Specific Detection of Viable *Salmonella* Cells by an Ethidium Monoazide-Loop Mediated Isothermal Amplification (EMA-LAMP) Method

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(Received April 16, 2009; Accepted August 7, 2009; Published online August 14, 2009)

The persistence of DNA after the cell death causes a major issue in aspects of medical or biological studies. The signal from viable bacterial cells cannot be distinguished from the dead cells in the conventional DNA-based detection methods. In the present study, the loop-mediated isothermal amplification (LAMP) method combined with the ethidium monoazide (EMA) treatment was applied for specific detection of viable, but not dead, *Salmonella* cells. For this method (EMA-LAMP), we designed a series of primers, which recognize six distinct sequences of the target *invA* gene conserved in *Salmonella*. The *invA* gene of the viable cells was remarkably amplified within 1 hr when as small amounts as 100 fg of DNA was subjected to EMA-LAMP. Because EMA selectively penetrated into the dead cells and bound covalently to DNA, the gene of the dead cells could not be amplified. This study offers a novel DNA-based method to distinguish the viable bacterial cells from the dead cells.

Key words —— ethidium monoazide, loop-mediated isothermal amplification, *Salmonella*, viable cell

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INTRODUCTION

Salmonella is a leading cause of food poisoning in humans and is also a major and important foodborne pathogen worldwide.[1,2] Many foods particularly those of animal origin or those subject to sewage are powerful vehicles for transmission of the pathogen to human beings and for spreading to the processing and kitchen environments.[3] Increased public awareness, which is related to human health and to economic impacts of food contamination, has evoked greater efforts to develop the more sensitive, rapid and inexpensive methods for detection and identification of the pathogenic microorganisms.[4]

The conventional bacteriological methods to detect *Salmonella* are too time-consuming while are less sensitive. Many DNA-based detection methods have been also developed in the recent years. The polymerase chain reaction (PCR) or real-time PCR technique has shown promising results due to the rapid, sensitive and specific detection of the pathogen.[5–8] Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method with ease of operation. By using this method, a few copies of the target DNA is specifically amplified to as high number as 10^9 within 1 hr under the isothermal (60 to 65°C) condition. However, due to the relatively long persistence of DNA after the cell death,[9] the DNA-based diagnostic methods tend to overestimate the cell numbers.[10]

A new DNA-intercalating dye, ethidium monoazide (EMA), can penetrate the cell walls or membranes of the dead cells and can bind covalently to DNA. The DNA associates with EMA is not amplified by the PCR or LAMP method.[11,12] So, the method combined with the EMA treatment is possible to distinguish rapidly and conveniently the viable bacterial cells from the dead cells. In this study, the living cells of *Salmonella* carrying the *invA* gene were selectively detected by the EMA treatment followed by the LAMP method (EMA-LAMP). This indicates that EMA-LAMP may be a very rapid, highly sensitive and cost-effective method for detection of the living bacterial cells.

MATERIAL AND METHODS

Bacterial Strains and Cultivation —— Two *Salmonella* strains, HB010 [*Salmonella enterica* (*S. enterica*) serovar Derby] and HB084 (*S. enter-*

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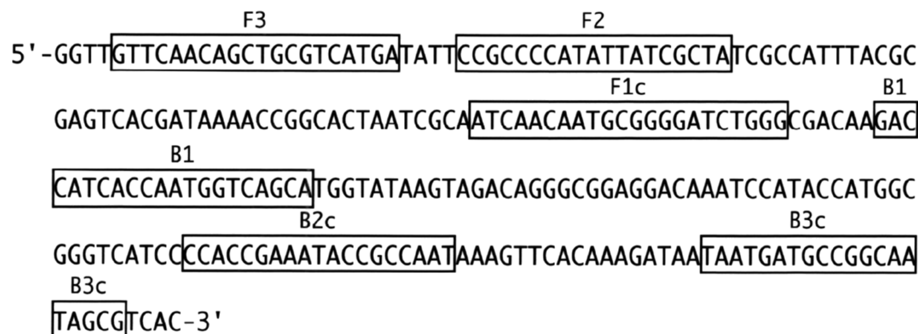

Fig. 1. The Nucleotide Sequence of the *invA* Gene and Primers Used for LAMP and PCR

ica serovar Indiana), which were isolated from raw chicken and raw pork respectively in Hebei province, China, were used in the present study. The bacterium was cultured overnight in 3 ml Luria-Bertani (LB) broth (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.5) with shaking (130 cycles/min) at 37°C. Thereafter, the bacterial culture was transferred to a centrifuge tube, and the bacterial cells were harvested, rinsed with and suspended into saline at a cell density of 5×10^8 CFU/ml.

Dead *Salmonella* Cells —— The dead cells were prepared by heat treatment or UV irradiation. For heat treatment, the living bacterial cells (1×10^9 CFU) in 2 ml saline were heated at 121°C for 15 min. For UV treatment, the living cells (5×10^9 CFU) in 10 ml saline were irradiated with a 40 W UVC lamp for 0 to 30 min in a petri dish.[13, 14] During irradiation, the lamp was placed at 35 cm from the bacterial cells, and the bacterial cell suspension was agitated to avoid sedimentation of the cells.

EMA Treatment —— The viable or dead cells in a centrifuge tube (5×10^8 cells/ml) were treated with EMA (Biotium Inc., Hayward, China) in the dark for 10 min and were subsequently exposed to a 650 W halogen lamp for 90 s. The lamp was placed at about 15 cm from the bacterial cells, which were cooled in the ice bath to minimize the elevation of temperature.[12] After EMA treatment, the bacterial cells were harvested and rinsed with saline.

Preparation of Bacterial DNA —— To prepare the bacterial DNA, the viable or dead *Salmonella* cells (1×10^9 cells in 2 ml saline) were collected by centrifugation and resuspended into 0.2 ml distilled water. Thereafter, the bacterial cell suspension was boiled for 10 min.

EMA-LAMP —— A serious of primers targeted six distinct regions in the *invA* gene, a *Salmonella* specific gene,[15–18] was designed for the EMA-LAMP method (Fig. 1). The sequences were analyzed by the Primer Explorer V4 software program (http://primerexplorer.jp/e/index.html) to design *invA*-F3: 5′-gttcaacagctgcgtcatga-3′ (a forward outer primer), *invA*-B3: 5′-cgctattgccggcatcatta-3′ (a backward outer primer), *invA*-FIP: 5′-cccagatc-cccgcattgttgat*tttt*ccgccccaatattatcgcta-3′ (a forward inner primer), and *invA*-BIP: 5′-gaccatcaccaatg-gtcagca*tttt*attggcggtatttcggtgg-3′ (a backward inner primer). The forward inner primer, *invA*-FIP, consisted of the complementary sequence of F1, a T-T-T-T linker and F2. The backward inner primer, *invA*-BIP, consisted of B1, a T-T-T-T linker and the complementary sequence of B2. The outer primers, *invA*-F3 and *invA*-B3, were located outside of the F2 and B2 regions, respectively.

The EMA-LAMP experiment was carried out in a total of 50 µl reaction mixture following the published procedure,[15] and the reaction was terminated by heating at 85°C for 5 min. Thereafter, SYBR Green I was added to the product to observe the color change. The green color represents the positive result, while orange color represents the negative result.

PCR —— The PCR experiment was performed with two outer primers, *invA*-F3 and *invA*-B3 (Fig. 1). The reaction mixture (50 µl) contained 5 µl of the buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3), 3 µl (30 pmol) of each of the primers, 4 µl of 2.5 mM dNTPs mixture, 3 µl of the template DNA and 0.25 µl (1.25 U) of rTaq DNA polymerase. The thermal profile was 94°C for 5 min (heat treatment), followed by 30 cycles of 94°C for 30 s (denaturation), 53°C for 30 s (annealing), and 72°C for 20 s (extension), and a final extension at 72°C for 7 min. The products were analyzed by electrophoresis in 1.2% agarose gel and visualized by staining with ethidium bromide.

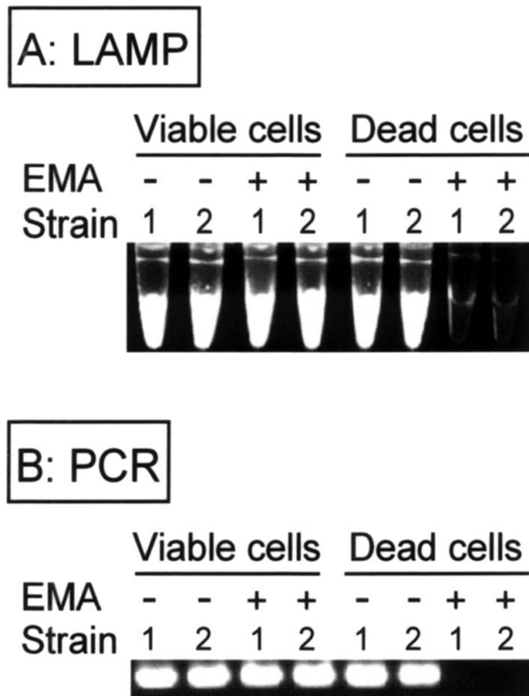

Fig. 2. Detection of the *Salmonella* Cells by LAMP or PCR

The viable or dead cells (5×10^8 cells/ml) were prepared from strain HB010 (1) or HB084 (2). Thereafter, the cells were treated with or without EMA and subjected to LAMP (A) or PCR (B).

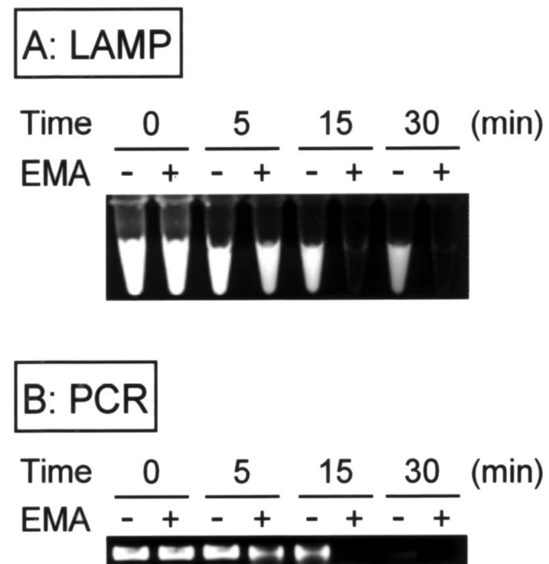

Fig. 3. Effect of UV Irradiation on the Detection of the *Salmonella* Cells by LAMP or PCR

The cell suspension of strain HB010 (5×10^8 CFU/ml) was irradiated with a UV lamp for 0 to 30 min. Thereafter, the cells were treated with or without EMA and subjected to LAMP (A) or PCR (B).

RESULTS

The dead cells of *Salmonella* were treated or untreated with EMA and subjected to LAMP or PCR. The *invA* gene of the EMA-untreated cells was apparently amplified; however, the gene of the EMA-treated cells was not amplified by LAMP as well as by PCR (Fig. 2). On the other hand, the *invA* gene of the viable cells was amplified even though treated with EMA (Fig. 2). These results indicate the EMA treatment is useful for the specific detection of the living cells by LAMP or PCR because EMA enters selectively to the dead cells. Although both EMA-LAMP and EMA-PCR showed sufficient amplification of the *invA* gene, the former offered the results more rapidly. In PCR, a total of 3 hr or more was needed for achievement of the experiments (DNA amplification, gel electrophoresis and visualizing with ethidium bromide). By contrast, the LAMP experiment including DNA amplification and visualizing with SYBR Green I was completed within 1 hr.

When the living cells of strain HB010 were irradiated with a UV lamp, the numbers of the cultivable cells were drastically decreased. At 5-min irradiation, the numbers of the colonies formed were reduced to only 0.1%, while at 15-min irradiation, no cultivable cell was detected (data not shown). As shown in Fig. 3, in both LAMP and PCR experiment, no amplification of the *invA* gene was observed at 15-min irradiation when pretreated with EMA, indicating the total killing of the *Salmonella* cells. The 5-min irradiated sample showed the positive results even when pretreated with EMA. However, when diluted 500-fold before the LAMP or PCR experiment, no amplicon was detected only in the EMA-pretreated sample (data not shown). The result also showed that the 30-min irradiation caused destruction and fragmentation of the bacterial DNA because the signal of the amplicon was apparently decreased in the sample without the EMA-pretreatment.

In order to compare the sensitivity, various amounts of DNA prepared from the EMA-treated living *Salmonella* cells were subjected to EMA-LAMP or EMA-PCR. The detection limit of the LAMP assay was as low as 100 fg DNA, which is equivalent to only 10 cells of the pure culture, while that of the PCR assay was 100 pg to 1 ng (Fig. 4).

DISCUSSION

Both PCR and real-time PCR have been very useful techniques for *Salmonella* detection. In the

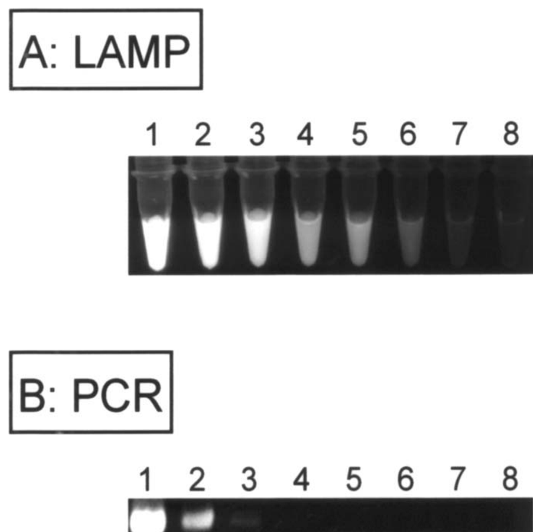

Fig. 4. Comparison of the Sensitivity of EMA-LAMP to EMA-PCR

The DNA prepared from strain HB010 was treated with EMA and subjected to LAMP (A) or PCR (B). Lane 1, 10 ng DNA; lane 2, 1.0 ng DNA; lane 3, 100 pg DNA; lane 4, 10 pg DNA; lane 5, 1.0 pg DNA; lane 6, 100 fg DNA; lane 7, 10 fg DNA; and lane 8, without DNA.

PCR experiment, the size of the product is revealed by electrophoresis using an agarose gel. Thus, the validity of the amplification is visually confirmed. On the other hand, the real-time PCR has many advantages such as rapidity, low contamination, higher sensitivity and easy standardization. However, because of requirement of the fluorogenic primers and probes as well as the expensive detection equipments, employment of the real-time PCR technique has restricted only in the laboratories with good financial resources.[19] Moreover, it should be emphasized that both PCR and real-time PCR can not distinguish the viable cells from the dead cells. The results of the present study demonstrated that the EMA treatment might be suitable for the selective detection of viable *Salmonella* cells by PCR.

The EMA-LAMP method developed herein requires no sophisticated equipment except for a water bath, and this technique can rapidly discriminate only the viable cells. Additionally, EMP-LAMP is more sensitive than other DNA-based detection techniques. It has been also documented that, during the EMA-LAMP experiment, much pyrophosphate ions are generated and white precipitates of magnesium pyrophosphate are formed through reaction with magnesium ions in the reaction mixture.[20, 21] This allows the easy and rapid confirmation of amplification of the target gene without visualizing by SYBR Green I.

The present study may be the first report on the application of EMA-LAMP for detection of the viable *Salmonella* cells. The EMA-LAMP method is potentially useful to all of the biological or medical diagnostic studies for distinction between the viable and dead cells.[22] Therefore, the EMA-LAMP technique may provide an efficient new approach for testing of the food safety. However, the enrichment cultivation using the selective broth,[23] as well as some modifications of the experimental procedures including DNA isolation, design of the primers and EMA treatment, might be required for suitable detection of a very low numbers of *Salmonella* cells from the food or feed sample.

Acknowledgements This study was supported by the National Natural Science Foundation of China (20877028), the Science Foundation of the Ministry of Education of China (706046) and the Technology Planning Project of Zhuhai City (PC20082034).

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