A Method to Detect only Live Bacteria during PCR Amplification

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Ethidium monoazide (EMA) is a DNA cross-linking agent and eukaorytic topoisomerase II poison. We previously reported that treatment of EMA with visible light irradiation (EMA+Light) directly cleaved chromosomal DNA of *E. coli* (Microbiol. Immunol. 51:763-775, 2007). Herein, we report that EMA+Light randomly cleaved chromosomal DNA of heat-treated, but not live, *Listeria monocytogenes* within 10 min of treatment. When polymerase chain reaction (PCR) amplified DNA of 894 bp, PCR final products from $10^8$ heat-treated *L. monocytogenes* were completely suppressed by EMA+Light. When target DNA was short (113 bp) like the *hly* gene of *L. monocytogenes*, DNA amplification was not completely suppressed by EMA+Light only. Thus, we used DNA gyrase/topoisomerase IV and mammalian topoisomerase poisons (here abbreviated as T-poisons) together with EMA+Light. T-poisons could penetrate heat-treated, but not live *L. monocytogenes* within 30 min, to cleave chromosomal DNA by poisoning activity. PCR product of the *hly* gene from $10^8$ heat-treated *L. monocytogenes* was inhibited by a combination of EMA+Light+T-poisons, but those from live bacteria were not suppressed. As a model for clinical application to bacteremia, we tried to discriminate live and antibiotic-treated *L. monocytogenes* present in human blood. EMA+Light+T-poisons completely suppressed PCR product from $10^3$ - $10^7$ antibiotic-treated *L. monocytogenes*, but could detect $10^2$ live bacteria. Considering the prevention and control of food poisoning, this method was applied to discriminate live and heat-treated *L. monocytogenes* spiked into pasteurized milk. EMA+Light+T-poisons inhibited PCR product from $10^3$ – $10^7$ heat-treated cells, but could detect $10^1$ live *L. monocytogenes*. Our method is useful in clinical as well as food-hygiene tests.
INTRODUCTION

Polymerase chain reaction (PCR) is widely used as an effective tool to detect bacteria in foods and clinical samples. The disadvantage of PCR is that it cannot discriminate dead from live bacteria. To overcome this disadvantage, reverse transcriptase (RT)-PCR targeting mRNA has been used. The mRNA derived from high levels of dead bacteria ($10^4$ - $10^7$/ml), however, cannot be removed from samples and subsequently the RT-PCR reaction becomes positive (31, 35). Measuring the RNA/DNA molar ratio is not sensitive enough to detect low levels of live bacteria in samples containing high levels of dead bacteria.

To discriminate live and dead bacteria by PCR, cross-linking agents such as psoralen, methylisopsoralen derivative (4'-AMDMIP), and ethidium monoazide (EMA) have been used (4, 5, 20, 23, 25, 27-29). They selectively permeate the cell walls of dead bacteria and irreversibly bind to chromosomal DNA by covalent attachment (20, 23, 27-29). It has been reported that EMA could cross-link to DNA at the rate of 1 agent per 10-80 base-pairs (bp) in vitro (17). PCR amplification of DNA from dead bacteria was inhibited by cross-linking action (23, 27-29), and the PCR signal from dead bacteria was reduced to 1/300 - 1/1000 (23, 27, 29). It has been reported that pasteurized milk contains $10^5$ - $10^7$ cells/ml of injured/dead bacteria (1, 30). When these methods are applied to the pasteurized samples, the PCR products from injured/dead bacteria are amplified. It is very difficult to judge whether the PCR product is derived only from live bacteria in test samples.

DNase was added to cleave the chromosomal DNA of dead bacteria (21), and PCR signal intensity from dead bacteria decreased to 1/10. External DNase, however, could not completely suppress PCR products from dead bacteria because DNase could not penetrate the cell membranes of dead bacteria due to its high molecular weight.

Developing rapid PCR methods to substitute the culture method is a pressing matter in clinical
and food hygiene tests. Most clinical samples are derived from patients administered with antibiotics. Various foods have been pasteurized to kill bacteria while minimizing the denaturation of food components such as proteins. Therefore, the bacteria present in clinical samples and foods may be injured. Hyperthermophilic enzymes are reported to be maintained in the bacteria of pasteurized milk (9, 11, 36). Activities of bacterial DNA gyrase and topoisomerase IV (16) are likely to be maintained. Here, we focused on active bacterial DNA gyrase and topoisomerase IV retained in heat-treated bacteria. Utilizing the enzyme activity, we could completely suppress the PCR end products of heat-injured bacteria. There have been no reports of inhibition of PCR products from heat-treated bacteria using DNA gyrase/topoisomerase IV poison (e.g. fluoroquinolones) and/or mammalian topoisomerase poisons (T-poisons). We employed EMA which cleaves DNA after photoactivation (32), DNA gyrase/topoisomerase IV poison (8, 15, 24, 37), and mammalian topoisomerase (I and II) poisons (3, 7, 14, 18, 19, 34). We examined whether the DNA of injured/dead bacteria are cleaved by EMA with the aid of ciprofloxacin or T-poisons, and whether PCR final products from the bacteria could be completely suppressed. We used the *Listeria monocytogenes* strain because this bacterium is important in both clinical and food hygiene application.
MATERIALS AND METHODS

Reagents. Ethidium monoazide (EMA, Sigma, St. Louis, MO, USA) was used for DNA cross-linking and DNA cleavage of bacteria. Ciprofloxacin (CIN) purchased from Fluka Chemie GmbH (Buchs, Germany) was dissolved in physiological saline. Ampicillin (AMP) and gentamicin (GEN) were from Sigma. Camptothecin (CAM), etoposide (ETP), ellipticine (ELP), mitoxantrone (MIT), and amsacrine (m-AMSA) were purchased from Sigma, and were dissolved in dimethylsulfoxide (DMSO).

Bacteria and culture. *Listeria monocytogenes* JCM 2873 was cultured at 30°C in Brain Heart Infusion (BHI) broth (Eiken KAGAKU, Tokyo, Japan). To prepare live bacterial suspension, bacteria in the logarithmic growth phase were suspended in physiological saline. The number of live bacteria was counted by plating the bacterial suspension on L-agar after appropriate dilution.

Preparation of heat-treated *L. monocytogenes*. The live bacterial suspension (1ml) was transferred to a 1.5 ml micro-tube (Eppendorf, Tokyo, Japan), and the tube was soaked in boiling water bath for 50 s. Thereafter, it was immediately chilled in an ice water bath. This treatment simulated High Temperature Short Time (HTST) Pasteurization and avoided denaturing DNA gyrase/topoisomerase IV of bacteria. Temperature of the content was measured by a thermal sensor (TX 10; Yokogawa M & C corp., Musashino, Japan).

Preparation of antibiotic-treated *L. monocytogenes*. *L. monocytogenes* (3.0 × 10^6 cells/ml) was treated in L-broth with AMP and GEN (final concentrations were 500 and 200 µg/ml, respectively) and incubated at 30°C for 3 weeks to bring *L. monocytogenes* completely to the injured/dead state, and/or to examine whether DNA gyrase/topoisomerase IV is retained during long term administration. The cell counts for the antibiotic-treated *L. monocytogenes* were done by a standard curve made from live bacterial counts and its OD_{600}.

EMA treatment and visible light irradiation (EMA + Light). EMA was dissolved in sterile...
water at the concentration of 1 mg/ml and filtrated through a 0.20 µm micro-filter (Minisart-plus; Sartorius AG, Gottingen, Germany). After EMA was added at the concentration of 10 µg/ml to each heat-/antibiotic-treated and live bacterial suspension, it was kept at 4°C for 5 min in the dark. Then, the suspension was set in an ice water bath and irradiated for 5 min with visible light (FLOOD PRF 100 V 500 W; Iwasaki Electric Co., Ltd., Tokyo, Japan) set 20 cm from the solution. The concentration of EMA was set at 10 µg/ml because >10 µg/ml of EMA could penetrate live *L. monocytogenes* as well.

**Use of topoisomerase poisons (T-poisons) together with EMA + Light.** After EMA + Light treatments, bacteria were washed by centrifugation. Topoisomerase poisons were used to make DNA more degraded by interfering with the breakage-reunion function of DNA gyrase/topoisomerase IV retained in the heat-treated cells. Topoisomerase poisons were added to 1 ml of bacterial suspensions at volumes and concentrations (in parenthesis) as follows: 8 µl of CIN (0.5 mg/ml), 10 µl of CAM (1 mg/ml), 10 µl of ETP (1 mg/ml), 5 µl of ELP (0.1 mg/ml), 10 µl of MIT (0.1 mg/ml), and 10 µl of m-AMSA (1 mg/ml). The bacterial suspensions were then incubated at 30°C for 30 min.

**Ciprofloxacin (CIN) treatment to confirm DNA gyrase and topoisomerase IV activities retained in heat-treated *L. monocytogenes*.** Live and heat-treated *L. monocytogenes* were suspended in fresh BHI broth and CIN was added at the final concentration of 20 µg/ml. Then, they were incubated at 30°C for 1.5, 3.5, 5, and 72 h. Simultaneously, the heat-treated bacterial suspension not treated with CIN was prepared as a control to examine the influence of DNase retained in heat-treated *L. monocytogenes* on chromosomal DNA. It was incubated for 72 h.

**Treatment of *L. monocytogenes*-added human blood.** Heparinized blood from a healthy human was cooled beforehand at 4°C. Live and antibiotic-treated *L. monocytogenes* was inoculated to the heparinized blood at concentrations of $1.8 \times 10^0 - 1.8 \times 10^7$ cells/ml. After diluting 2-fold with physiological saline, 1 ml was slowly overlaid on 1.0 ml of Ficoll-Paque PLUS
GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in a sterilized micro-tube (2 ml of volume). Then, the layers were subjected to centrifugation at 100 × g for 5 min at 4°C, and the blood plasma containing microorganism was collected. EMA + Light treatment along with the washing of the bacteria were carried out as mentioned above. When T-poison was added, the bacterial suspensions were incubated for 45 min at 30°C followed by the same washing step. As a control, sterilized water and 0.5%(v/v) DMSO were used to substitute EMA and T-poisons, respectively.

Treatment of commercial milk spiked with *L. monocytogenes*. Live and heat-treated *L. monocytogenes* (2.2 × 10⁰ – 2.2 × 10⁷ cells/ml) was spiked into pasteurized milk (125°C, 2 sec) in which no live *L. monocytogenes* was beforehand detected by culture and no *L. monocytogenes* was detected by PCR without EMA as described below (detection limit for live *L. monocytogenes* in milk: 2.2 × 10¹ cells/ml). T-poisons were initially added to 1 ml of milk inoculated with *L. monocytogenes* and then incubated at 30°C for 3 h. 1 ml of 1% Triton X-100/2 mM EDTA solution (pH 8.0) was added and centrifuged at 3,000 × g for 5 min at room temperature. After the lipid and supernatant were removed, the washing step (at 15,000 × g for 10 min at room temperature) was done with 2 ml of physiological saline, and then 1 ml of physiological saline was added. EMA + Light treatment and washing of bacteria were performed as mentioned above. As a control, 0.5% DMSO and sterile water were added instead of T-poisons and EMA, respectively.

DNA extraction from bacteria. After 0.5 ml of 5 mM EDTA was added to bacterial pellets in a micro-tube (2 ml), 20 µl of achromopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved at 5 mg/ml in 10 mM NaCl, was added and incubated at 50°C for 30 min. Then, 0.5 ml of 10 mM Tris-HCl (pH 8.0), 20 µl of 1250 U/ml proteinase K (Sigma, St. Louis, MO, USA), and 400 µl of 10% (w/v) lauryl sulfate sodium salt (SDS) solution were added one after another. The solution was incubated at 50°C overnight. The chromosomal DNA was purified by usual phenol/chloroform extraction, and ethanol precipitation. 150 µl of TE buffer (10mM Tris-HCl, 1 mM EDTA·2Na) was added to the purified DNA. The concentration of DNA was calculated from
OD$_{260}$, and purity was evaluated from the ratio of OD$_{260}$/OD$_{280}$. Furthermore, usual RNase treatment was carried out successively for PCR.

**Gel electrophoresis of chromosomal DNA.** Seakem GTG agarose (FMC BioProducts, Rockland, ME, USA) was dissolved in 0.8% by TAE buffer. After 1 µg of purified DNA was applied to wells, electrophoresis was performed at 100 V. λ-EcoT14 I digest and/or 100 bp DNA ladder (Takara-Bio, Ohtsu, Japan) were used as DNA markers. After the gel was stained with 1 µg/ml ethidium bromide, the result was visualized with an UV-trans-illuminator at 254 nm of UV light and recorded on Polaroid film (type 667; Nippon Polaroid, Tokyo, Japan).

**Real-time PCR.** Reactions were performed in the real-time PCR system (i cycler iQ; Bio-rad, Hercules, CA, USA). The fluorescence threshold was set at a value of 10 × the standard deviation (SD) calculated from the fluorescence values, from 0 to 10 cycles. Then the first cycle, where the signals of real-time PCR amplification were above the threshold fluorescence value was set as C$_T$ value.

**Targeting genes and primers used for PCR.** The 23S rDNA primers to precisely discriminate live and heat-treated *L. monocytogenes* were 23S-MF (5’-ACCAGGATTTTGGCTTAGAAG-3’) and 23S-MR (5’-CACTTACCCCGACAAGGAAT-3’) (12). The length of PCR product was 894 bp.

The listeriolysin O gene (*hly*) was also targeted to discriminate live *L. monocytogenes* from heat-/antibiotic-treated *L. monocytogenes*. The *hly* primers were *hly*-F (5’-TGCAAGTCCTAAGACGCCA-3’) and *hly*-R (5’-CACTGCATCTCCGTGGTATACTAA-3’) (22). The length of PCR product was 113 bp.

**Amplification of 23S rDNA and hly by real-time PCR.** 50 µl of PCR master-mix was prepared containing 150 ng of template DNA, 5 µl of 10 x Ex-Taq Buffer (Takara-Bio), 200 µM each of dNTPs (Takara-Bio), 0.25 µM of 23S rDNA or *hly* gene primers (Takara-Bio), 0.4 × SYBR Green (BMA, Rockland, ME, USA), and 1.25 U of Ex-Taq polymerase (Takara-Bio).
The PCR protocol for 23S rDNA of *L. monocytogenes* was 1 cycle at 4°C for 3 min, 1 cycle at 94°C for 30 s, and 40 cycles at 94°C for 20 s, 46°C for 30 s, and 72°C for 1 min. After PCR, the Tm pattern analysis (melting point measurement) of PCR product was carried out with 1 cycle at 95°C for 3 min, followed by cooling at 60°C, and heating to 95°C at the rate of 0.75°C per min.

The PCR protocol for *hly* was 1 cycle at 4°C for 3 min, 1 cycle at 94°C for 30 s, and 40 cycles at 95°C for 20 s followed by 60°C for 1 min. After PCR, the Tm pattern analysis of PCR product was performed with the same procedures as that of 23S rDNA.

In an experimental procedure applied to blood and milk, direct PCR cocktail (G & g Science) was added to the bacterial pellet suspended with sterilized water (10 µl) after EMA + Light + T-poison treatment and successive washing, taking into consideration the simplification of DNA extraction. That is, 5µl of bacterial suspension treated by EMA + Light + T-poison was added to 50.5 µl of PCR cocktail. The thermal cycle profile, which was the same as the PCR protocol for *hly*, was utilized.

Electrophoresis of PCR final products amplified by real-time PCR. A 0.8% or 3% agarose gel was made from the Seakem GTG agarose and TAE buffer for PCR final products from 23S rDNA and *hly*, respectively. The λ-EcoT14 I digest and 100 bp DNA ladder (Takara-Bio) were used as DNA markers. After 10 µl of PCR product was applied to the wells, it was separated at 100 V.
RESULTS

The time course of temperature and colony forming units of bacteria when bacterial suspension was inserted in boiling bath. The relationship between insertion-time into boiling bath, and temperature of contents (time, temperature) was (0 s, 25.0 ± 0.15°C; 27 s, 65.0 ± 0.20°C; 34 s, 70.0 ± 0.10°C; 47 s, 80.0 ± 0.15°C; 70 s, 90.0 ± 0.25°C; 90 s, 93.8 ± 0.60°C; 120 s, 99.0 ± 0.45°C) (n = 3). The relation of immersion-time and viable cell counts (Log_{10} CFU/ml) of L. monocytogenes JCM 2873 (time, counts) was (0 s, 8.1 ± 0.20; 10 s, 7.5 ± 0.10; 20 s, 6.1 ± 0.10; 30 s, 4.7 ± 0.15; 40 s, 2.4 ± 0.10; 50 s, no counts) (n = 3). The detection limit was 5 CFU/ml. Insertion for 50 s offered the condition similar to High Temperature Short Time (HTST) Pasteurization, that is, 72 to 75°C for 15 to 16 s.

Effects of EMA on the cleavage of chromosomal DNA of heat-treated and live L. monocytogenes. Live and heat-treated cells of L. monocytogenes was treated with EMA + Light (4°C for 5 min in the dark; irradiation time, 5 min), and chromosomal DNA was purified. The gel electrophoresis patterns are shown in Fig. 1. When EMA + Light was performed on live bacteria, upper long fragments near 19,329 bp (derived from chromosomal DNA because the band is fuzzy on the lower site, i.e. anode side of the gel) were detected. As for the heat-treated L. monocytogenes, the long fragments did not appear but smear bands were detectable at a range of less than 1,489 bp when EMA + Light was done.

Discrimination by PCR of live and heat-treated L. monocytogenes after EMA + Light using 23S rDNA. PCR was performed targeting 23S rDNA of L. monocytogenes after being treated with EMA + Light. PCR band from live L. monocytogenes was observed after EMA + Light, but that from heat-treated cells was not observed by EMA + Light (Fig. 2). Evidently, the discrimination of live from heat-treated L. monocytogenes could be done.

Discrimination of live and heat-treated L. monocytogenes targeting short DNA. When PCR
targets pathogenic bacteria, short genes specific for the organism are often amplified. Our method
tested whether hly gene (113 bp) can be used for discrimination between live and heat-treated L
monocytogenes. The PCR final product from heat-treated cells was not suppressed after processing
by EMA + Light, thus, discrimination between live and heat-treated L. monocytogenes was not
successful (Fig. 3, lanes N and E). Therefore, combination methods (EMA + Light + T-poison)
were performed (Fig. 3). When etoposide (ETP), mitoxantrone (MIT), and amsacrine
(m-AMSA) were treated at 30°C for 30 min after EMA + Light, the PCR final products from
heat-treated cells were greatly inhibited, though those from live cells were not.

Involvement of DNA gyrase/topoisomerase IV in DNA cleavage. Topoisomerase poisons
impair by accelerating the forward rate (breakage) and inhibiting the reunion of breakage-reunion
activity of topoisomerases. CIN is one topoisomerase poison. The effect of CIN on the cleavage
of chromosomal DNA was examined using live and heat-treated L. monocytogenes. The results
were shown in CIN (+) lanes with live and heat-treated L. monocytogenes in Fig. 4. When live L
monocytogenes was treated with CIN, long fragments decreased time-dependently during 0-3.5 h of
incubation. The intensity of long fragments increased at 5 h, but decreased to near the detection
limit at 72 h. Next, heat-treated L. monocytogenes was incubated at 30 °C, for 72 h with and
without CIN. With CIN, band intensity of long fragments decreased in a time dependent manner,
and was near the detection limit at 72 h. Without CIN, band intensity of long fragments (near
19,329 bp) did not decrease. These results show that DNA gyrase/topoisomerase IV is active in
heat-treated L. monocytogenes because topoisomerase poisons work only when topoisomerase is
active. Besides, the results imply that the influence of DNase retained in heat-treated L.
monocytogenes on DNA cleavage is minimal.

Discrimination of live and heat-treated L. monocytogenes by CIN using real-time PCR
targeting 23S rDNA. As far as we compare the thickness of bands in gels (Fig. 3), we cannot see
the additive effect of CIN on EMA + Light. To make the slight difference in discrimination power
more distinct, the effect of CIN on the degree of PCR suppression was evaluated by the C_T value of real-time PCR. The higher the C_T value, the greater the PCR inhibition. \( \Delta C_T \) means the degree of PCR suppression, which is represented as C_T (at treatment time) minus C_T (0 h). For live cells, \( \Delta C_T \) (0 to 72 h) was 0.0 \( \pm \) 0.00 (0 h), 0.0 \( \pm \) 0.00 (1.5 h), 1.6 \( \pm \) 0.10 (3.5 h), 1.0 \( \pm \) 0.08 (5 h), and 2.9 \( \pm \) 0.10 (72 h) (mean \( \pm \) SD; n = 3). As for heat-treated L. monocytogenes, \( \Delta C_T \) (0 to 72 h) was 0.0 \( \pm \) 0.00 (0 h), 0.0 \( \pm \) 0.00 (1.5 h), 1.0 \( \pm \) 0.10 (3.5 h), 1.4 \( \pm \) 0.10 (5 h), and 6.1 \( \pm \) 0.15 (72 h).

The \( \Delta C_T \) value (2.9 \( \pm \) 0.01) after 72 h of CIN treatment of live organisms was significantly different from the \( \Delta C_T \) value (6.1 \( \pm \) 0.15) of heat-treated cells (p < 0.05 by the t-test). Hence, PCR was suppressed more greatly in heat-treated cells than live L. monocytogenes by CIN treatment for 72 h.

Detection limit of live L. monocytogenes in human blood with PCR targeting hly gene. Fig. 5A shows the results of detection for live L. monocytogenes inoculated into healthy human blood using PCR targeting the hly gene. The PCR bands stemming from \( 1.8 \times 10^2 \) – \( 10^7 \) cells/ml of L. monocytogenes in blood were clear, but were not detectable from L. monocytogenes at concentrations of \( 1.8 \times 10^0 \) – \( 1.8 \times 10^1 \) cells/ml.

PCR after EMA + Light + T-poison on live and antibiotic-treated L. monocytogenes inoculated to healthy human blood. Live L. monocytogenes was treated with 500 \( \mu \)g/ml of AMP plus 200 \( \mu \)g/ml of GEN. Live and antibiotic-treated L. monocytogenes were inoculated into healthy human blood at concentrations of \( 1.8 \times 10^7 \), \( 2.9 \times 10^4 \) and \( 2.9 \times 10^3 \) cells/ml. Figs. 5B-D present the amplified hly gene by PCR after EMA + Light + T-poisons (CAM, ETP, ELP, and m-AMSA) treatment. In the case of non-treatment and EMA + Light (\( 1.8 \times 10^7 \) cells/ml; Fig. 5B), bands of live and antibiotic treated L. monocytogenes were clearly detected. However, the intensity of the bands of antibiotic-treated L. monocytogenes decreased to under or near the detection limit after adding CAM, ETP, or m-AMSA. The effect of ELP treatment was weak.
When $2.9 \times 10^4$ cells/ml of *L. monocytogenes* was mixed in blood (Fig. 5C), the PCR bands of live *L. monocytogenes* clearly appeared without treatment (lane N), after EMA + Light (lane E) and EMA + Light + T-poisons treatments. In the case of antibiotic-treated cells, the effects of T-poisons were clear, that is, PCR bands did not appear after EMA + Light + T-poisons treatments.

Even when the *L. monocytogenes* dose was lowered to $2.9 \times 10^3$ cells/ml in blood (Fig. 5D), the effects of T-poisons were almost the same as those of Fig. 5C.

**Detection limit of live *L. monocytogenes*-spiked commercially available milk with PCR targeting *hly* gene.** Fig. 6A shows the results of detection for live *L. monocytogenes* inoculated into pasteurized commercial milk using PCR targeting *hly* gene. The PCR bands derived from $2.2 \times 10^1$ to $2.2 \times 10^7$ cells/ml of *L. monocytogenes* in milk were detectable. No band of *L. monocytogenes* for $2.2 \times 10^0$ cells/ml was detected, which means that detection limit is between $2.2 \times 10^1$ and $2.2 \times 10^0$ cells/ml.

**Amplifications of PCR targeting *hly* gene after T-poison + EMA + Light to live and heat-treated *L. monocytogenes*-spiked commercial milk.** *L. monocytogenes* was inoculated into milk at concentrations of $2.2 \times 10^7$ (Fig. 6B) and $2.2 \times 10^3$ cells/ml (Fig. 6C). Figs. 6B and C indicate the final products (*hly*) amplified by PCR performed after T-poisons (CAM, ETP, ELP, and m-AMSA) + EMA + Light treatment on live and heat-treated *L. monocytogenes*. In the case of $2.2 \times 10^7$ cells/ml (Fig. 6B), bands of live and heat-treated *L. monocytogenes* were apparently detected both without treatment (lane N) and after EMA + Light (lane E) treatment. As for CAM + EMA + Light and m-AMSA + EMA + Light, the bands of heat-treated *L. monocytogenes* were near or under the detection limit, although bands of live cells were clearly detected and the intensity was near that of non-treated live cells. In ETP or ELP + EMA + Light treatment, PCR bands of live and heat-treated bacteria appeared, thus the effectiveness of the discrimination of ETP and ELP was weak when milk was used.

For $2.2 \times 10^3$ cells/ml of *L. monocytogenes* in milk (Fig. 6C), the PCR bands of live *L. monocytogenes* were clearly detected without treatment (lane N), after EMA + Light (lane E) and EMA + Light + T-poisons treatments. In the case of antibiotic-treated cells, the effects of T-poisons were clear, that is, PCR bands did not appear after EMA + Light + T-poisons treatments.
*monocytogenes* obviously appeared in non-treatment, EMA + Light, and T-poisons + EMA + Light (four kind of agents). However, PCR bands did not appear after EMA + Light and T-poisons (four agents) + EMA + Light.
DISCUSSION

Rapid PCR methods which detect only viable and culturable bacteria are required in food hygiene and clinical tests, in substitution for the culture method. Rudi et al. (27, 29) applied EMA as a cross-linking agent to discriminate between live and dead pathogens, and targeted 85 bp DNA for *Campylobacter jejuni* and 113 bp DNA of *hly* gene of *L. monocytogenes*. PCR signals from dead, but not live bacteria, were suppressed to 1/1000 in dead cell counts, which means that the CT value of dead bacteria, would increase by 10 cycles, compared with no use of cross-linking EMA. Rudi et al., however, could not suppress real-time PCR final products from dead pathogens. In the case of food or clinical samples, in which the concentration of live and dead pathogen is unknown, it is very difficult to judge whether CT values of test samples are derived from live or dead bacteria.

No end product of PCR from dead cells is necessary in the factory or bedside, and Food and Drug Administration (FDA) requests complete inhibition of PCR product from dead bacteria.

We have completely suppressed PCR signals from $10^7$ - $10^8$ cells of heat-/antibiotics-treated bacteria considered as a background signal in many food and clinical samples. Furthermore PCR products derived from $10^2$ cells/ml (in blood) and $10^1$ cells/ml (in pasteurized milk) of live bacteria were detected. It is a very important consequence in clinical diagnostics and food testing that a high level of heat-/antibiotics-treated bacteria was not detected but a low level of live bacteria was detectable by our PCR method. Although gel electrophoresis was used as the detection step for PCR amplified genes in the present study, automated melting point analysis (using real-time PCR apparatus) for PCR products shown in MATERIALS AND METHODS may be used, considering rapidity, simplicity, and sensitivity to dose of live bacteria during the detection steps. If EMA + Light or EMA + Light + T-poison were combined with melting point analysis after real-time PCR, $10^2$ and $10^1$ cells/ml of live bacteria in blood or milk, respectively, could be detected within 3 – 6 h.

We recently demonstrated that 1 to 10 µg/ml of EMA has the direct DNA cleavage function
without mediating enzymes in bacteria, and that 50-100 ng/ml of EMA has the function of single-stranded breaks (32). For heat-treated *L. monocytogenes*, PCR targeting long DNA (894 bp in 23S rDNA) was suppressed by EMA + Light more greatly than short DNA (113bp in *hly*) (Fig. 2 and 3). This may be because there are more cleavage sites in long DNA. It was reported that nano-gram per ml level EMA as topoisomerase II poison could be cross-linked to DNA at the rate of 1 agent per 10-80 bp in vitro (17). In the present study using 10 µg/ml of EMA, direct DNA cleavage was not induced in the *hly* region of heat-treated *L. monocytogenes* (Fig. 3). We suppose that Rudi et al. (27) did not notice that PCR inhibition by EMA was not satisfactory when short DNA had been targeted. As seen above, the main cause of failure to suppress PCR product is that the PCR target gene was short. Even if PCR were targeted to short DNA such as *hly*, live *L. monocytogenes* could be clearly discriminated from heat-treated *L. monocytogenes* (10^8 cells/ml) by combining EMA + Light together with T-poisons (Fig. 3).

The mechanism of our method for discriminating live from dead bacteria is shown in Fig. 7. After EMA (dark red bar) penetrates heat-/antibiotic-treated cells and intercalates to the chromosomal DNA (23, 27-29), the cleavage of DNA is greatly induced by irradiation of visible light (32). The cleavage sites are shown in dsDNA. When the targeted gene is short as is *hly*, DNA cleavage sites are not likely to be contained in every bacterial cell. The cell membranes of heat-/antibiotic-treated *L. monocytogenes* are physiologically injured. Therefore, when a T-poison (e.g. m-AMSA, yellow bar) is added, m-AMSA randomly cross-links to chromosomal DNA and DNA cleavage is accelerated by inhibiting reunion in breakage-reunion by DNA gyrase and/or topoisomerase IV retained in heat-/antibiotic-treated cells. As EMA and m-AMSA randomly cross-link, more cleavage is induced by the combination of EMA + Light + m-AMSA (Fig. 7). Hence, the disappearance of bands in Fig. 5 and 6 is thought to be due mainly to the poisoning effects of T-poisons.

Effectiveness of EMA + Light + T-poison (mainly CAM, ETP, and m-AMSA) was demonstrated
in a model of bacteremia (Fig. 5). In adult bacteremia patients, numbers of microorganisms present
in blood are fewer than 10 cells/ml, and 30 ml of blood is used for culture to maximize microbial
recovery. A 30 ml volume of blood could be concentrated to approximately 1 ml for PCR testing.
Thus, the concentration of live bacteria would be approximately $3 \times 10^2$ cells/ml (26). In case of
bacteremia in infants, the number of bacteria existing in blood is often more than $1.0 \times 10^3$ cells/ml,
but only 1 to 4.5 ml of blood should be cultured, taking into consideration the weight of the infant
(13). Bacteria injured or killed by antibiotics are supposed to exist in blood together with live
bacteria. In the present study, therefore, the live and antibiotic-treated *L. monocytogenes* were
spiked into healthy human blood at the concentration of $2.9 \times 10^4$ (Fig. 5C), and $2.9 \times 10^3$ cells/ml
(Fig. 5D). On the other hand, live and antibiotic-treated *L. monocytogenes* were inoculated to
blood at a concentration of $1.8 \times 10^7$ cells/ml, considering the presence of high levels of
injured/dead bacteria in urine from urinary tract infection and sputa of tuberculosis patients given
anti-tuberculosis agents (6). EMA + Light + T-poison may be effective to rapidly discriminate live
from injured/dead pathogen.

Pasteurized milk contains $10^5 - 10^7$ cells/ml of injured/dead bacteria, and approximately half
of the bacteria are gram-positive (1, 30). If *L. monocytogenes* was estimated to be the major
contaminant, only live *L. monocytogenes* must be detected by PCR among a high level of
background injured/dead *L. monocytogenes*. Effectiveness of EMA + Light and T-poison (mainly
m-AMSA and CAM) + EMA + Light was tested in food hygiene tests of dairy products. Hence, as
shown in Fig. 6B, live and heat-treated *L. monocytogenes* $(2.2 \times 10^7$ cells/ml) were inoculated to
pasteurized milk. The discriminating power of T-poisons + EMA + Light was conceivable to be
inferior in milk compared to blood (Fig. 5B) except for m-AMSA + EMA + Light. It has been
reported that 2.4 – 7.5% of raw milk is contaminated by live *L. monocytogenes* (10) and live *L.
monocytogenes* exists at a concentration of $2.0 \times 10^2$ cells/g in raw milk cheese (2). Hence, live
and heat-treated *L. monocytogenes* $(2.2 \times 10^3$ cells/ml) was also inoculated to pasteurized milk (Fig.
When *L. monocytogenes* in milk is of low concentration (live and heat-treated cells: $2.2 \times 10^3$ cells/ml), EMA + Light without T-poisons could discriminate live from heat-treated *L. monocytogenes* as well (Fig. 6C).

Verification of active DNA gyrase/topoisomerase IV retained in heat-treated bacteria is speculated as follows. As shown in Fig. 4, when live and heat-treated *L. monocytogenes* is treated with CIN for 72 h, the long fragments derived from chromosomal DNA (close to 19,329 bp) obviously decrease. Fluoroquinolones, such as CIN, cause inhibition of DNA synthesis and trigger cell killing by interfering with breakage-reunion mediated by DNA gyrase (33). The reason for this phenomenon is thought to be that DNA scission is stimulated by inhibiting the reunion of breakage-reunion or enhancing the forward DNA breakage rate by DNA gyrase and/or topoisomerase IV persistent in heat-treated and live bacteria.

Last, our EMA + Light + T-poison method will be applied to boiled foods. When foods are treated by higher temperatures and for longer periods, the foods contain mainly dead bacteria in which no activity of DNA gyrase/topoisomerase IV is retained. In such cases, at least CAM and m-AMSA, among the T-poisons, would cross-link to chromosomal DNA, and might specifically suppress PCR final products from dead bacteria (7). In this case, however, the PCR suppression is due to the cross-linking effect but not poisoning activity (7). EMA could function as a random and direct cleavage agent of chromosomal DNA with irradiation of visible light, even if DNA gyrase and/or topoisomerase IV are completely denatured in dead cells (32).

**ACKNOWLEDGMENTS**

We are grateful to Dr. Hiroaki Nakayama for expertise and advice regarding the mechanism of DNA gyrase poison and topoisomerase IV poisons.
REFERENCES


components and DNA topology requirements of *Pyrococcus* transcription.

Genetics. **152**: 1325-1333.


Figure legends

FIG. 1. Gel electrophoresis patterns of chromosomal DNA purified from live and heat-treated *L. monocytogenes* before and after EMA + Light treatment. N: no EMA + Light treatment; E: EMA + Light treatment (at 4°C in the dark for 5 min; irradiation for 5 min). M1: λ-EcoT14 I digest; M2: 100 bp DNA ladder. The experiments for DNA extraction were performed in duplicate, and the electrophoresis patterns were the same.

FIG. 2. Amplification results of 23S rDNA-PCR after no EMA + Light or EMA + Light treatment using live and heat-treated *L. monocytogenes*. The amplification results for 23S rDNA (894 bp) of *L. monocytogenes* are represented. N: no EMA + Light treatment, E: EMA + Light treatment (at 4°C in the dark for 5 min; irradiation for 5 min). M1: λ-EcoT14 I digest; M2: 100 bp DNA ladder. The PCR experiments were done in three replicates, and the results were the same.

FIG. 3. Amplification results of PCR targeting *hly* gene after combination treatment of EMA + Light and ciprofloxacin/mammalian topoisomerase (I and II) poisons on live and heat-treated *L. monocytogenes*. Targeted *hly* gene was 113 bp.
Topoisomerase poison treatments were carried out for 30 min after EMA + Light.

N: no EMA + Light treatment; E: EMA + Light treatment (at 4°C in the dark for 5 min; irradiation for 5 min); M: 100 bp DNA ladder; CIN: ciprofloxacin (4 µg/ml); CAM: camptothecin (10 µg/ml); ETP: etoposide (10 µg/ml); ELP: ellipticine (0.5 µg/ml); MIT: mitoxantrone (1 µg/ml); m-AMSA: amsacrine (10 µg/ml). The concentrations of CIN to m-AMSA represent final concentrations. The experiments were carried out in triplicate, and the electrophoresis images were the same.

**FIG. 4.** Effects of ciprofloxacin on the retaining activities of DNA gyrase and topoisomerase IV in heat-treated *L. monocytogenes*. The influence of ciprofloxacin on the chromosomal DNA of live and heat-treated *L. monocytogenes* and the effect of DNase retained in heat-treated bacteria on the chromosomal DNA of heat-treated *L. monocytogenes* is shown. N: non-treatment; CIN (+): ciprofloxacin treatment. CIN (-) represents that heat-treated *L. monocytogenes* were incubated at 30°C for 24, 48, or 72 h without ciprofloxacin. The evaluations were done in duplicate, and the same electrophoresis patterns were obtained.
FIG. 5. Detection limit of *L. monocytogenes* and discrimination of the live/antibiotic-treated *L. monocytogenes* in human blood by PCR targeting *hly* gene. (A) Live *L. monocytogenes* \((1.8 \times 10^0 – 10^7 \text{ cells/ml})\) inoculated into heparinized healthy human blood was harvested and *hly* (listeriolysin O) gene \((113 \text{ bp: short DNA})\) was targeted by PCR. (B) to (D). Live or antibiotic-treated *L. monocytogenes* were mixed in human blood, and then treatments by EMA + Light + T-poisons and PCR methods were carried out. M: 100 bp DNA ladder; *hly*: listeriolysin O gene \((113 \text{ bp: short DNA})\) of *L. monocytogenes*; N: non-treatment as a control; E: EMA + Light (at 4°C in the dark for 5 min; irradiation for 5 min); CAM: camptothecin \((25 \mu \text{g/ml})\); ETP: etoposide \((25 \mu \text{g/ml})\); ELP: ellipticine \((2.5 \mu \text{g/ml})\); m-AMSA: amsacrine \((25 \mu \text{g/ml})\). The concentrations of CAM to m-AMSA represent final concentrations. Each examination was performed in duplicate, and the same results were observed.

FIG. 6. Detection limit of *L. monocytogenes* and discrimination of live/heat-treated cells spiked into pasteurized milk with PCR targeting *hly* gene. (A) Live *L. monocytogenes* spiked into pasteurized \((125°C \text{ for } 2s)\) commercial milk \((2.2 \times 10^0 – 2.2 \times 10^7 \text{ cells/ml})\) was recovered as bacterial pellets, and supplied to direct PCR.
(hly) cocktail. The hly (listeriolysin O) gene (113 bp: short DNA) was targeted by PCR. (B) and (C). Live or heat-treated *L. monocytogenes* was spiked in milk, and then treated by T-poisons + EMA + Light and PCR methods. M: 100 bp DNA ladder; hly: listeriolysin O gene (113 bp: short DNA) of *L. monocytogenes*; N: non-treatment as a control; E: EMA + Light (at 4°C in the dark for 5 min; irradiation for 5 min); CAM: camptothecin (25 µg/ml); ETP: etoposide (25 µg/ml); ELP: ellipticine (2.5 µg/ml); m-AMSA: amsacrine (25 µg/ml). The concentrations of CAM to m-AMSA represent final concentrations. The experiments were carried out in duplicate, and the results were reproducible.

**FIG. 7.** The left panel is the scheme for PCR suppression by EMA, psoralen, and methylisopsoralen derivative (4’-AMDMIP) as a DNA cross-linking agent (current scheme). The right panel is the scheme for PCR suppression through DNA cleavage by new function of EMA and topoisomerase poisons containing fluoroquinolone (new scheme). EMA, psoralen, or 4’-AMDMIP: dark red bar; Topoisomerase poison: yellow bar (m-AMSA etc.); Topo IV: bacterial topoisomerase IV. The cleavage points are represented in double strand DNA.
FIG. 1

*L. monocytogenes* (1.3 × 10⁸ cells/ml)

<table>
<thead>
<tr>
<th>bp</th>
<th>Live</th>
<th>Heat-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>19329</td>
<td>M1</td>
<td>M2</td>
</tr>
<tr>
<td>1882</td>
<td>N</td>
<td>E</td>
</tr>
<tr>
<td>1489</td>
<td></td>
<td></td>
</tr>
<tr>
<td>925</td>
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<tr>
<td>421</td>
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</tr>
</tbody>
</table>

- Chromosomal DNA
- 23S rRNA
- 16S rRNA
FIG. 2

*L. monocytogenes* (1.3 × 10^8 cells/ml)

<table>
<thead>
<tr>
<th>bp</th>
<th>M1 M2</th>
<th>Live N</th>
<th>Heat-Treated N</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>421</td>
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</tbody>
</table>

23S for *L. monocytogenes*
FIG. 3

*L. monocytogenes* (1.3 × 10^8 cells/ml)
**FIG. 4**

Listeria monocytogenes

<table>
<thead>
<tr>
<th></th>
<th>Live CIN (+)</th>
<th>Heat-Treated CIN (+)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N 1.5 3.5 5 72</td>
<td>N 1.5 3.5 5 72 24 48 72</td>
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<tr>
<td>bp</td>
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Chromosomal DNA
FIG. 5

A

Live *L. monocytogenes* inoculated to healthy human blood

<table>
<thead>
<tr>
<th>M</th>
<th>10⁷</th>
<th>10⁶</th>
<th>10⁵</th>
<th>10⁴</th>
<th>10³</th>
<th>10²</th>
<th>10¹</th>
<th>10⁰</th>
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B

*L. monocytogenes* (1.8 × 10⁷) in healthy human blood

<table>
<thead>
<tr>
<th>M</th>
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<th>E</th>
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<th>E</th>
<th>E</th>
<th>E</th>
<th>+CAM</th>
<th>+ETP</th>
<th>+ELP</th>
<th>+m-AMSA</th>
<th>Live</th>
</tr>
</thead>
</table>

Antibiotic-Treated (AMP + GEN)
FIG. 5

**C**

**L. monocytogenes** (2.9 \( \times \) 10^4) in healthy human blood

<table>
<thead>
<tr>
<th></th>
<th>M</th>
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CAM  ETP  ELP  m-AMSA

**hly** (113 bp)

**D**

**L. monocytogenes** (2.9 \( \times \) 10^3) in healthy human blood

<table>
<thead>
<tr>
<th></th>
<th>M</th>
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CAM  ETP  ELP  m-AMSA

**hly** (113 bp)
FIG. 6

**A**

```
Live *L. monocytogenes* inoculated to UHT milk

<table>
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<tr>
<th>M</th>
<th>10^7</th>
<th>10^6</th>
<th>10^5</th>
<th>10^4</th>
<th>10^3</th>
<th>10^2</th>
<th>10^1</th>
<th>10^0</th>
<th>× 2.2</th>
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<tbody>
<tr>
<td>hly (113 bp)</td>
<td>BP 200</td>
<td>BP 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

**B**

```
*L. monocytogenes* (2.2 × 10^7) in UHT milk

<table>
<thead>
<tr>
<th>M</th>
<th>N</th>
<th>E</th>
<th>CAM</th>
<th>ETP</th>
<th>ELP</th>
<th>m-AMSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hly (113 bp)</td>
<td>BP 200</td>
<td>BP 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

**C**

```
*L. monocytogenes* (2.2 × 10^3) in UHT milk

<table>
<thead>
<tr>
<th>M</th>
<th>N</th>
<th>E</th>
<th>CAM</th>
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<td>BP 200</td>
<td>BP 100</td>
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</tr>
</tbody>
</table>
```
Live

Dead

EMA, psoralen, 4'-AMDMIP

Current scheme

Injured/Dead

EMA

Our proposed scheme

Topoisomerase poison (amsacrine)

DNA extraction

DNA denaturation (PCR)

PCR band

DNA extraction

DNA denaturation (PCR)

PCR band

Idea 1

Idea 2

DNA gyrase Topo IV

PCR (Long DNA)

PCR (Short DNA)