Rapid On-chip flow Cytometric Detection of *Listeria monocytogenes* in Milk

Masafumi Ikeda, Nobuyasu Yamaguchi, and Masao Nasu*

Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan

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***Listeria monocytogenes* (*L. monocytogenes*) is a Gram-positive, intracellular bacterium that can cause severe infections in humans. Contamination by *L. monocytogenes* in foods represents a potential public health problem. Here we describe a rapid method for the detection of *L. monocytogenes* in milk by flow cytometry based on microfluidics (on-chip flow cytometry). This rapid identification of *L. monocytogenes* is based on fluorescence *in situ* hybridization (FISH) with a Cy5-labeled rRNA-targeting oligonucleotide probe. FISH experiments were successfully analyzed using a commercially available on-chip flow cytometry. FISH results with bacterial cultures indicated that the *L. monocytogenes* probe RL-2 was hybridized with 4 major strains of *L. monocytogenes* (serotype 1/2a, 1/2b, 1/2c and 4b) but not with other *Listeria* strains or milk spoilage bacteria. Specific FISH detection of *L. monocytogenes* was accomplished using the probe RL-2 when the milk contained a mixture of other bacterial species. The positive identification of *L. monocytogenes* in milk was completed within 5 hr (milk clearing: 40 min, hybridization: 3.5 hr, on-chip analysis: 30 min). The method presented in this study allows the specific and rapid identification of *L. monocytogenes* in milk.**

Key words——food contamination, *Listeria monocytogenes*, on-chip flow cytometry, microbial monitoring, milk, fluorescence *in situ* hybridization

INTRODUCTION

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive intracellular organism causing severe infections that primarily affect pregnant women, newborns, and immunocompromised individuals.[1] This pathogenic bacterium has been incriminated in food-borne diseases disseminated by a wide variety of food products in many countries since the 1980s.[2]

The development of rapid detection methods for *L. monocytogenes* in food is therefore critical to ensure food safety. Traditional plating methods are well established, but complex and time consuming. For *L. monocytogenes*, positive detection can take as long as 5 to 7 days using the culture method.[3]

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes is a powerful tool to detect targeted microbial cells based on their rRNA sequences[4] and to analyze in a cultivation-independent way the structure and dynamics of complex microbial communities.[5] FISH with rRNA-targeted oligonucleotide probes is widely used to identify various bacteria. A FISH protocol for the specific detection of *L. monocytogenes* in milk has been developed by Oliveira *et al.*, but in that method the enumeration of *L. monocytogenes* was performed with an epifluorescence microscope.[6] The application of this methodology to quantify pathogenic bacteria needs to be optimized. Moreover, the enumeration of bacteria using an epifluorescence microscope is laborious and time consuming.[7] To solve this problem, flow cytometry has been applied to detect bacterial cells in milk;[8,9] however, flow cytometers are relatively expensive, and maintenance is rather complicated for an unskilled operator. Microfluidic chips are an effective alternative to this problem.

Microfluidic chips have many advantages: microfluidic chip-based analysis is small scale and can be completed more quickly than analysis using conventional devices, consumption of sample and reagent is low, analysis is performed automatically with high reproducibility and risk of biohazard

*To whom correspondence should be addressed: Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan. Tel.: +81-6879-8170; Fax: +81-6879-8174; E-mail: nasu@phs.osaka-u.ac.jp

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can be reduced because of the closed system. Microfluidic chip-based devices have been employed for many analyses, such as capillary electrophoresis,[10] PCR,[11] and flow cytometry (on-chip flow cytometry).[12] We have applied commercially available on-chip flow cytometry to analyze hybridization results by beads assay for the detection of food-poisoning bacteria.[13] On-chip flow cytometry has also been applied for the enumeration of freshwater bacteria.[14]

Here we report the application of commercially available on-chip flow cytometry for FISH analysis to identify *L. monocytogenes* in milk.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions —— The following reference strains were used: *L. monocytogenes* 1/2a RIMD 1205022, *L. monocytogenes* 1/2c RIMD 1205001, *L. monocytogenes* 1/2b ATCC 51780, *L. monocytogenes* 4b RIMD 1205021, *L. ivanovii* JCM 7681, *Enterococcus hirae* (*Ent. hirae*) GTC 0459 and *Escherichia coli* (*E. coli*) W3110. All *Listeria* spp. and *Ent. hirae* cultures were grown aerobically at 37°C in brain-heart infusion broth (Becton, Dickinson and Company, NJ, U.S.A.). *E. coli* culture was grown aerobically in Luria-Bertani broth. Incubation was performed at 37°C for 17 hr.

Fixation of Bacteria —— Aliquots (100 µl) of bacterial cultures (approximately 10^8 cells) were centrifuged at 10000 *g* for 5 min. Bacterial cell pellets were suspended with 20 µl of 50% solution of absolute ethanol in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4 [pH 7.2]). Ethanol-fixed cells were stored at −20°C until use.

Oligonucleotide Probes —— Four oligonucleotide probes were used: the universal bacterial probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′);[15] *L. monocytogenes* probe RL-2 (5′-ATAGTTTTATGGGATTAGC-3′);[16] *E. coli* probe ES445 (5′-CTTTACTCCCTTCCTCCCC-3′);[17] and the nonsense probe complementary to EUB338 ("non-EUB338"; 5′-ACTCCTACGGGAGGCAGC-3′).[15] Probes EUB338 and non-EUB338 were used as positive and negative controls of hybridization, respectively. All oligonucleotide probes were synthesized and labeled with Cy5 (excitation, 633 nm; emission, 670 nm) at the 5′end (Tsukuba Oligo Service, Ibaraki, Japan). Probes were reconstituted with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Prepared probes were stored at −20°C until use.

Bacteria Inoculation and Milk Clearing —— The specificity of the FISH probe was tested after inoculating the bacteria into milk (heat treated at 140°C for 2 s; fat > 3.5%, protein > 3.0%; pH 6.8) purchased from a retail store and used immediately. Milk protein and fat were removed using the clearing method as described in Gunasekera *et al.*[8] Ten microliters of savinase (EC 3.4.21.52; Sigma-Aldrich, St. Louis, MO, U.S.A.) were added to 100 µl of artificially contaminated milk, incubated at 30°C for 30 min, 900 µl of 150 mM NaCl was added, and samples centrifuged at 10000 *g* for 5 min at 22°C. Digested proteins and the top layer containing lipids were drawn off with a micropipette and the bacterial pellet was suspended with 20 µl of 50% solution of absolute ethanol in PBS (pH 7.2).

FISH —— Ethanol-fixed bacterial cells (10^6–10^7) were added to 10 µl of pre-warmed hybridization solution containing 900 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.01% sodium dodecyl sulfate (SDS), and 1 ng/µl Cy5-labeled oligonucleotide probe in a small tube. The tube was incubated at 46°C. After 3 hr, the hybridization reaction was stopped by adding 40 µl of cold PBS (pH 8.4). Bacterial cells were counterstained with SYBR Green I (excitation, 494 nm; emission, 521 nm; Invitrogen, Carlsbad, CA, U.S.A.). Bacterial cells were stained for 5 min at room temperature (approximately 25°C) under dark conditions, precipitated by centrifugation (10000 *g*, 5 min), and suspended in 50 µl of PBS (pH 8.4) for on-chip flow cytometric analysis.

On-chip Flow Cytometry and Data Analysis —— An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.) was used for on-chip flow cytometry. This system is a commercially available microchip-based analysis system which has been used primarily for the analysis of eukaryotic cells. The apparatus is relatively inexpensive and does not require substantial user training or experience. This instrument is capable of two-color fluorescence detection. The blue Light Emitting Dicde (LED) has a maximum emission at 470 nm. The maximum emission of the red laser diode is 630 nm. The detection windows are 525 nm for the green and 680 nm for the far-red channel. The measured fluorescence values and event numbers are displayed as histograms or dot plots on a personal computer. Hybridized bacterial cells were analyzed with a Cell Fluorescence LabChip kit (Ag-

ilent Technologies). This kit supplies microchips, priming solution, focusing dye and cell buffer. This microchip design has no cross-point between samples; therefore, it could analyze 6 samples in one chip. Cell numbers could be counted up to approximately 2500 cells per sample in 4 min, and the total time for analysis was within 30 min for 6 samples (*i.e.*, one full microchip). The instrumentation for this chip-based system and the design of the microfluidic chip have been described in detail by Preckel *et al.*[18)]

Flow cytometric data were analyzed using 2100 Bioanalyzer expert software (Agilent Technologies). Gates for the enumeration of bacteria were set in dot plots of events detected in the blue and red channels of the Bioanalyzer. For each individual set of hybridizations of one sample, the gate "all bacteria" was defined on the basis of the SYBR Green I-stained sample, whereas the gate "hybridized cells" was defined on the basis of the SYBR Green I-stained sample hybridized with the each probe (Fig. 1; SYBR Green I-fluorescence, 1–50; Cy5-fluorescence, 1–100).

RESULTS

In situ Detection of *L. monocytogenes* with 16S rRNA-targeted Oligonucleotide Probe

The specificity of the FISH probes (non-EUB338, EUB338, RL-2 and ES445) in on-chip flow cytometry was tested against 7 reference strains of different bacteria species including 4 serotypes of *L. monocytogenes* (1/2a, 1/2c, 1/2b, and 4b) and other milk spoilage bacteria. The results are summarized in Table 1. The RL-2 probe was revealed to be specific for *L. monocytogenes*, since all 4 serotypes of *L. monocytogenes* strains showed positive hybridization, and no cross hybridization was observed to any of the other non-*L. monocytogenes* strains tested. All 7 reference strains could be hybridized with the EUB388 probe (positive control), and none was hybridized with the non-EUB338 probe (negative control). Only the *E. coli* W3110 strain could be hybridized with the ES445 probe.

On-chip Flow Cytometric Detection of *L. monocytogenes* in Milk

To identify *L. monocytogenes* 4b in milk using

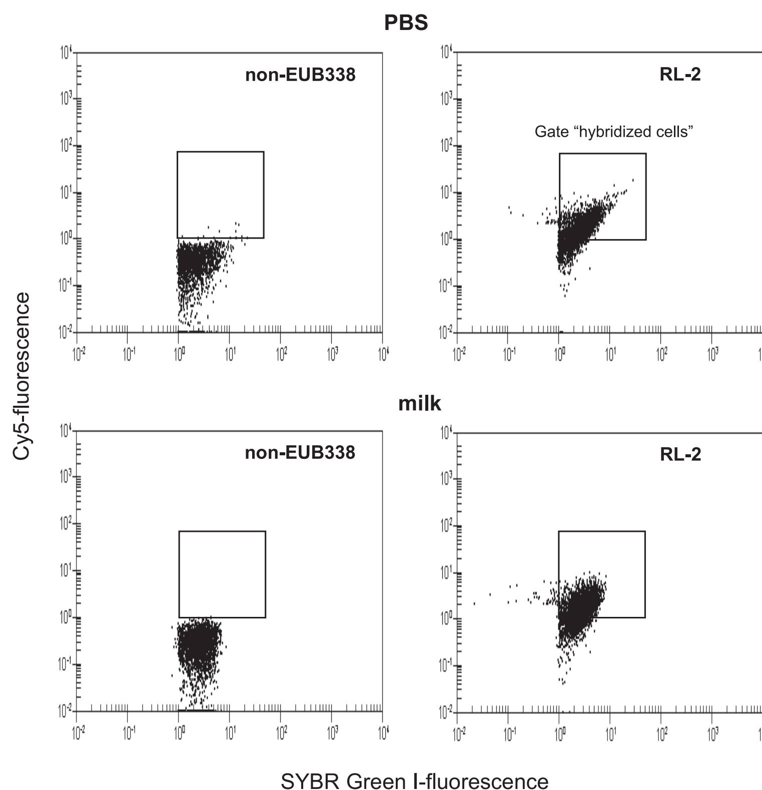

Fig. 1. On-chip Flow Cytometric Detection of *L. monocytogenes* 4b in PBS (control) or Artificially Contaminated Milk

The milk sample was treated with savinase to remove milk protein and fat prior to hybridization as described in Materials and Methods.

Table 1. Specificity of Oligonucleotide Probes Tested with 7 Reference Bacterial Strains Determined by On-chip Flow Cytometry

Species	Serotype	Strain	Probe			
			non-EUB338	EUB338	RL-2	ES445
L. monocytogenes	1/2a	RIMD 1205022	−	+	+	−
	1/2c	RIMD 1205001	−	+	+	−
	1/2b	ATCC 51780	−	+	+	−
	4b	RIMD 1205021	−	+	+	−
L. ivanovii		JCM 7681	−	+	−	−
Ent. hirae		GTC 0459	−	+	−	−
E. coli		W3110	−	+	−	+

+: FISH positive, −: FISH negative.

on-chip flow cytometry, milk was artificially contaminated with suspensions of *L. monocytogenes* 4b (10^6 cells) and hybridized with the RL-2 probe as described in Materials and Methods. *L. monocytogenes* 4b inoculated in PBS was also tested as control. Figure 1 shows dot plots of *L. monocytogenes* inoculated into PBS (pH 7.2) and milk hybridized with the probes non-EUB338 and RL-2. The differences of the intensity of Cy5 fluorescence of the cells hybridized with the non-EUB338 probe and the RL-2 probe were measured. *L. monocytogenes* 4b in milk appeared in exactly the same positions on dot plots as *L. monocytogenes* 4b in PBS.

To test the specific FISH detection of *L. monocytogenes* in milk, mixed bacterial cells of *L. monocytogenes* 4b and *L. ivanovii* (*L. monocytogenes* 4b: *L. ivanovii* ratios of 10 : 0, 8 : 2, 5 : 5, 2 : 8, and 0 : 10) were suspended in milk. Event numbers in the gate were automatically counted and recorded; those derived from cells hybridized with the RL-2 probe increased with the presence of *L. monocytogenes* 4b, and decreased in accordance with the diminution of *L. monocytogenes* 4b cells in the mixed cultures (Fig. 2). This suggests that on-chip flow cytometry can be used to specifically detect *L. monocytogenes* in milk.

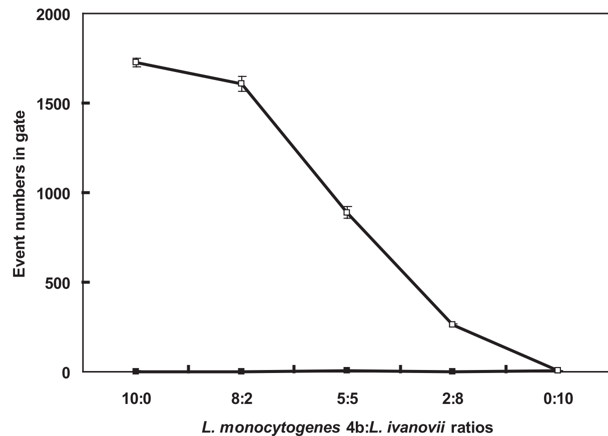

Fig. 2. On-chip Flow Cytometric Detection of *L. monocytogenes* 4b in Milk Artificially Contaminated with Mixed Suspensions of *L. monocytogenes* 4b and *L. ivanovii*

■: non-EUB338 probe, □: RL-2 probe. The plots and error bars show the mean values and the standard deviations ($n = 3$).

DISCUSSION

The traditional culture method for the detection of *L. monocytogenes* is time and labor intensive; positive detection of *L. monocytogenes* in foods can take as long as 5 to 7 days using culture methods. New tools for the rapid and direct detection of *L. monocytogenes* are needed to help ensure food safety. This study focused on a specific and rapid technique for the identification of *L. monocytogenes* using the FISH technique in combination with on-chip flow cytometric analysis. Evaluation of *L. monocytogenes* contamination in milk was completed within a single working day (milk clearing: 40 min, hybridization: 3.5 hr, on-chip analysis: 30 min).

The RL-2 probe (19-mer) is a specific probe for *L. monocytogenes*, and the target is 16S rRNA.[16] The RL-2 probe had two bases for which the corresponding sequences differ in *L. monocytogenes vs. L. innocua* and *L. ivanovii*. Although this probe was originally used in a dot-blot hybridization technique, its characteristics were ideal for application in the specific FISH detection of *L. monocytogenes*. Oliveira *et al.* evaluated the specificity of this probe with 68 reference strains including 24 *Listeria* strains, 17 of which corresponded to different serotypes of *L. monocytogenes* by fluorescent microscopy.[6] Only *L. monocytogenes* strains could be hybridized with the RL-2 probe, and no cross hybridization was observed with any of the other

strains tested. In this study, we were also able to check the specificity of the RL-2 probe in on-chip flow cytometry using pure cultures in PBS and those in milk. The results demonstrated that the RL-2 probe was useful for specific FISH detection of *L. monocytogenes* in milk by on-chip flow cytometry.

In this study, satisfactory FISH results were obtained from 50% ethanol-fixed *L. monocytogenes* cells. For fixation of Gram-positive bacteria, 50% ethanol, ethanol/formalin (9 : 1 v/v) or heat treatment is recommended.[19] The same FISH results were obtained from *L. monocytogenes* cells fixed with 8% (w/v) paraformaldehyde-PBS (pH 7.2) (data not shown). Fixation in 50% ethanol is simpler and there are fewer concerns regarding fixative toxicity.

We have already investigated quantification of bacteria using this on-chip flow cytometer.[14] The bacterial numbers of *E. coli* O157:H7 determined by this technique were similar to those obtained by direct microscopic count (within 4×10^5 to 5×10^6 cells/ml, $r^2 = 0.95$). In this study, we also tested the measurement range for the analysis of bacterial cells using this on-chip flow cytometry, and found a similar measurement range (lower detection limit: 10^2 CFU/ml).

In conclusion, a specific and rapid assay for the detection of *L. monocytogenes* in milk was developed. The assay technique can be easily adapted for the detection of other important pathogenic bacteria. Our findings suggest that the FISH technique in combination with on-chip flow cytometric analysis can play an important role in the biological safety of milk.

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REFERENCES

1) Seeliger, H. P. R. (1961) *Listeriosis*, Hafner Press, New York.
2) Ryser, E. T. (1999) Foodborne listeriosis. In *Listeria, Listeriosis and Food Safety* (Ryser, E. T. and Marth, E. H. Eds.), Marcel Dekker Inc., New York, pp. 299–358.
3) Norton, D. M. (2002) Polymerase chain reaction-based methods for detection of *Listeria monocytogenes*: toward real-time screening for food and environmental samples. *Journal of AOAC International*, **85**, 505–515.
4) Milko, J., Yamaguchi, N., Tani, K. and Nasu, M. (2007) Stimulatory effect of glutamine and pyruvate on plasmid transfer between *Pseudomonas* strains. *Microbes and Environments*, **22**, 320–326.
5) Amann, R., Fuchs, B. M. and Behrens, S. (2001) The identification of microorganisms by fluorescence in situ hybridization. *Curr. Opin. Biotechnol.*, **12**, 231–236.
6) Oliveira, M., Andrade, G. and Bernardo, F. (2003). Development of a fluorescent in situ hybridization protocol for the rapid detection and enumeration of *Listeria monocytogenes* in milk. *Revista Portuguesa de Ciencias Veterinarias*, **98**, 119–124.
7) Yamaguchi, N. and Nasu, M. (1997) Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *J. Appl. Microbiol.*, **83**, 43–52.
8) Gunasekera, T. S., Veal, D. A. and Attfield, P. V. (2003) Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk. *Int. J. Food Microbiol.*, **85**, 269–279.
9) Yamaguchi, N., Sasada, M., Yamanaka, M. and Nasu, M. (2003) Rapid detection of respiring *Escherichia coli* O157:H7 in apple juice, milk, and ground beef by flow cytometry. *Cytometry*, **54A**, 27–35.
10) Harrison, D. J., Fluri, K., Seiler, K., Fan, Z., Effenhauser, C. S. and Manz, A. (1993) Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip. *Science*, **261**, 895–897.
11) Kopp, M. U., DeMello, A. J. and Manz, A. (1998) Chemical amplification: continuous-flow PCR on a chip. *Science*, **280**, 1046–1048.
12) Sakamoto, C., Yamaguchi, N., Yamada, M., Nagase, H., Seki, M. and Nasu, M. (2007) Rapid quantification of bacterial cells in potable water using a simplified microfluidic device. *J. Microbiol. Methods*, **68**, 643–647.
13) Ikeda, M., Yamaguchi, N., Tani, K. and Nasu, M. (2006) Rapid and simple detection of food poisoning bacteria by bead assay with a microfluidic chip-based system. *J. Microbiol. Methods*, **67**, 241–247.
14) Sakamoto, C., Yamaguchi, N. and Nasu, M. (2005) Rapid and simple quantification of bacterial cells by using a microfluidic device. *Appl. Environ. Microbiol.*, **71**, 1117–1121.
15) Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes

with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.*, **56**, 1919–1925.

16) Wang, R.-F., Cao, W.-W. and Johnson, M. G. (1991) Development of a 16S rRNA-based oligomer probe specific for *Listeria monocytogenes*. *Appl. Environ. Microbiol.*, **57**, 3666–3670.

17) Kenzaka, T., Yamaguchi, N., Prapagdee, B., Mikami, E. and Nasu, M. (2001) Bacterial community composition and activity in urban rivers in Thailand and Malaysia. *J. Health Sci.*, **47**, 353–361.

18) Preckel, T., Luedke, G., Chan, S. D. H., Wang, B. N., Dubrow, R. and Buhlmann, C. (2002) Detection of cellular parameters using a microfluidic chip based system. *Journal of the Association for Laboratory Automation*, **7**, 85–89.

19) Moter, A. and Gobel, U. B. (2000) Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Methods.*, **41**, 85–112.