Miami, FL) equipped with an argon laser. The power output was 15 mW, and the 488 nm wavelength was used to excite the fluorescent probes. Light scattered in the forward light scatter was filtered by a 488 nm narrow band-pass filter, and collected by photomultiplier. The fluorescent emission from the FITC-labeled cells, excited at 488 nm, was detected at 505–545 nm with a FL1 photomultiplier. The fluorescent signal was gated using manual-gating regions. To establish optimum conditions for cell counting, the value of the discriminator was varied from 0 to 100. The stop time for the passage of the sample was set at 60 or 120 s, and an event number (number of particles passing through the laser beam) was measured as the number of bacteria.

RESULTS AND DISCUSSION

The results indicate that the method is highly specific for the rapid detection of *L. garvieae* organisms from among several species of bacteria. The antiserum containing *L. garvieae* Ab (first Ab) reacts with this organism specifically. The rabbit Ab binding to *L. garvieae* can react with the FITC-labeled second Ab (second Ab), and consequently the resultant FITC-labeled cells fluoresce. The number of FITC-labeled cells could be measured by counting the event number, which is the number of particles passing through the laser beam of FCM. Thus, it was possible to determine the cell number of *L. garvieae*.

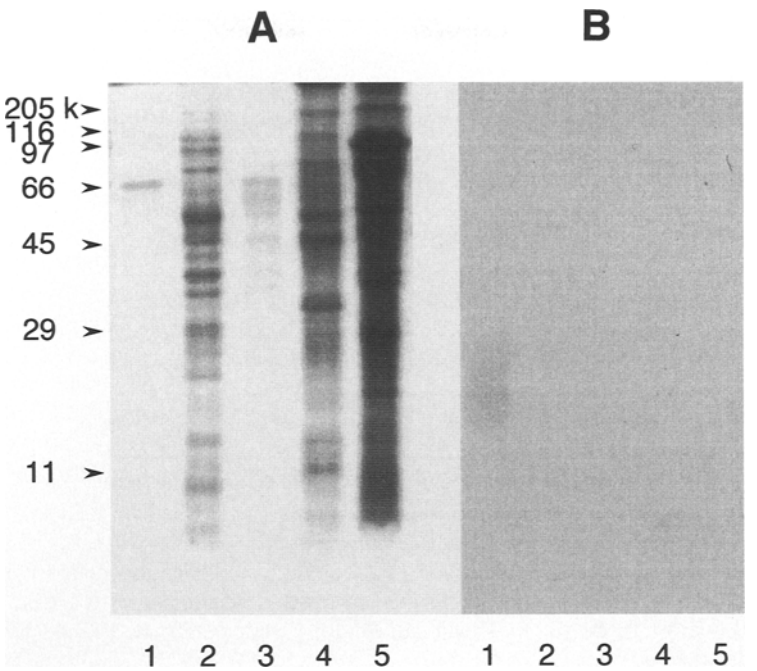

Fig. 1. Electrophoretic analyses. **(A)** SDS-PAGE; **(B)** immunoblotting analysis. 1, *L. garvieae*; 2, *E. coli*; 3, *S. aureus*; 4, *P. fluorescens*; 5, *Vibrio* sp.

Immunological Property

The specificity of the antiserum against *L. garvieae* was examined by SDS-PAGE and by immunoblotting (Fig. 1). Although samples with similar cell counts were applied to the gels, much lower intensities were observed in lanes occupied by *L. garvieae* and *S. aureus* than in others when stained with Coomassie brilliant blue (Fig. 1A). SDS probably failed to extract proteins from the bacteria, because these bacteria are Gram-negatives and have a SDS-tolerant outer membrane containing peptidoglycans and teichoic acids.

In the immunoblotting analysis, the Ab strongly reacted with *L. garvieae* protein(s) of about 20,000 in mol wt, in spite of the low extractability with SDS (Fig. 1B). Thus, it is revealed that the present Ab would recognize some epitopes of *L. garvieae* with high specificity.

Correlation Between Event Number and Cell Number for Various Values of the Discriminator

In this study, the number of bacteria was measured by counting an event number of FCM. However, the value of the event number was read-

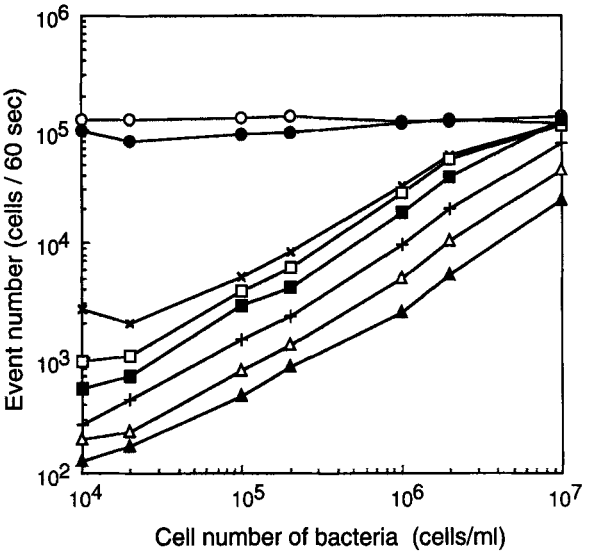

Fig. 2. Correlation between the event number and the cell number for various values of the discriminator. *L. garvieae* cells was used. The values of the discriminator were as follows: ○, 0; ●, 10; ×, 15; □, 20; ■, 40; +, 60; △, 80; ▲, 100. A stop time of FCM was set at 60 s.

ily influenced by the debris in a sample and by light scattering. For this reason, it was necessary to establish favorable assay conditions by varying the discriminator. A discriminator is a channel setting for a parameter allowing events below the setting to be ignored, and it can eliminate signal caused by debris. Figure 2 shows a correlation between the event number (cells/60 s) and the cell number (cells/mL) of *L. garvieae* for various values of the discriminator. The event number is seen to increase as the value of the discriminator is decreased at low concentrations of the cells. When the discriminator was set at 0, the event number did not change with the cell concentration, and was represented by a straight line. This observation may have been caused by the effects of the cell debris or light scattering. On the other hand, when the value of the discriminator was set above 20, the event number increased with increasing cell concentration. A linear correlation was observed between both the parameters when the discriminator was set at 60. Therefore, all subsequent experiments were performed at this value.

Fluorescence Scattergrams and Histograms for *L. garvieae* and *E. coli*

Figure 3 shows typical fluorescence scattergrams (3.1a–d) and histograms (3.2a–d) of bacterial samples. *E. coli* was used as main reference microorganism, because it is frequently found in marine aquacultures.

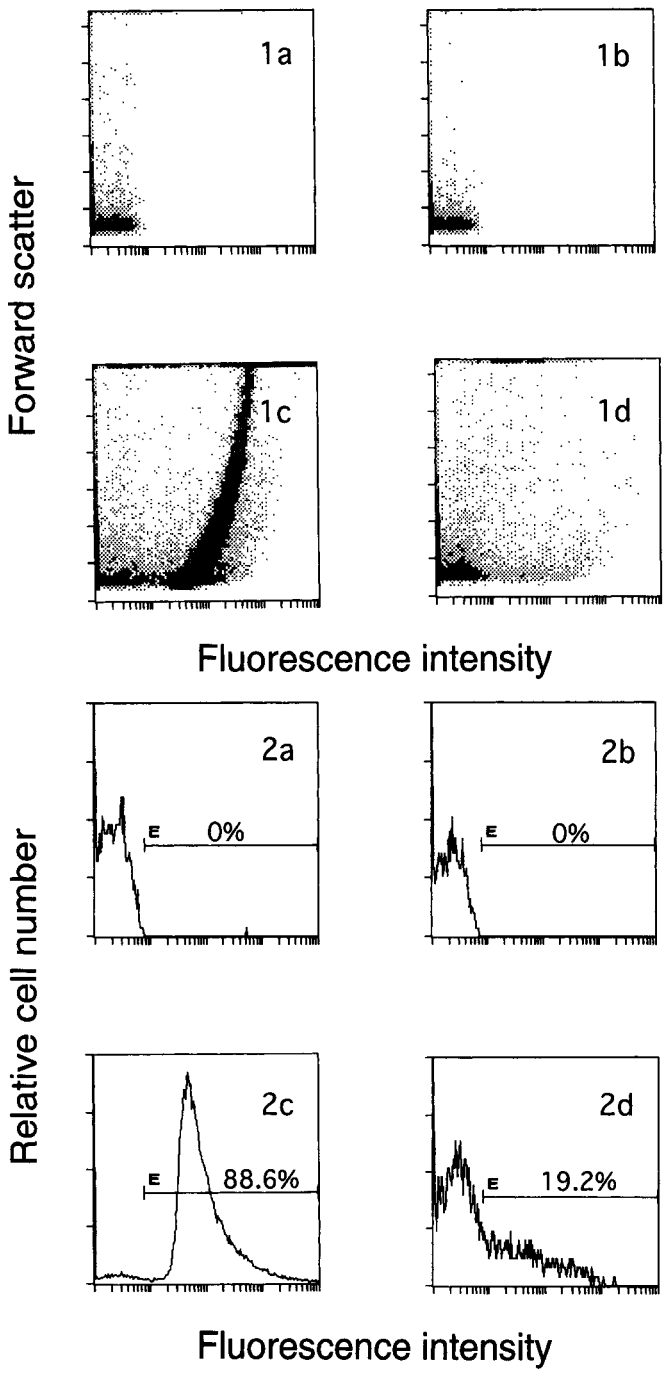

Fig. 3. Fluorescence scattergrams and histograms for *L. garvieae* and *E. coli*. 1, scattergram for FITC fluorescence intensity and the forward-scatter intensity; 2, histogram for FITC fluorescence intensity and relative cell number. a, control for *L. garvieae*; b, control for *E. coli*; c, *L. garvieae* labeled with FITC; d, *E. coli* labeled with FITC. Each cell concentration was prepared at 1×10^6 cells/mL. Antiserum and FITC-labeled second Ab were diluted 1×10^4-fold and 1×10^2-fold, respectively. The conditions for the FCM assay were as follows: discriminate value, 60; stop time, 60 s.

In Fig. 3.1a–d, the horizontal axis represents FITC fluorescence intensity, and the forward scatter intensity is plotted along the vertical axis. The fluorescence scattergrams of the control samples, which do not react with the antiserum against *L. garvieae,* are shown in Fig. 3.1a, b. In both cases, the fluorescence intensity was very low, and the forward scatter reflected in the cell size exhibited similar values. Figure 3.1c, d show the fluorescence scattergrams for *L. garvieae* and *E. coli,* respectively, reacted with Ab to *L. garvieae,* and later with the FITC-labeled second Ab. The control samples all possessed low fluorescence intensity, whereas high fluorescence intensity was observed in the sample of *L. garvieae* (Fig. 3.1c). Furthermore, when the FITC-labeled second Ab solution was added to *L. garvieae* cells that had not reacted with antiserum to *L. garvieae,* very low fluorescence was observed (data not shown). Thus, the FITC-labeled second Ab did not react with *L. garvieae* cells. From these results, it is presumed that, since the antiserum reacted with *L. garvieae* specifically, the FITC-labeled second Ab could bind to the Ab to *L. garvieae,* and fluoresce. In the case of *E. coli,* because the antiserum did not react with the cells, the FITC-labeled second Ab also could not bind to it. Thus, this accounts for the low level of fluorescence in the sample of *E. coli.*

Figure 3.2a–d shows the fluorescence histograms for the same bacterial samples. The horizontal axis represents FITC fluorescence intensity, and a vertical axis represents relative cell number. In Fig. 3.2a and b, the maximum values for the number of the cells were observed at low level of fluorescence intensity. A linear region (E) was drawn in the histograms and was adjusted to distinguish between the cells; that is, the cells within region (E) are regarded as cells that reacted with the antiserum, and outside of (E) are considered the cells that did not react with the antiserum. The FITC-labeled cells in the region (E) was 88.6% (Fig. 3.2c), but only 19.2% of cells were contained in region (E) in the case of *E. coli,* as shown in Fig. 3.2d. Thus, it is possible to discriminate between *L. garvieae* and *E. coli* cells, using the FCM method.

Optimum Concentration of Antiserum on Antigen–Antibody Reaction

In general, the Ag–Ab reaction was influenced by the Ab concentration. Figure 4 shows the effect of concentration of antiserum to *L. garvieae* on the Ag–Ab reaction of *L. garvieae* and *E. coli.* Each cell concentration was 1×10^6 cells/mL. The number of FITC-labeled cells between *L. garvieae* and *E. coli* was almost the same when diluted 5.0×10^2-fold. This phenomenon might be induced by nonspecific reaction of the Ab with *E. coli* cells or by interference with Ag–Ab reaction, caused by the high concentration of the antiserum against Ag. Upon further dilution of the antiserum, the difference between ratio of labeled and nonlabeled cells extended to 4–5 × at 1.0

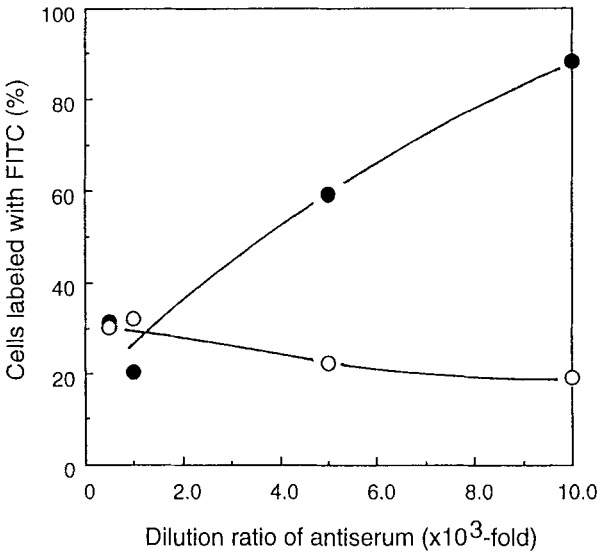

Fig. 4. Effect of antiserum concentration on the Ag–Ab reaction. ●, *L. garvieae*; ○, *E. coli*. Each cell concentration was prepared at 1×10^6 cells/mL. The FITC-labeled second Ab was 1×10^2-fold. The FCM assay conditions were the same as in Fig. 3.

$\times 10^4$ of dilution ratio of the antiserum. Therefore, the subsequent experiments were performed at 1.0×10^4 of dilution ratio of the antiserum.

Specificity of Antiserum for Several Species of Bacteria

The specificity of the antiserum was also examined against *S. aureus*, *P. fluorescens*, and *Vibrio* sp. (Fig. 5). Each cell concentration was prepared at 1×10^6–2×10^6 cells/mL. The antiserum reacted with *L. garvieae*, specifically, reaching a maximum value of 90%, but not against *E. coli*, *S. aureus*, *P. fluorescens*, and *Vibrio* sp. The difference extended to 4–5 × between *L. garvieae* and two bacterial strains, such as *E. coli* and *P. fluorescens*.

At present, the antiserum does not perfectly distinguish between *L. garvieae* and other species of bacteria cells. However, it is possible to estimate the existence of *L. garvieae* cells in aquaculture to some extent by using a shape of scattergram. For instance, in Fig. 3, noticeable difference in scattergram between *L. garvieae* and *E. coli* cells was observed, although a nonspecific binding was obtained 19.2%.

Calibration Curve for *L. garvieae*

Figure 6 shows a plot of the event number of FCM against cell number of *L. garvieae* labeled with the FITC. The curve was linear in the range of 2.4×10^4–1.5×10^7 cells/mL (relative error, ±2%). One FCM assay could be completed within 120 s. Although it was difficult to measure sample

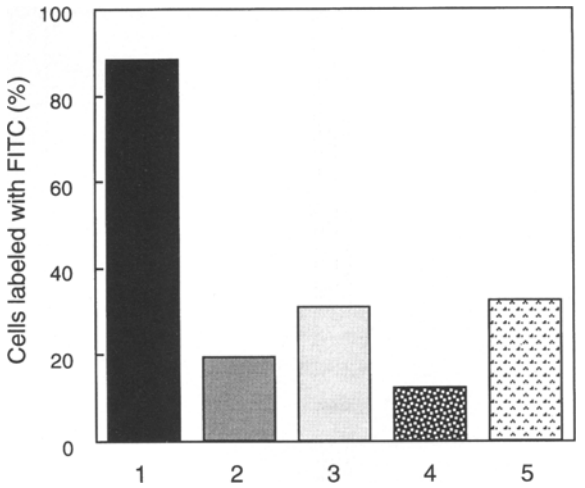

Fig. 5. Specificity of antiserum to *L. garvieae* and other species of bacteria. 1, *L. garvieae*; 2, *E. coli*; 3, *S. aureus*; 4, *P. fluorescens*; 5, *Vibrio* sp. Each cell concentration was prepared to 1×10^5–2×10^6 cells/mL. Antiserum and FITC-labeled second Ab were diluted 1×10^4-fold and 1×10^2-fold, respectively. The FCM assay conditions were the same as in Fig. 3.

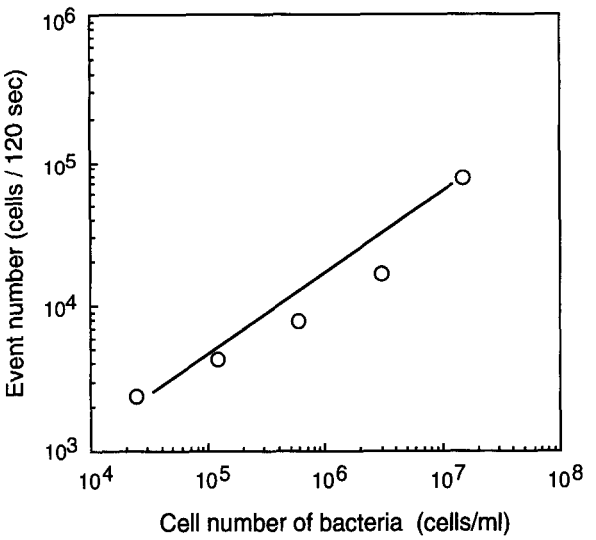

Fig. 6. Calibration curve for *L. garvieae*. Antiserum and FITC-labeled second Ab were diluted 1×10^4-fold and 1×10^2-fold, respectively. The FCM assay conditions were the same as in Fig. 3.

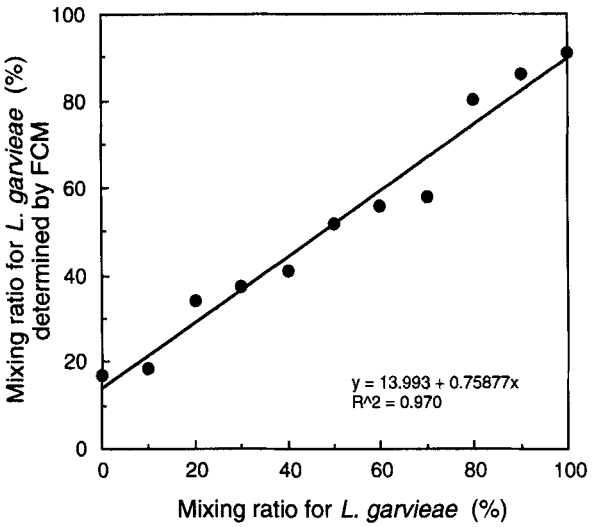

Fig. 7. Measurement of the mixing ratio of *L. garvieae* in cell suspensions by FCM method. All samples contained 10^5–10^6 cells/mL. Antiserum and FITC-labeled second Ab were diluted 1×10^4-fold and 1×10^2-fold, respectively. The FCM assay conditions were the same as in Fig. 3.

concentrations less than 10^4 cells/mL, it was possible to determine low concentrations by increasing the sample volume. For example, if 1 mL of sample, containing 10^3 bacterial cells, was passed through the cytometer at 100 μL/min, then it would be possible to detect 1000 events in 10 min. Therefore, the FCM method may be applied for the rapid determination of *L. garvieae* cell number.

Detection of *L. garvieae* in Cell Suspensions Contaminated with *E. coli*

The FCM method was applied to the detection of *L. garvieae* in cell suspensions contaminated with *E. coli*. *L. garvieae* cultivated in BPG medium and *E. coli* cultivated in LBS medium were mixed in differing proportions. All samples contained 10^5–10^6 cells/mL (logarithmic phase cells). Figure 7 shows a plot of the mixing ratio of *L. garvieae* determined by FCM assay. A good correlation is seen in the range of 20–90% for the mixing ratio of *L. garvieae*. However, at lower levels, the correlation was not good, because of antiserum binding to 19.2% of *E. coli*, as described previously. Although the detection of *L. garvieae* was not possible when the mixing ratio was below 20%, the method was successful when the mixing ratio was between 20 and 90%. One FCM assay could be completed within 120 s and the total assay time, including the preparation of bacteria, was within 3 h.

CONCLUSION

The proposed assay of *L. garvieae* using FCM required significantly less time than the traditional colony-counting methods, and was applicable for the rapid detection of this organism. Work is presently in progress toward widespread application of the FCM assay for the detection of bacteria in marine aquaculture.

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