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螢光顯微鏡、流式細胞儀與定量 PCR 用於生物氣膠之分析 (1/3)

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計畫主持人：李芝珊

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行政院國家科學委員會專題研究計畫成果報告

螢光顯微鏡、流式細胞儀與定量 PCR 用於生物氣膠之分析(1/3)

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個別型計畫： 計畫主持人：李芝珊

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執行單位：台灣大學環境衛生研究所
中華民國 93 年 5 月 21 日

行政院國家科學委員會專題研究計畫成果報告

螢光顯微鏡、流式細胞儀與定量 PCR 用於生物氣膠之分析(1/3)
REAL-TIME QUANITITATIVE PCR WITH GENE PROBE, FLUOROCHROME, AND FLOW CYTOMETRY FOR BIOAEROSOL ANALYSIS

計畫編號：92-2320-B-002-149-

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一、中文摘要

生物氣膠至今的主要分析方法仍是培養，但此方法有些許限制。因此，本研究便朝向建立與評估非培養方法在生物氣膠分析運用上的可行性與相互間的比較。在本研究中，選取大腸桿菌為測試標的進行流式細胞儀、螢光顯微鏡、real-time qPCR 與培養方法之比較，並運用 AO 與 PI 兩種螢光染劑進行全部細胞與死亡細胞之染色，結果顯示，流式細胞儀得到之結果較螢光顯微鏡要大(2.62 – 4.94 倍)，此結果應與螢光顯微鏡方法之前處理步驟較多而產生之損失有關。在活性上，流式細胞儀得到之活性較螢光顯微鏡為大，而培養的方法所得到的活性為最小，此外，流式細胞儀的精確度與正確性都較螢光顯微鏡為佳，因此，流式細胞儀提供生物氣膠分析一不錯的選擇。在 real-time qPCR 方面，其測量到的值也與其他兩種方法所測到之所有的細胞濃度呈良好相關，因此，本研究顯示，對於所有微生物濃度與微生物活性的分析，非培養方法能提供更快速與精確的資訊。

關鍵詞：生物氣膠、非培養之分析、流式細胞儀、螢光顯微鏡、PCR、方法比較

Abstract

Currently, culture-based analysis still remains the primary methods for bioaerosol analysis. For better understanding and quantifying microorganism, both culture and nonculture-based methods should play equally important role. In this investigation, *E. coli* was used to compare three nonculture methods, flow cytometry (FCM), epifluorescence microscopy (EFM), and real-time qPCR with gene probe, with culture method. Moreover, acridine orange (AO) and propidium iodide (PI) were used as fluorescent dyes to determine the viabilities of microorganisms. Our results indicated that the total cell concentrations counted by FCM were statistically higher than the yield of EFM (2.62 – 4.94 folds). This might be related to cell losses by extensive preparations for EFM. Regarding viability, measured viabilities ranged from high to low in the order of that from FCM, EFM, and culture method. In addition, FCM performed better on both precision and accuracy than EFM. Therefore, it was demonstrated that FCM provides a better choice than EFM for microorganism analysis, and traditional culture-based method underestimates microorganism viability. In addition, strong correlations between EFM and FCM were demonstrated for both concentrations and viability. Furthermore, it was also found high associations between DNA obtained by real-time quantitative polymerase chain reaction (qPCR) and total number concentration by AO from both EFM and FCM. In summary, non-culture methods could provide rapid and accurate information for microorganism analysis regarding microorganism concentrations and viabilities.

Keywords:bioaerosol, nonculture-based method, real-time qPCR, epifluorescence

microscopy, flow cytometry, methods comparison, viability

二、緣由與目的

Recently, exposure assessment of bioaerosols has become an important issue. Microorganism consists of culturable, non-culturable, viable or non-viable components. Currently, culture-based analysis still remains the primary method for microorganism analysis. However, several important pathogenic microorganisms are viable but nonculturable in the air such as *Mycobacterium tuberculosis* and *Legionella pneumophila* (Ballard et al., 2000; Schafer et al., 1999). Fail to identify non-viable or non-culturable microorganisms, analytical methods could not accurately measure microorganism levels and underestimate human exposure to bioaerosols. This might be the reason why field evaluations demonstrated very weak associations between colony-forming unit (CFU) by culture-based method and human health effects (Li et al., 1997; Wan and Li, 1999). Furthermore, culture-based method can take several days to weeks to perform. These disadvantages may cause effects ranging from much more time-consuming in researches to serious results in some special situations, such as germ warfare. For better understanding and quantifying microorganisms, both culture-based and nonculture-based methods should play equally important role to provide more information of the whole story.

Regarding nonculture-based methods, there are primary microscopic, immunological, biochemical, chemical, flow cytometric (FCM), and molecular biological techniques. Among these methods, chemical analysis, immunoassay, and biochemical assay are commonly used to detect either the component or the fragment of microorganisms. In addition, ELISA (enzyme linked immunosorbant assay) had also successfully detected total concentration of airborne bacteria (Speight et al., 1997). However, the analytical methods mentioned above cannot distinguish viable from nonviable microorganisms. Microorganism viability is an important characteristic related to human health effects. The understandings of the differences in culturibility, viability, and total counts of microorganism should provide more insights of microorganism characteristics and related health risk. Therefore, epifluorescence microscopy (EFM), FCM and molecular biological methods are demonstrated to be the most potential nonculture-based means to be evaluated.

EFM is a well-developed method available for the enumeration of bacteria in environment samples (Kepner et al., 1994). The viability measurements of EFM and FCM include a variety of stain-based methods. The so-called vital stains for estimating microbial viability fall into three broad categories. The first one is membrane integrity, such as Propidium iodide (PI), which is excluded by the intact membranes of viable cells. Therefore, the presence of the dye within the cell indicates disruption of the cell membrane and may be correlated to cell death. The second one is metabolic activity, such as 5-Cyano-2, 3-ditolytetrazolium chloride (CTC). The third one is enzymatic activity, such as fluorescein diacetate (FDA). FDA dye is a membrane-permeant nonfluorescent precursor converted to a membrane-impermeant fluorescent molecule by the activity of intracellular enzymes, and thus is an indicator of enzymatically active cells. For molecular biological methods, the fluorescent probe was used to anneal target nucleic acid. Moreover, it was observed that direct microscopic enumeration has demonstrated that numbers of bacteria capable of forming colonies on nonselective media are usually several orders of magnitude fewer than numbers actually present and metabolically active in freshwater, marine, and soil environment (Roszak et al., 1987). These results demonstrated that the majority of bacteria existing in environmental medium are either nonviable, or viable but nonculturable. In regard to microorganism application, it was reported that DTAF and CTC dyes by EFM were used to evaluate absolute measurements

of total and viable airborne microorganisms under in situ conditions (Hernandez et al., 1999). The results indicated that this direct microscopic technique, capable of characterizing microorganisms, is considered to be a laborious approach.

Regarding FCM, many researches have already applied FCM to measure the total, viable or metabolically active microorganisms in seawater, fresh water, lake, biofilm, or waste water samples. For FCM, the greatest advantage is the automatically rapid counting nature with a counting speed of 1000 cells per second. Moreover, basic cell functions such as reproductive ability, metabolic activity, and membrane integrity, to characterize the physiological state or degree of bacterial viability can be also determined by various fluorescent dyes (Nebe-von-Caron et al., 2000). All these characteristics of FCM are very powerful for understanding the generation stress, sampling stress with different samplers, and the physiological states of microorganisms by different indicators such as viability, metabolic activity, or culturability. Therefore, FCM should be a valuable new toolbox to microorganism analysis.

Among molecular biological methods, polymerase chain reaction (PCR) was successfully applied to characterize microorganisms with relatively low detection limits (Mukoda et al., 1994). Moreover, PCR used for detecting airborne *Mycobacterium tuberculosis* H37Ra particles (surrogate of Mycobacterium tuberculosis) demonstrated to be a very sensitive technique (Shafer et al., 1998; Schafer et al., 1999). In the previous investigations (Alvarez et al, 1994; Alvarez et al., 1995), the PCR method is only a qualitative method. Recently, real-time quantitative polymerase chain reaction (qPC)R using TaqMan system is accomplished by the in-tube, real-time detection of PCR accumulation during each amplification cycle by utilizing an internal probe in addition to standard PCR amplification primers. In clinical studies, TaqMan real-time qPCR was popularly used to detect *M. tuberculosis* (Desjardin et al., 1998) and *E. Coli* O157:H7 (Oberst et al., 1998; Sharma et al., 1999). The real-time qPCR demonstrated several remarkable advantages over quantitative PCR approaches (Heid et al., 1996). The fluorogenic assay is a convenient and self-contained process with steps of reaction setup and tube sealing. Unlike other quantitative PCR method, real-time qPCR does not require post PCR sample handling, thus preventing potential PCR product carryover contamination and resulting in much faster (2.5 hr) and higher throughput (96 samples/reaction) assays. Therefore, the real-time qPCR has a very large dynamic range of starting target molecule determination (at least 5 orders of magnitude), as well as is extremely accurate and less labor-intensive than current quantitative PCR methods.

Regarding environmental samples, there were levels of chemical and genetic complexity that not normally encountered in tissue and/or physiological samples or pure cultures. These complexities affect the ability of TaqMan PCR to quantity RNA and DNA in these matrices. From several recent reports (Becker et al., 2000; Suzuki et al., 2000; Takai et al., 2000), it was suggested that TaqMan chemistry could be successfully applied to DNA analysis in concentrated water samples. Moreover, *Staphylococcus aereus* cells were also quantified from food sample by real-time quantification PCR in cheese (Hein et al., 2001). Taking the advantages of the simple, sensitive, specific, fast, and automatic characteristics, the real-time qPCR technique should be a very promising technique for microorganism analysis.

Until now, no data was available regarding intercomparisons among culture method, real-time qPCR, FCM, and epifluorescence microscopy for evaluating total number concentrations and viabilities of microorganisms. In our current investigation, three nonculture methods, EFM, FCM, and the real-time quantitative PCR with gene probe, were compared with culture method regarding microorganism concentrations and viabilities.

三、材料方法

Test microorganism

Escerichia coli (E. coli, ATCC10675*)* from Taiwan Food Industrial Research and Development Institute (FIRDI) were used in this evaluation. An active *E. coli* culture was inoculated into nutrient broth (Difco) and incubated for 24 h at 37 ^{o}C. The broth were later aseptically washed by sterile phosphate buffered saline (PBS) into a 15-ml sterile conical centrifuge tube, capped, and centrifuged at 4,000 rpm (Model 2010, Kubota, Japan) for 5 min twice. The cell suspension (5x10^{8}/ml) was divided into two equal aliquots of 1 ml. Cells in one aliquot were exposed to 20 ml 70% isopropanol solution for 1 hr and another aliquot was exposed to 20 ml PBS. These two aliquots were separately resuspended in a total volume of 5 ml PBS (Karelyants et al., 1992). The suspension exposed to isopropanol was defined as 0% controlled-viability samples, and another aliquot exposed to PBS was defined as 100% controlled-viability samples. Four different controlled-viability samples (90%, 60%, 30% and 10%) made by these two aliquots were used in this study. In addition, the colony forming unit (CFU) numbers in suspension samples were also determined on TSA (trypticase soy agar, Difco) plates, plated with 10 serial dilutions and incubated for 24 h at 37 ^{o}C (Jensen et al. 1992).

Dye Chosen

Viability assays using fluorescent dyes AO and PI have been developed for the visualization of both viable and nonviable human cells (Bank et al., 1987). AO is a membrane-permeable, and cationic dye that binds to nucleic acids of both viable and nonviable cells with green fluorescence. PI is impermeable to intact membranes, but readily penetrates the membranes of nonviable cells and binds to DNA or RNA with orange fluorescence. When AO and PI were used separately, total (viable and nonviable) cells (fluoresce green) and nonviable cells (fluoresce orange) were observed.

Optimization of staining methods for EFM and FCM

In order to evaluate the AO optimization conditions for EFM and FCM, the *E coli* counts were measured at different stain concentrations (5, 10, 50, 100 μg/ml for EFM, and 1, 3, 5, 10, 15 μg/ml for FCM) and incubation times (3, 5, 10, and 15 min). In regard to PI staining, the optimal condition was 1.5 μl of 20 mM PI for 15 min which was previously reported (Kaprelyants et al., 1992).

EFM

The *E.coli* suspension was observed under EFM (model HAL 100, Laborlux K, Leica, Germany) fitted with a mercury 50 W lamp, a BP 450-490 nm excitation filters and an LP 515 nm emission filter. Aliquots of each suspension were filtered through a 0.2 μm black Nuclepore membrane filter and the filter was placed between slide and cover slide. For AO, green staining cells were counted, as well as red staining cells were counted by PI. The counting conditions are 30 to 50 bacteria per field and 15 fields per sample. All of the EFM samples were analyzed in triplicate.

FCM

FCM was performed with *E. coli* cell suspensions labeled with AO and PI, with AO only, with PI only, and with non-labeled cells. Cell suspensions were diluted in PBS that had been filtered through a 0.22 μm pore-size-filter. Monodisperse fluorescein-tagged 1.0-μm-diameter spherical polystyrene beads (Fluoresbrite; Polyscience, Inc., Warrington, PA) were used to enable enumerations of cells in the FCM samples. FCM samples were prepared by mixing 1 ml cell suspension stained with AO or PI, and 20 μl of fluorescent bead suspension (7.37 x 10^{7} beads/ml). In addition, T$_{RU}$count^{TM} tubes (Bection Dickinson, Inc., U.S.A) with a known concentration (548,950/ml) were used as standard solutions for evaluating FCM accuracy.

Analysis by FCM immediately after staining was completed with a FacsCalibur flow cytometer (Becton Diskinson, San Jose, CA) equipped with air-cooled argon laser (488 nm, 15 mW). The samples were vortexed

prior to analysis. A side scatter (SSC) threshold level was used to reduce background noise. The sample was delivered at low flow rate, which gave 300 to 600 events per second. In this study, 100,000 counts were used for data acquisition. For each cell, forward scatter (FSC), SSC, green fluorescence (515 to 545 nm), yellow-orange fluorescence (564 to 606 nm), and red fluorescence (< 670 nm) were recorded. Stained microorganisms were discriminated and enumerated by SSC (related to cell granule) and green fluorescence (FL1) (530 ± 30 nm). A four-log decade was used for all cytograms. Viable cells were indicated in a plot of SSC versus FL1, and nonviable cell concentrations were demonstrated in a plot of SSC versus red fluorescence (FL3). The parameters of FSC and SSC were collected as logarithmic signals. Basically, the volume of a bacterial cell is highly correlated to the parameter of FSC (Robertson et al., 1989). However, *E. coli* cell cannot be separated from the granule of PBS by FSC (data not shown). The size of *E. coli* cell is very close to size detection limit (0.5 μm) of FSC. Therefore, the parameters of SSC and fluorescence intensity were used to distinguish cells from debris and beads in this investigation.

FCM was evaluated for its capacity of distinguishing bacteria from background events of instrument noise and granules in buffer. A dot plot of a microbial suspension was used to identify the region of background events. The coordinates of the region were confirmed with a blank sample, for which all events were within the demarcated background region. By AO/PI dyes and SSC, the population of bacteria cells could be clearly discriminated from background of similar density (i.e., equivalent side scatter values), as well as from the fluorescent polystyrene beads added to the sample for enumeration purpose. This region of bacterial cells was gated for enumeration of microbial suspension. All of the samples analyzed with FCM were done in triplicate.

DNA extraction/purification method

Bacteria isolates were used for PCR. Approximately 10^7 CFU/ml of overnight culture bacteria were extracted by Bacteria DNA extraction Kit (Hexwater Inc., Germany). For bacterial cultures, 200 μl standardized suspension was added to 1 ml of a WB1 lysis buffer, vortexed, and then pelleted by centrifugation at 12,000 rpm for 1 min. After removing the supernatant, the pellet was suspended in 200 μl buffer WB2 (included beads on the bottom of the buffer) and incubated at 55 °C for 30 min. After vortexing for 5 sec, and spinning down, it was boiled at 100 °C for 8 min to release the DNA. The samples were pelleted by centrifugation at 12,000 rpm for 3 min. Finally, 100 μl supernatant of each sample was saved for PCR and stored at -20 °C.

Real-time qPCR with gene probe

The primers and probes (ABI, applied biosystem inc., Forster City, CA) of our target microorganism were reviewed from a previous investigation (Corless et al., 2000) and was checked with Primer Express 1.0 software (PE Biosystem, Forster City, CA), which produces a 94-bp amplicon (CCATG AAGTC GGAAT CGCTA GTAAT CGTGG ATCAG AATGC CACGG TGAAT ACGTT CCCGG GCCTT GTACA CACCG CCCGT CACAC CATGG GAGT). The primer sequences were CCATGAAGTCGGAATCGCTAG (forward) and ACTCCCATGGTGTGACGG (reverse). The probe sequences was 6-FAM-5' CGGTGAATACGTTCCCGGGCCTTGTA3' -TAMRA.

The PCR assay with TaqMan System uses a fluorogenic probe labeled at the 5' end with a reporter dye (6-FAM) and at the 3' end with the quencher dye (TAMRA). When the sequence-specific probe is cleaved by *Taq* DNA polymerase 5' nuclease activity, the reporter dye is separated from the quencher dye, generating a fluorescent and sequence-specific signal. The TaqMan system monitors fluorescence level at every cycle. In this way, the threshold cycle (Ct) value can be determined and the real-time progress of the PCR can be monitored. The

5' nuclease PCR with fluorogenic probe is run under generic cycling conditions, so the optimization of primer concentration was required to take account of real differences in primer melting temperature. For *E. coli* probe, a primer concentration of 300 nM was found to be the most efficient, giving a high endpoint fluorescence and low Ct.

PCR was performed in 50 µl volume using MicroAmp Optical 96-well reaction plates and MicroAmp Optical Caps (PE Biosystems) for each well. All reagents were obtained from the TaqMan Core PCR Reagent kit (PE Biosystems). The PCR mixture was as following: 5 µl of cell lysate; 25 µl of 2 x TaqMan universal Master Mix; 300 nM (each) forward and reverse primers; 200 nM probe. The sequence detector system (ABI Prism 7700; Applied Biosystem) was used for amplification and fluorescence measurement. All cycles began with 2 min at 50 ^{o}C for UNG enzyme incubation, and then AmpliTaq Gold was activated at 95 ^{o}C for 10 min. The subsequent PCR conditions consisted of 50 cycles of denaturation at 95 ^{o}C for 20 s, and annealing and extension at 60 ^{o}C for 1 min per cycle. All the samples analyzed with real-time qPCR were done in triplate.

During the PCR amplification, the amplified products were measured continuously by determination of fluorescence emission. After real-time data acquisition, Ct value for each sample was calculated by determining the point at which fluorescence exceeded a threshold limit (10 times the baseline standard deviation). For establishing the standard curve, a standard DNA solution was constructed. The construct was used as the primary standard after quantification by optical density determination. A standard curve by serially diluted external DNA Standard was prepared. For dilution of the standards, distilled water was used, and the standard was stored at –20 ^{o}C. Ct values obtained from the samples were plotted on the standard curve, and the number of copies was calculated.

Statistical methods

Software programs (Excel for Windows XP, version 7.0, Microsoft, Redmond, WA; SigmaPlot for Windows, version 3.06, SPSS, Chicago, IL) were used for data analysis. Measurements performed by each method were analyzed by linear regression, regression coefficients were compared with the ideal values of 0 and 1, and 95% CIs were determined. In addition, the comparisons of different methods for microorganisms were performed using regression analysis.

四、結果

Measured-viability

The percentage of CFU (CFU%, the CFU grown from each of the three plating concentrations at different controlled-viability samples divided by that of 100% controlled-viability samples) was used to evaluate the viability of the fresh culture of *E. coli*. It was observed that CFU% was extremely linear with controlled viability consistently for the entire range of viability measurement, from 10% to 90% (Fig. 1). The linear regression equation for CFU% was Y=0.92X+1.93, with R =0.97. Slope and intercept of this regression line were not significant from 1 and 0, respectively, and the regression line for predicted viability fell within the 95% CI.

Optimal concentrations of AO

By EFM, at 50 µg/ml AO concentration, the cell concentrations were the same at different incubation time (3, 5, 10, 15 min) (data not shown). Regarding the optimal AO concentrations of EFM, the best quantity of stained cell was obtained at 50 µg/ml. The cell concentrations of 50 µg/ml and 100 µg/ml were found to be 1.7 folds higher than those of 10 µg/ml and 5 µg/ml with a coefficient percentage of variation (CV%) ranging from 1.7% to 32.6%. Therefore, the optimal staining protocol of AO for EFM detection was 50 µg/ml AO for 5 minutes. Regarding FCM optimization, AO concentration higher than 10 µg/ml was found not to separate *E. coli* cells and background. Moreover, no differences of cell concentrations in 1, 3, 5,

and 10 μg/ml of AO were observed (data not shown). In addition, cell concentrations at 3 μg/ml AO concentration were similar for the incubation time of 2, 5, 10, and 15 min (data not shown). Therefore, the optimal staining protocol for AO with FCM was 3 μg/ml AO for 5 minutes.

EFM

By using EFM, the total cell concentrations stained with AO were observed to range from 7.9 x 10^6 /ml to 1.3 x 10^7 /ml, whereas the determinations of nonviable cell concentrations stained with PI range from 3.2 x 10^6 /ml to 1.3 x 10^7 /ml (Table 1). Regarding viability measured by EFM, it was found that measured and controlled viability was linear which is consistently in agreement for the entire range of viability measurement (Fig. 1). The linear regression equation for controlled-viability and EFM-measured viability was Y=0.75X+6.03 (R = 0.9). In addition, the measured CFU% in each sample was also found to be proportionately linear to its predicted percentage of controlled-viability samples. The percentage of viable cells measured by EFM was correlated well to CFU% (R=0.98). Furthermore, the percentage of viable cells measured by EFM was pretty closed to CFU%. Regarding the precision of EFM, the CV% of *E. coli* cells stained with AO was 17.5 % (6.9 % to 30.9 %). In regard to PI staining, the average value of CV% was found to be 23.7 % (10 % to 56.5 %).

FCM

By using AO and PI to stain microbial cells, as well as SSC, the population of bacteria cells could be clearly discriminated from background of similar density, as well as from the fluorescent polystyrene beads added to the sample for enumeration purposes (Fig. 2). For analysis with FCM, the differences in the measured concentrations of bacteria from dilution to dilution were only correlated linearly from 10^4 /ml to 10^7 /ml (R=0.96). It was demonstrated that the detection limit of FCM for *E. coli* was 10^4 /ml. Speaking of FCM accuracy, it was found that the relative error between measured values (556,351/ml with standard deviation of 2,780/ml) and true values (548,950/ml) was 1.35%. Regarding FCM precision, the CV % values were in the range of 0.6% to 5.3% for AO and 2.1% to 6.9% for PI, respectively. It was clearly demonstrated that FCM has very high accuracy and precision.

Regarding the total cell concentrations, it was found that counts by FCM (2.2 x 10^7 /ml to 6.1 x 10^7 /ml) were 2.62 – 4.69–folds (p=0.029) of the yield of EFM (Table 1). Moreover, the nonviable cell concentrations by FCM (4.5 x 10^6 /ml to 4.3 x 10^7 /ml) were 1.41 – 3.3-folds (p=0.41) of the yield of EFM. These observations indicated that the yields of FCM are all higher than those of EFM and the amount of difference depend on dyes. Regarding viability measured by FCM, the measured-controlled viability regression line was y=0.98x+16, with a correlation coefficient (R) of 0.84 (Fig. 1).

Real-time quantitative PCR (qPCR) with gene probe

In order to establish a real-time quantitative PCR, we first developed a standard curve of serial 10-fold dilutions of *E. coli* DNA. It was revealed high reproducibility of the standards in the range of 4.24 x 10^5 DNA copies/μl (4.5 x 10^4 /μl) to 4.24 x 10^{10} DNA copies/μl (4.5 x 10^9/μl), when 300 nM forward and 300 nM reverse primer were used for 250 nM TaqMan probe with CV% range of 0.2 % and 3.4 % (Fig. 3). The observed variations in our study were similar to those (0.6 % - 3.4 %) found in the previous study (Wellinghausen et al., 2001). For quantitative determination of *E. coli* by real-time qPCR, four viabilities (90%, 60%, 30% and 10%) were investigated. The DNA detected with external standard curve was ranging from 3.1 x 10^9 to 9.1 x 10^9 DNA copies/μl . The detection and quantification were indicated to be linear over the range of DNA concentration examined in water sterilized and filtered by 0.22 μm filters. In addition, no false-positive signal was detected when primers and probe diluted in sterile water were amplified in the reaction plate.

五、討論

Optimal concentrations of AO

The optimal staining protocols for AO were determined for pure culture *E. coli*. In comparison, the optimal AO concentration of EFM was ten times of those of FCM. These differences might be related to better detection limit of fluorescence by FCM. In a previous review paper (Kepner et al., 1994), 100 μg/ml AO was widely used for short duration (< 5 min). In Fry's study (1990), a lower concentration of 5 μg/ml AO was used for samples from Chesapeake Bay. However, the concentration was too low to be detected. The similar results were also observed in our study. The cell concentrations with staining 5 μg/ml and 10 μg/ml AO have smaller quantity than those of 50 μg/ml and 100 μg/ml AO. Until now, there was no standard staining protocol of EFM for environmental medium samples, such as freshwater, saltwater, soil/sediment, and surface. The stain concentrations could vary by as much as 3 orders of magnitude even for the same sample type (Kepner et al., 1994).

EFM

Our results of precision were well agreed with those of *E. coli* (17 %) with ethidium bromide (Henningson et al., 1997), and of *Bacillus subtilus*, *E. coli*, and *Micrococcus leteus* (8 % to 35 %) with DTAF and CTC (Hernandez et al., 1999). Generally speaking, biases in EFM were related to uneven distribution of cells on the filter, improper cell concentration, dye concentration, and staining time (Kepner et al., 1994). In our current study, mixing the stained cells with 10 ml buffer thoroughly before filtration and counting more fields (15 fields) was performed to reduce uneven distribution. When cell concentrations were too high, errors from investigator fatigue would probably increase and quenching of fluorochromes following extended UV illumination would decrease the sensitivity of the method (Kirchman et al., 1982). Counting of stained microorganisms in EFM is one of the most commonly used methods to analyze environmental air samples because it is well-developed, inexpensive of instrument and easy to perform. However, the disadvantage of EFM is time and labor consuming. Furthermore, microscopic methods generally rely upon even distribution and an optimal concentration of cells on the filter, because only a small number of cells or particles are counted.

FCM

The yields of FCM are found to be all higher than those of EFM. Moreover, the amount of differences depended on dyes. Henningson and his coworkers indicated that coulter count (another kind of flow cytometry) gave 1.03 to 1.35-fold of the yield of EFM for *E. coli* stained *with* ethidium bromide (1997). Another investigation demonstrated that FCM overestimated ten times of EFM for using 5-(and 6-)-carboxyfluorescein diacetate, succinimidyl ester (FDA/SE) to stain groundwater microorganism (Fuller et al., 2000). These differences might be related to cell losses of extensive preparations for EFM, such as filtration of stained sample, filter mounting onto the glass slide, and cover-slip application. Regarding viability, measured-viability by FCM are found to be insignificantly different from those of EFM (p=0.052). In comparison of CFU%, the FCM-measured viability correlated well with CFU% (R=0.95), but the values were consistently higher than that of EFM. Furthermore, the determinations of viability obtained by FCM were significantly higher than the CFU% (p=0.005), but the slope was similar to 1.

Regarding the precision of FCM, the variation within samples was found to be significantly smaller (p=0.03) from FCM than those from EFM. It was also observed previously that the variation within samples from the coulter counter (another kind of flow cytometry) was significantly smaller (p=0.02) than that from EFM (Henningson et al., 1997). Therefore, the precision was better for FCM analysis. FCM was more rapid and gave a higher precision and a larger yield than EFM.

The large numbers of cells counted, up to 100,000 in less than a minute, increase the precision of the method. Acceptable statistical confidence can be achieved with only two analyses of a sample, provided that more than 1000 particles are counted (British Standards, 1983). Our analysis gave CV% of 0.6 % to 6.9 % when 3 analyses of 20,000 particles each were calculated per sample.

Comparison of total concentration and viability from EFM and FCM

Regarding total concentration measurement, our results indicated that EFM method has a higher degree of linearity and it results more closely resemble controlled-viability than those of the FCM method. Regarding correlation of total cell concentrations between EFM and FCM, the linear regression for AO-stained cells measured by EFM and FCM was Y=4.6X-5.25 (R=0.89). For PI-stained cells, the linear regression was Y=4.25X-1.07, with R=1.00. Therefore, it was demonstrated that cell concentrations measured by FCM were well correlated with those of EFM. The correlation between EFM and FCM of PI-stained cells was better than that of AO-stained ones. Regarding the measured-viability, correlation between FCM and EFM, the equation of fitting linear was Y=0.71X-2.6 (R=0.99). Further evaluation between microorganism viability and health effects/sampling efficiency/control efficiency is needed. In addition, EFM might underestimate cell concentrations due to sample handling loss, such as filtration of stained sample, filter mounting onto the glass slide, and cover-slip application (Henningson et al., 1997). Furthermore, with nature characteristics of rapid counting ability, low variation and good reproducibility, FCM permits a good alternative for microorganism analysis.

Real-time quantitative PCR (qPCR) with gene probe

Real-time qPCR is robust, rapid, automated, and quantitative, with high sample throughput potential, sample preparation, DNA isolation, and analysis up to 96 samples in 5 h. Because of proper primers and probes designation, high specificity by qPCR could be achieved. Avoidance of laborious post-PCR gel electrophoresis, and greatly reduced opportunity for contamination of reaction mixtures with target DNA further increased the suitability of this assay for microorganism analysis. Additionally, great sensitivity is also a noticeable advantage of real-time qPCR due to amplification process. In laboratory studies of cheese, *S. aureus* can be detected in pure cultures as low as 6 DNA copies/μl (Hein et al., 2001). Moreover, detection limit of *Legionella* spp. detected by LightCycler (another real-time qPCR) was observed to be 2 fg DNA/μl (Wellinghausen et al., 2001). In the field samples, low detection limit of 1 fg DNA/μl was also achieved in sediment samples (Stults et al., 2001). However, the interferences, such as other bacteria or other inhibitor existed in the air, need further investigation. In summary, the real-time quantitative PCR with gene probe provided an excellent choice to detect some important bacteria in the air.

Comparison of total cell concentrations from FCM and real-time qPCR

Until now, there was very few data available concerning the correlations between real-time qPCR and FCM/EFM. Regarding relationships among qPCR, EFM, and FCM (Fig. 4), it was demonstrated there were high correlations between DNA obtained by real-time qPCR and the total number concentration by AO from EFM and FCM (R=0.94 for FCM and R=0.82 for EFM). In comparison with FCM, real-time qPCR has high specificity and sensitivity. In addition, real-time qPCR provides a good way for safety-concerned microorganisms because of its simple and safe preparation (only DNA extraction). In summary, real-time qPCR should provide a good alternative for microorganism analysis.

六、結論

The intercomparisons of three nonculture methods (EFM, FCM, and qPCR) and culture method were evaluated. Our results

demonstrated that concentrations by FCM are higher than those of EFM. These differences might be related to cell losses through filtration of stained samples, filter mounting onto the glass slide, and cover-slip of EFM. In addition, strong associations between EFM and FCM were demonstrated for both concentrations and viabilities. Furthermore, it was also found there were strong relations between DNA obtained by real-time qPCR and the total number concentrations by AO from EFM and FCM. Regarding viability by staining dyes, the high correlation of FCM and EFM was demonstrated. Moreover, FCM measured viability using staining dyes was observed to be higher than culturability. In the future, microorganism viability, not just culturability, related to health effects should be assessed.

七、計畫成果自評

本計畫已在實驗室中建立螢光顯微鏡、流式細胞儀與 real-time qPCR 對生物氣膠之分析方法，並與傳統培養方法作比較，此成果可運用於環境中生物氣膠分析方法的選擇，並提供更完整的生物氣膠暴露評估結果。

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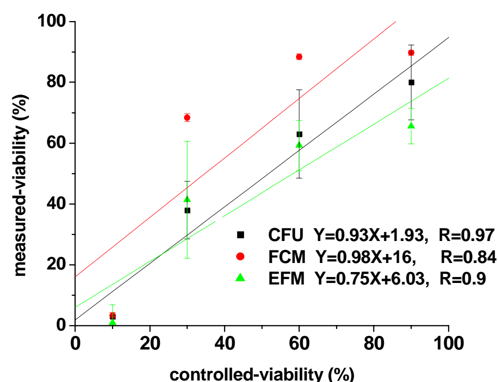

Fig. 1 Correlations between measured-viability and controlled-viability by epifluorescence microscopy (EFM), flow cytometric (FCM), and colony forming unit (CFU) methods.

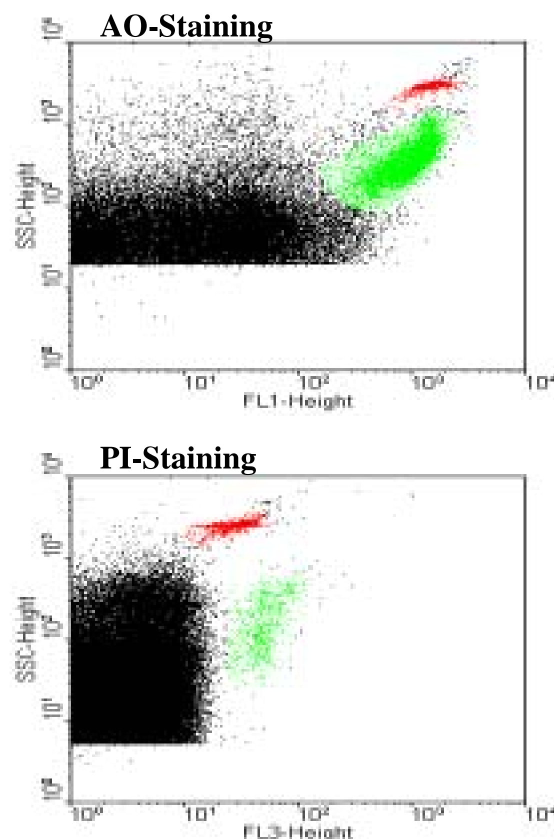

Fig. 2 Dot plots of *E. coli* cells (green region)

stained with acridine orange (AO) and propidium iodide (PI), fluorescent beads (red region), and background (black region).

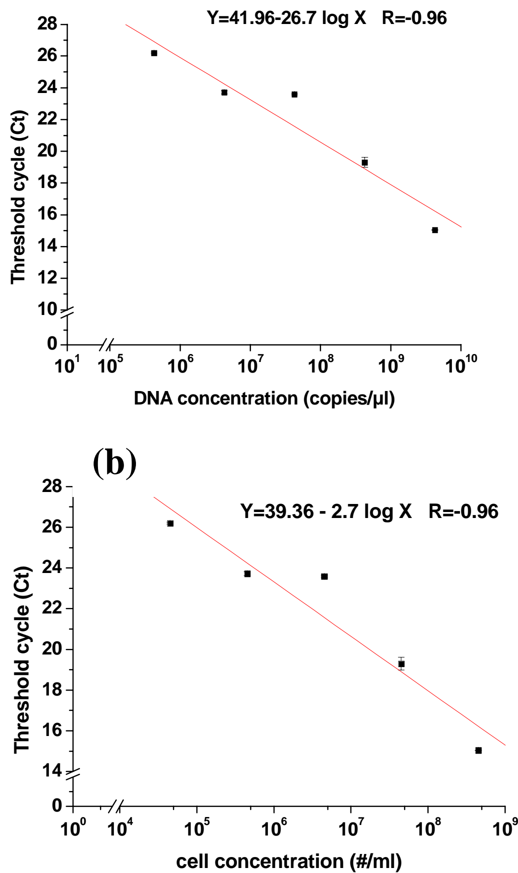

Fig. 3 (a). Calibration curve of DNA concentrations and threshold cycle (Ct) by real-time qPCR. (b). Calibration curve of cell concentrations and threshold cycle (Ct) by real-time qPCR.

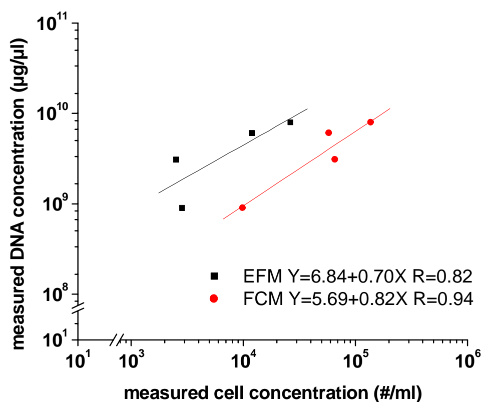

Fig. 4 Correlations of measured total cell concentrations with AO staining between EFM/FCM and DNA by real-time qPCR.

Table 1. Total and nonviable cell concentrations by epifluorescence microscopy (EFM) and flow cytometry (FCM) methods using acridine orange (AO) and propidium iodide (PI) staining. AO is used for total counting and PI is used for counting of nonviable cells.

Controlled viability (%)	Total conc. -AO stain (#/ml)			Nonviable conc. -PI stain (#/ml)			Measured Viability(%)		
	FCM	EFM	FCM /EFM[b]	FCM	EFM	FCM /EFM	FCM	EFM	FCM /EFM
90	$6.1 \times 10^7 \pm 3.7 \times 10^6$ [a]	$1.2 \times 10^7 \pm 8.1 \times 10^6$	4.69	$6.3 \times 10^6 \pm 4.3 \times 10^5$	$4.0 \times 10^6 \pm 4.3 \times 10^5$	1.58	89.7± 0.77	65.6± 5.82	1.37
60	$3.9 \times 10^7 \pm 2.0 \times 10^6$	$7.9 \times 10^6 \pm 1.4 \times 10^6$	4.94	$4.5 \times 10^6 \pm 1.4 \times 10^5$	$3.2 \times 10^6 \pm 4.1 \times 10^5$	1.41	88.4± 0.91	59.2± 8.2	1.49
30	$2.2 \times 10^7 \pm 9.0 \times 10^5$	$8.4 \times 10^6 \pm 2.6 \times 10^6$	2.62	$6.9 \times 10^6 \pm 1.4 \times 10^5$	$4.6 \times 10^6 \pm 2.6 \times 10^6$	1.5	68.4± 1.33	46.2± 19.2	1.48
10	$4.4 \times 10^7 \pm 2.0 \times 10^6$	$1.3 \times 10^7 \pm 1.9 \times 10^6$	3.38	$4.3 \times 10^7 \pm 1.5 \times 10^6$	$1.3 \times 10^7 \pm 2.0 \times 10^6$	3.3	3.4± 0.98	0.97± 0.76	3.51

a. mean ± standard deviation

b. FCM/EFM ratio