Fluorescent Viability Assay of *Mycobacterium avium* subsp. *paratuberculosis* by carboxyfluorescein diacetate succinimidyl ester and carboxyfluorescein diacetate

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KEY WORDS: CFDA, Mycobacterium, paratuberculosis, fluorescence, viability

ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (*Map*) is an important pathogen causing ruminant paratuberculosis. Paratuberculosis is characterized by chronic granulomatous enteritis, persistent diarrhea, progressive wasting, and finally death, and as resulted in significant economic losses to the dairy and cattle industries worldwide. Furthermore, *Map* is speculated to be the cause of human Crohn's disease. One of the difficulties of *Map* in diagnosis and research is its significant slow growth. Therefore, we investigated the application of a viability test of *Map* by Carboxyfluorescein diacetate succinimidyl (CFDA) and carboxyfluorescein diacetate succinimidyl ester (CFDA/SE) labeling as a quick method. The results revealed that CFDA is a useful reagent as a fluorescent probe to determine the quantitative viability of Map but unsatisfactory results obtained with CFDA/SE. Incubation of *Map* with CFDA at 100μM for 30min was practically the optimal condition for the viability test. We suggest that this method will be used widely as a simplified semi-quantitative viability assay in evaluation of effect of anti-bacterial substance, disinfectant or bactericidal activity of macrophages.

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (*Map*) is the etiological agent of

granulomatous enteritis in ruminants, known as paratuberculosis (Johne's disease).[1, 2] This infection is characterized by chronic granulomatous enteritis, persistent diarrhea, progressive wasting, and finally death.[3] Paratuberculosis has resulted in significant economic losses to the dairy and cattle industries.[2] Furthermore, the bacteria are speculated to be the cause of human Crohn's disease, an incurable chronic inflammatory bowel disease.[4, 5, 6, 7] Since Map can be shed into milk in infected cows, contamination of milk by Map is also an important problem in food hygiene. [8, 9]

Fluorescent staining with Auramine O[10] or acridine orange[11]has been used to detect mycobacteria in tissue. Fluorescent vital dyes, fatty acid ester fluorescein diacetate (FDA) and ethidium bromide (EB) (FDA/EB) and R123/EB were used to detect the viability of *Mycobacterium leprae* that is not culturable; [12, 13] however, fluorescent measuring of *Map* viability has not yet been reported. Before the discovery of mycobactin, *Map* was impossible to culture, like *M. leprae* ,[14] and field isolates take 3 to 4 months to see visible colonies on the Herrold's egg yolk medium (HEYM) containing 2 µg/mL mycobactin J at present.[15] A liquid culture system shortens the period, but it still takes 2 weeks in laboratory strain and more than 1 month in field isolates.[16] This super-slow growth is a unique character of *Map*, but it has obstructed the promotion of diagnosis and research. PCR is very convenient to detect the presence of *Map*, but the viability of the bacteria is not estimated.[17]

CFDA/SE (carboxyfluorescein diacetate succinimidyl ester) is an esterified fluorogenic substrate and has been used to label both primary cells and cell lines,[18] as well as to determine metabolically active bacteria.[19, 20, 21,22] Recently, Hoefel et al. (2003) reported a comparative study of carboxyfluorescein diacetate (CFDA) and CFDA/SE[23] Their study on *Aeromonas hydrophila, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus epidermidis* and bacteria from environmental waters showed that CFDA has an advantage over CFDA/SE in the evaluation of metabolic activity.[23] This work was undertaken to evaluate Map labelling with CFDA/SE and CFDA fluorescent stains and to develop an easy and effective semiquantitative method.

MATERIALS AND METHODS

Bacterial Strain and Culture

Mycobacterium avium subspecies *paratuberculosis* (ATCC 10698) was grown in Middlebrook 7H9 liquid medium (Difco Laboratories, MD, USA) enriched with BBL Middlebrook OADC (Becton Dickinson, ML, USA) and 2 mg/L of mycobactin J (Allied Laboratory, MO, USA) for two weeks. Bacilli were washed and re-suspended in phosphate-buffered saline (PBS) containing 0.01% Tween 80 to prevent bacterial clumping. The colony-forming units (CFU) were counted and expressed per ml following serial dilution and culture on Middlebrook 7H10 agar plates (Difco Laboratories, MD, and USA) containing mycobactin J for 1 month.

Fluorescent Reagent

CFDA and CFDA/SE stock solution s were prepared at 10mM in 100% methanol, and aliquots of stock solution were stored in single-usage tubes at –20°C.

Labelling conditions and assay.

To evaluate the condition of fluorescent staining, 8x108 live and heat-killed *Map* was prepared. Aliquots of live bacteria suspension were incubated at 72°C for 30 min for heat killing. Suspensions were incubated with 10, 50 or 100µM of CFDA or CFDA/SE in PBS pH6.8 for 10, 30 and 60min at 37°C, respectively. The bacterial suspension was diluted 2.5-fold with PBS containing 0.1% Tween80. Since Map showed strong nature of clamping, tween 80 was added to prevent it and acquire homogenous *Map* suspension. To evaluate the optimal pH of the reaction, pH6.0, 7.0, 7.6, and 8.0 of PBS were prepared to make staining solution of CFDA/SA and CFDA. A 100µl sample was dispensed into 3 wells of a 96-well plate (Falcon 3915, Beckton Dickinson, USA)

for fluorescent reading and intensity was measured by Cytofluor 2300 (Millipore®) with excitation at 485 nm and emission at 530 nm. Data are expressed as fluorescence intensity.

Colony-forming unit assay for viability of Map treated with CFDA and CFDA/SE

For CFU culture, 100µl of the remaining CFDA-treated samples were serially 10-fold diluted with phosphate-buffered saline (PBS) containing 0.1% Tween 80 to prevent bacterial clumping. Bacterial suspension treated with the same concentration of methanol without CFDA was cultured as the control. The colony-forming units (CFU) were counted and expressed as CFU/ml following serial dilution and culture on Middlebrook 7H10 agar plates enriched with BBL Middlebrook OADC and 2 mg/L mycobactin J at 37°C for 1 month.

Fluorescent microscopy

To evaluate the practical usefulness of fluorescence labelling as a tracer, we tested the distribution of labelled *Map* in milk after centrifugation. This was conducted to determine the optimal sampling of *Map* from milk samples. Fresh non-processed milk (kindly provided by the National Institute of Livestock and Grassland Science) was mixed with 8x108 live *Map*. 200µl of the spiked milk in 1.5 ml Eppendorf microtubes were heated in a digital heat block, THERMO BLOCK, (No.801, Nissin, Japan) at 65°C for 30 min and then chilled on ice. The milk was serially diluted 10 times and incubated with Middlebrook 7H10 agar with OECD compound and 2% mycobactin J at 37°C for 1 month. To confirm viability by the CFDA method quickly, the milk sample was transfer to a microtube, mixed with 100µM CFDA solution, and incubated for 37°C for 60 min. After incubation, a smear was made using Cytospin4 (Thermo Electron Corporation, USA) and observed under a fluorescent microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan). To test the application of CFDA as a fluorescent probe, 109 CFU Map was treated with 100µM CFDA for 30 min, and 1µl was mixed with 100µl of fresh milk. The 30 µl spiked milk was absorbed in hematocrit capillary tubes (VC-H075P, Thermo, Tokyo, Japan) and centrifuged at 12000rpm for 10 min by Kubota 3200 with an HT/15 rotor (Kubota, Tokyo, Japan) . The tubes were then observed under fluorescent microscope, Olympus BX51, at EX: 495, EM: 520.

Statistics analysis

All values are expressed as the mean ± standard error and statistical significance was analyzed using Student's t-test. Statistical significance was set at $P < 0.05$.

RESULTS

Fluorescence stainability of CFDA and CFDA/SE: The results of fluorescent intensity and CFDA are shown in Fig. 1. Fluorescent intensity was highest in 100µM CFDA with 60min incubation (Fig. 1A), and fell according to the concentration (Fig. 1A-C). In heat-killed *Map*, fluorescence with 100µM CFDA was faint (Fig. 1D). The results of CFDA/SE showed fluorescence intensity, according to the concentration of CFDA and the incubation period in live and heat-killed Map (Figs. 2 and 3).

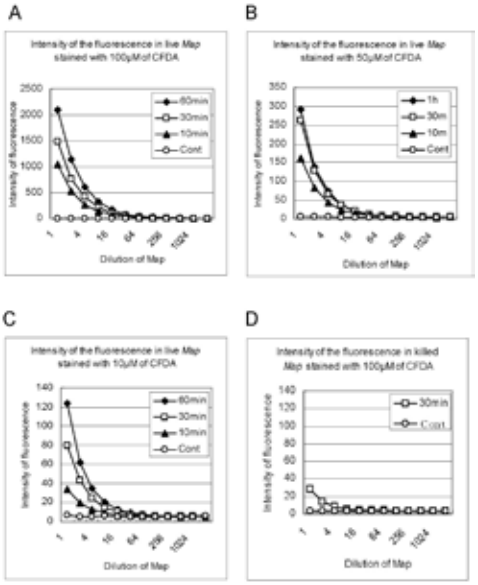
No effect on viability of Map by CFDA and CFDA/SE: Cultural examination revealed that 100, 50 and 10µM of CFDA and CFDA/SE did not influence the viability or growth rate of *Map* with treatment for 1, 3 and 10 hours, respectively. About 2-7x107CFU *Map* was isolated in all samples. There was no statistical difference in the data of CFU among the different concentrations and different times of staining with CFDA (Table 1) or CFDA/SE (Table 2).

Evaluation of the fate of Map in milk by heat-sterilization: CFDA labelling of spiked *Map* in fresh milk un-heated *Map* in the milk revealed clear fluorescence (Fig. 4, A, but in heated milk at 65°C for 30 min showed no fluorescent bacteria (Fig. 4, B).

Distribution of fluorescent-labelled Map: By fluorescent microscopic observation (at EX:495, EM: 520), CFDA-labelled Map was observed in the superficial layer of accumulated milk protein at the bottom of the

Figure 1 - Fluorescent intensity in Live and heat killed Map stained with CFDA

Live Map (108CFU) was stained with different concentrations of CFDA, 100μM, (1-A); 50μlM (1-B) and 10μM (1-C) for 10, 30 and 60min, respectively. The same CFU of killed Map were stained with 100μM CFDA for 30 min. Non-treated control sample was diluted in the same manner. Live Map stained with CFDA showed fluorescence intensity according to the bacterial number. Heat-inactivated Map showed faint fluorescence of significantly weaker than those in live Map (1-D). Fluorescent intensities shown in Fig. 1 were measured in the same setting of the intensity.



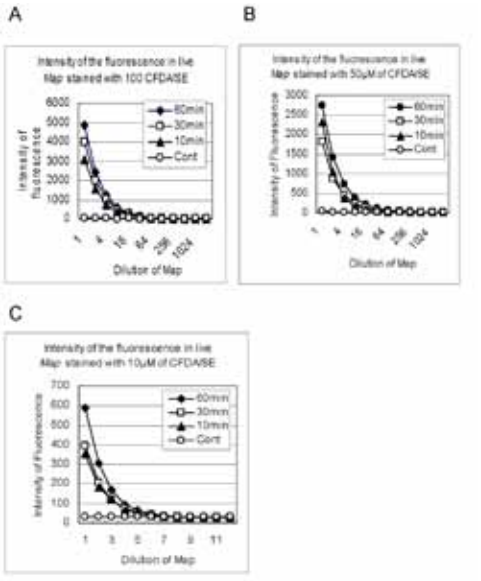
capillary tube after centrifugation (Fig. 5 A, B).

DISCUSSION

The present study demonstrated that CFDA stain live *Map* well and hard to stain heat killed *Map*, however CFDA staining showed very faint fluorescent in heat killed one. This might be caused by few survived live *Map* or other reason. These results strongly suggest that CFDA is a useful fluorescent reagent to measure the viability of *Map*. The method will required a much shorter time to measure viability in evaluation of the effect of antibiotics, disinfectants, however further

Figure 2 - Fluorescence intensity in Live Map stained with CFDA/SE

Live Map (108CFU) were stained with different concentrations of CFDA/SE, 100μM, (2-A); 50μlM (1-B) and 10μM (2-C) for 10, 30 and 60min. Non-treated control samples with CFDA/SE was diluted in the same manner. Live Map stained with CFDA showed fluorescence intensity according to the bacterial number. Fluorescent intensities shown in Fig.2-B and C were measured at more sensitive setting than Fig2-A.

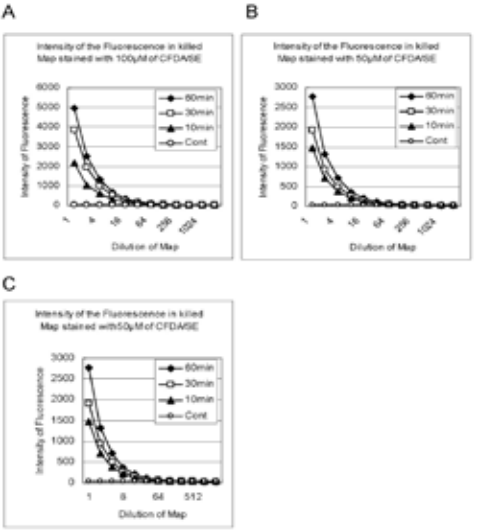


analyses in case to case evaluation. Hoefel et al (2003) reported similar results in other bacteria than *Mycobacterium* and speculated that the specific mode of action of the succinimidyl ester (SE) group in combination with the nonenzymatic aqueous hydrolysis of the CFDA moiety results in the nonspecific labelling of all cells, irrespective of their metabolic state.[23] The efficacy of heat-sterilization of milk is an important issue in relation to *Map* contamination in daily products [8, 24] and human Crohn disease. [2, 4, 6]

An advantage of this method is the measurement of viability using a reagent, microplate and fluorescent plate reader. By comparing with untreated control *Map* suspension and a serially diluted control of known concentration, we can determine the

Figure 3 - Fluorescence intensity in heat-inactivated Map stained with CFDA/SE

Heat-inactivated Map (108CFU) was stained with different concentrations of CFDA/SE, 100μM, (3-A); 50μlM (3-B) and 10μM 3-C) for 10, 30 and 60min. Non-treated control sample was diluted in the same manner. Heat-inactivated Map stained with CFDA/SE showed fluorescence intensity according to the bacterial number. Fluorescent intensities shown in Fig.3 were measured at more sensitive setting than those in Fig 1 and Fig 2-A.



ratio of killed/live *Map* and the semi-quantitative result of live bacilli in a few hours. This is a very convenient semi-quantitative method to quantify the approximate number of *Map* before experimental infection or after examination of the bactericidal effect of various drugs, reagents or heat-inactivation of *Map*. Since we usually need to culture laboratory strain of *Map* for 2 weeks and wild strains for 3 to 4 months or more onspecial medium to obtain the CFU/ml, 25 this shorter method will accelerate various research studies on *Map*. Quantitative-PCR (Q-PCR) is a quick, specific and reliable method to determine the approximate number of *Map* by converting total DNA concentration, [26] but the data does not indicate the number of live bacteria.

The present study revealed the possibility of using the CFDA method as a tracer to detect distribution in milk and culture macrophages as well as other bacteria in experiments. Frozen stock of *Map* suspension with CFU data at -80°C will be used as a CFU standard to calibrate fluorescence intensity. Flow cytometrical analysis was applied to measure lactic acid bacteria.[27] Likewise, determining the number of *Map* by CFDA staining using flow cytometry seems attractive, but *Map* aggregate well when suspended in milk or CFDA solution. Further studies to effectively prevent aggregation are therefore necessary.

The present study indicated that CFDA specifically stains live *Map* and can be used in the viability measurement of *Map*, a

Figure 4 - Fluorescence microscopic findings of Map in milk

Map spiked unheated milk sample showed fluorescent Map by CFDA staining (Fig. 4A). Arrows suggest fluorescent Map those show clamping nature. But heated milk did not show fluorescent Map (Fig. 4B) suggesting heat killing was achieved.

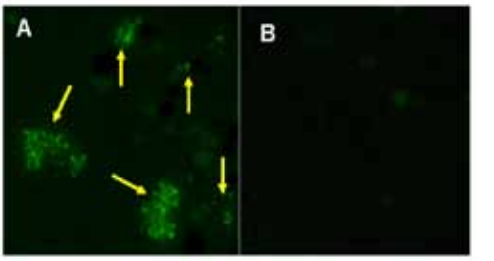
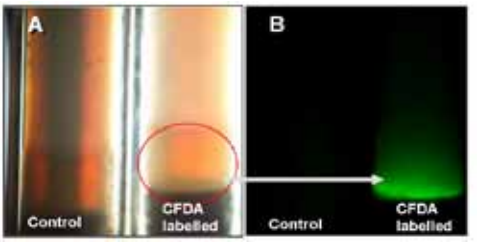


Figure 5 - Distribution of fluorescence positive Map in milk in capillary

To observe the distribution of Map after centrifugation of milk, CFDA-labelled Map was observed in the superficial layer of accumulated milk protein at the bottom of the capillary tube after centrifugation (Fig.5 A, B)).



very slow-growing Mycobacteria, however, CFDA/SE is not suitable for that. We further demonstrated the application of the rapid method to determine heat killing of *Map* in milk. This method can accelerate various research studies on *Map* to measure their viability and clarify the practical issues associated with the very slow growth of *Map*. Therefore, we suggest that this method will be used widely as a simplified semi-quantitative viability assay.

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