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A Novel Method for Estimating Viable *Salmonella* Cell Counts Using Real-Time PCR

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A novel method for estimating viable *Salmonella* Enteritidis cell counts with 5'-nuclease real-time PCR was developed in this study. Our method was based on the increase kinetics of the target DNA region (*invA*) of the microorganism growing in a food/clinical sample in a culture medium during incubation. The index of increase in the target DNA region studied here was threshold cycle, C_T . A test *Salmonella* strain was grown in buffered peptone water at the optimal temperature (39°C). As *Salmonella* cells were grown, the value of C_T decreased with time, generating a downward sigmoidal curve. The slope of the curve was constant at various initial cell concentrations. With higher initial cell concentration, the C_T value evaluated from the slope at a given time was lower. With this relationship, a novel method for estimating the initial viable cell concentration of a sample was developed. Dead *Salmonella* cells or bacteria other than the target cell caused deviation in the C_T curve. Incubation in a selective media suppressed the deviation caused by other bacterial cells. We think that this method could be applied to many other microorganisms cultivable in a suitable medium.

Key words: real-time PCR; viable cell counts; *Salmonella*; estimation method; threshold cycle

Introduction

Recently polymerase chain reaction (PCR) technologies in the microbiological field, as well as other biological fields, have progressed remarkably. In conventional PCR replication of the target DNA fragment is qualitatively detected as an electrophoretic band. Real-time PCR, especially TaqMan real-time PCR, has some great advantages over conventional PCR¹⁾. The advantages include the ability to detect replication quantitatively with a high specificity, by the introduction of the TaqMan probe labeled with reporter and quencher dyes into the PCR system. A number of researchers have developed TaqMan real-time PCR methods to detect microbiological pathogens in foods and/or clinical samples²⁾⁻⁵⁾.

However, some problems remain in PCR, even the TaqMan real-time PCR. One of them is an inability to distinguish between live and dead cells¹⁾. That is, when a food or clinical sample contains both dead and live target cells, the TaqMan real-time PCR detects both. Thus, there is a possibility that the cell counts estimated with the real-time PCR are higher than the real live cell counts. Even if the target cells in the sample were all dead cells, the PCR would give a positive reaction. This can be a serious problem in foods, where processing may destroy microbial cells, but leave their DNA relatively intact¹⁾. DNA itself is heat-stable; samples are heated for DNA extraction prior to PCR, as

shown in Materials and Methods. If this problem can be solved, PCR would be a much more reliable tool.

On the other hand, enrichment of microorganisms in a sample with a nutrient medium (broth) has been recognized to be a useful procedure for subsequent real-time PCR^{4), 6), 7)}. The procedure is effective to detect a small number of target cells in a sample, but this is still qualitative. No studies have indicated that enrichment might have the potential to allow estimation of the number of viable target cells in a sample. Recently, we found that the total viable cell counts of bacteria contaminating a food sample can be successfully estimated by measuring the increase kinetics of the ATP level of the sample incubated in nutrient broth^{8), 9)}. Here, the ATP level of the sample increases with the bacterial growth in the sample during the incubation. Using this estimation method, in the present study, we aimed to develop a novel procedure for viable cell counts estimation of the target microorganism with the TaqMan real-time PCR. We chose *Salmonella enterica* serovar Enteritidis cells as the target, since it is one of the major agents of bacterial food poisoning outbreaks worldwide.

Materials and Methods

Bacterial strain

A *Salmonella enterica* serovar Enteritidis strain 04-137 was isolated from a *Salmonella* food poisoning outbreak that occurred in Tokyo, Japan, in 2004. *Staphylo-*

coccus aureus, *Escherichia coli*, and *Proteus* sp. strains were isolated from commercial foods in our institute.

Incubation

A *Salmonella* strain 04-137 was isolated on a nutrient agar plate (Nissui Pharmaceuticals, Tokyo, Japan) and incubated at 37°C for about 20 hr. Colonies that grew well on the plate were inoculated in trypticase soy broth (Becton, Dickinson, and Company, Franklin Lakes, U.S.A.) and incubated with shaking at 100 strokes/min and 37°C for about 20 hr. Cultured cells (10^{10} CFU/mL) were washed twice with buffered sodium chloride solution (Nissui) containing 0.005% (v/v) Tween 80 at $6,200\times g$ and 10°C for 15 min and finally suspended in buffered peptone water (BPW), pH 7.2 (Oxoid, Basingstoke, UK) to afford the desired cell concentrations. *S. aureus*, *E. coli*, and *Proteus* cells were also prepared in the same manner as *Salmonella* cells.

The prepared cell suspension was diluted at various cell concentrations with pre-cooled BPW and seven-milliliter portions of the dilution were then inoculated into glass test tubes (15 mm inner diameter $\times$ 180 mm height). The sample suspensions were incubated at 39°C in an incubator with shaking at 100 strokes/min. At each one-hour interval a test tube was removed and cooled in ice water. In the selective medium experiment, Enterobacteriaceae Enrichment Mannitol (EEM) broth (Merck, Darmstadt, Germany) was used as the broth instead of BPW. Bacterial cell counts inoculated into the samples were measured with a plating method using nutrient agar plates (Nissui).

Dead *Salmonella* cells were prepared by heating the cell suspension prepared above (10^8 CFU/mL) at 75°C for 10 min. The heated cell suspension was diluted appropriately with saline (0.85% (w/v) sodium chloride solution) and then mixed into a viable cell suspension with a ratio of 1/100 (v/v), to make the final dead cell concentrations of 10^4 , 10^5 , and 10^6 cell/mL.

DNA extraction

DNA extraction from a sample suspension was performed in an alkaline solution¹⁰. Namely, a sample suspension prepared above (0.2 mL each) was centrifuged at $6,200\times g$ and 4°C for 10 min and the resultant supernatant was removed with a capillary. Cells were mixed with 25 mM NaOH (0.1 mL) and heated at 100°C for 5 min. The mixture was neutralized with 80 mM Tris-HCl, pH 7.0 (0.1 mL) and centrifuged at $6,200\times g$ and 4°C for 10 minutes. The supernatant of the sample was used for real-time PCR as described below.

Oligonucleotides

The primers and fluorogenic probe for TaqMan real-time PCR were obtained from Sigma-Aldrich, Japan (Tokyo). The *Salmonella*-specific primers (Styinv-JHO-2-left and -right) and probe [dual-labelled with 6-carboxyfluorescein and 6-carboxytetramethylrhodamine] were those designed by Hoofar *et al.*¹¹ to amplify a 119-base pair fragment of the invasion (*invA*) gene

(AN: M90846)¹².

TaqMan real-time PCR

The *Salmonella* TaqMan real-time PCR was performed in a 25-microliter reaction volume. A sample (2.5 microliter) and a PCR mixture (total 22.5 microliters, finally consisting of 250 nM Taqman probe, 300 nM each primer, $1\times$ Taqman Universal PCR Master Mix) (Applied Biosystems, Foster City, U.S.A.), prepared following the users' manual (Applied Biosystems), was plated in duplicate into wells of a microwell plate (Applied Biosystems). Two nontemplate controls were also added to the plate. Amplification was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems). The operating conditions were the same as those of Knutsson *et al.*⁷ The value of threshold cycle (C_T), the cycle number at which the fluorescence is greater the fixed threshold, was estimated at the threshold of 0.2.

Statistical analysis

Linear regression analysis, including slope and intercept estimations, was done using spreadsheet software, Microsoft Excel.

Results

Increase kinetics of DNA during incubation

When a *Salmonella* cell suspension was incubated in BPW, the growth curve of cells was an ordinary sigmoidal one (Fig. 1). In contrast with this curve, the C_T value of the suspension was reduced with time, generating a downward sigmoidal curve (Fig. 1). The straight portion, the slope, in the C_T curve was located between 2 to 6 hours of incubation in Fig. 1. That portion just corresponded to the logarithmic phase in the growth curve (Fig. 1). These results showed that the change in the C_T value was related to that in the number of the target cells in the suspension.

With lower initial viable cell number, the slope in the

Fig. 1. Kinetics of cell growth and the C_T value of a *Salmonella* cell suspension in BPW during incubation

The initial cell concentration was $10^{5.1}$ CFU/mL. Symbols: ■, C_T ; ●, cell concentration, N . Arrows show the vertical axes corresponding to the curves.

Fig. 2. Kinetics of the C_T value of a *Salmonella* cell suspension at various initial concentrations

The initial cell concentrations were $10^{5.1}$ (▲), $10^{4.1}$ (□), $10^{3.1}$ (◆), $10^{2.1}$ (○), and $10^{1.1}$ (■) CFU/mL. Straight lines are the regression lines for the data points at those initial cell concentrations. Data at $10^{5.1}$ CFU/mL are the same as those in Fig. 1. Point A is a point at 5 hr on the line and point B is the intercept of the line with the vertical axis at $10^{5.1}$ CFU/mL.

Fig. 3. Linear relationship between C_{TE} and the initial cell concentration

Closed circles are obtained from the data in Fig. 2. The line is the regression line for the data points. Points and arrows are described in the text.

C_T curve appeared later during the incubation (Fig. 2). The slopes at various initial cell concentrations were almost constant, being parallel to each other (Fig. 2); the values were -2.9 , -2.8 , -2.9 , -2.7 , and -2.8 at the initial cell concentrations of $10^{5.1}$, $10^{4.1}$, $10^{3.1}$, $10^{2.1}$, and $10^{1.1}$ CFU/mL, respectively. Here, the slopes with correlation coefficients over 0.99 were chosen. Since the C_T value over 40 was unstable, such high values were not analyzed in this study.

Cell count estimation algorithm

Based on the above findings, a novel method for estimation of viable *Salmonella* cell counts was developed. That is, the C_T value from the straight portion C_{TE} at a given time during the incubation was estimated using linear regression analysis. Let us name the time of estimation as T_E . For example, in Fig. 2, point A

on the regression line shows the estimated C_{TE} value at $T_E=5$ hr at the initial cell concentration of $10^{5.1}$ CFU/mL. C_{TE} was found to be linearly related to the initial cell concentration with a correlation coefficient of 0.999 (Fig. 3). The regression line was $C_{TE} = -3.27 \times \log N_0 + 37.4$. Here N_0 is the initial cell concentration (CFU/mL) of a sample. With this equation, the initial viable cell concentration of a sample can be estimated from the observed C_{TE} value. To actually estimate the initial concentration, the above equation needs to be transformed to $\log N_0 = -0.31 \times C_{TE} + 11.4$. For example, when the C_{TE} value of a sample is 28, the estimated cell concentration is $10^{2.7}$ CFU/mL. Graphically, the estimation procedure is shown as arrows in Fig. 3. Point A shows an imaginary value of C_{TE} of a sample at $T_E=5$ hr and point B shows the initial cell concentration estimated with the method.

Effect of dead cells

When a sample cell suspension contained both viable and dead *Salmonella* cells, the kinetics of C_T changed with the concentration of dead cells (Fig. 4). With a higher dead cell concentration in the sample, C_T started at a lower value, because such a sample initially had more of the target DNA derived from the dead cells. But, as the viable cells grew with increasing incubation time, the value of C_T decreased linearly and converged into a single value.

The (downward) slope in the C_T curve was identical in all samples (Fig. 4). This is because the slope is based on the increase in the target DNA of the viable cells. Here the initial viable cell concentrations of all samples were the same. Thus, the slope in the decreasing C_T curve could be used for the viable cell concentration estimation as described above. As shown in a curve with an extremely high dead cell concentration ($10^{6.1}$ cell/mL) in Fig. 4, when the slope was horizontal or almost horizontal during the incubation time, that linear por-

Fig. 4. Effect of dead *Salmonella* cells on the kinetics of the C_T value

The initial viable *Salmonella* cell concentrations were $10^{3.0}$ CFU/mL for all samples. Dead cell concentrations are $10^{6.1}$ (◆), $10^{5.1}$ (■), and $10^{4.1}$ (▲) CFU/mL. The control (●) does not contain dead cells.

Fig. 5. Effect of bacteria other than *Salmonella* on the kinetics of the C_T value

The initial viable *Salmonella* cell concentration was $10^{2.9}$ CFU/mL for all samples. Combinations of *E. coli* and *S. aureus* cell concentrations are $10^{8.2}$ and $10^{7.5}$ (A), $10^{6.2}$ and $10^{5.5}$ (B), and $10^{4.2}$ and $10^{3.5}$ (C) CFU/mL, respectively. The control (D) contains *Salmonella* cells only.

tion could not be used for the estimation.

Effect of bacteria other than *Salmonella*

The effect of presence of bacteria other than *Salmonella* at high cell concentrations in a sample cell suspension on the kinetics of C_T for *Salmonella* was studied, because food and clinical samples that are suspected to contain *Salmonella* are usually contaminated with other bacteria as well, at levels much higher than that of *Salmonella*. When a sample cell suspension contained *E. coli* as a gram-negative bacterium and *S. aureus* as a gram-positive one at concentrations much higher than that of *Salmonella* cells, the kinetics of C_T of the target microorganism changed with the concentrations of those bacteria (Fig. 5). The concentration ratios of those bacteria to *Salmonella* were about 10^5 (A), 10^3 (B), and 10^1 (C) in Fig. 5. As the concentrations of *E. coli* and *S. aureus* cells increased, the C_T curve deviated from the slope of the control with *Salmonella* only; the values of C_{TE} estimated from the slope of the C_T curve for samples B, C, and D in Fig. 5 were 32, 31, and 30, respectively, at $T_E = 4$ hr. For sample A, the estimation was not done, because there was no downward slope in the C_T curve.

Effect of selective medium

Based on the results shown in Fig. 5, we tried to modify the culture medium to obtain a longer downward slope in the C_T curve for the estimation. A selective medium for the target organism, EEM, was studied as the culture medium at the presence of other bacteria, *E. coli*, *Proteus* sp., and *S. aureus* cells. Compared with the period of the slope in BPW (2 hr, from 2 to 4 hr of incubation) (Fig. 6A), EEM prolonged the period (3 hr, from 2 to 5 hr of incubation) (Fig. 6B). The four regression lines in Figs. 6A and B were almost identical. Thus, the C_{TE} values at $T_E = 4$ hr estimated from the regres-

Fig. 6. Comparison between BPW (A) and EEM (B) in the kinetics of the C_T value

The initial viable *Salmonella* cell concentration was $10^{2.0}$ CFU/mL in all cases. Samples (○, □) contain *E. coli*, *Proteus* sp., and *S. aureus* cells at concentrations of $10^{7.1}$, $10^{7.2}$, and $10^{6.6}$ CFU/mL, respectively, as well. The control (●, ■) contains *Salmonella* cells only. Gray and solid lines are linear regression lines for the samples and the controls, respectively.

sion lines were the same, being 33 for all the samples in BPW and EEM. These results showed that EEM made the slope in the C_T curve longer, suggesting that EEM could be a useful culture medium for this estimation method.

Discussion

The optimal growth temperature for the test strain in BPW prior to the real-time PCR procedure was first studied. Growth of cells suspended in BPW at the concentration of 10^4 CFU/mL with shaking was recorded in terms of turbidity at 660 nm with a temperature gradient incubator (TN-2612, Advantec Toyo, Osaka, Japan) at constant temperatures ranging from 30 to 45°C. As a result, the optimal value was 39.5°C, followed by 38.5°C, 40.8°C, and 37.5°C in that order. From this result, the preferred incubation temperature of the test strain was determined to be 39°C. When four other *Salmonella* strains isolated from different food poisoning outbreaks in Tokyo were tested, their optimal growth temperatures were also all about 39°C.

In this study we developed a new estimation proce-

ture for the initial viable cell concentration of the target microorganism in a sample (broth), using a quantitative real-time PCR method. We also established some characteristics of the C_T curve for the sample, as follows. When a sample contained only viable target cells, the slope in the C_T curve was long enough to estimate viable target cell counts, as shown in Figs. 2 and 3. With lower initial viable cell numbers, the slope appeared later during the incubation. Dead target cells in a sample increased the initial value of C_T . For a sample containing bacteria other than the target microorganism, the value of C_T deviated from the slope of the control as the incubation time progressed.

The key point to accurately estimate C_{TE} in the C_T curve is how to obtain a "genuine" downward slope that is not affected by the factors described above or other unknown factors. Real food or clinical samples might include several kinds of microorganisms, dead target cells, and chemical or biological substances that affect the PCR reaction of the target DNA fragment. There might be several ways to avoid the effects due to those factors. Since the effect of microorganisms other than the target organism could not be ignored, use of a selective medium is expected to be very useful.

In our preliminary study, highly selective media for *Salmonella* such as Rappaport–Vassiliadis Enrichment Broth medium (Oxoid) and Selenite Cystine Broth (Nissui) were also studied as the incubation medium. In those media, however, the growth of *Salmonella* itself was slower than that in BPW and EEM, giving C_T values higher than those with BPW and EEM. Moreover, there is a possibility that injured *Salmonella* cells might not grow in those highly selective media. Thus, those media cannot be recommended as the incubation medium at present.

This study revealed that EEM could be a good candidate for the incubation medium to estimate viable *Salmonella* cells in food/clinical samples with our method. That is, EEM did not affect the C_{TE} values (with or without other bacteria) in comparison with the control (BPW), and it prolonged the slope (Figs. 6A, B). EEM contains effective ingredients to grow *Salmonella* well and selectively; bile and brilliant green inhibit gram-positive bacteria and mannitol promotes *Salmonella* growth and inhibits *Proteus* and *Providencia* growth¹³. Never the less, a better selective medium for *Salmonella* may be found in the future.

The value of T_E slightly affected the linearity between N_0 and C_{TE} , as shown in Fig. 3. The optimal T_E which gives the highest linearity between them was used to estimate C_{TE} in this study. The optimal T_E was 5 hr in Fig. 3 and 4 hr in Figs. 5 and 6. From these results, it was thought that the optimal T_E would be located at the second half of the slope in the C_T curves.

We found that the lower limit of this estimation method was 10^0 ($=1$) CFU/mL (data not shown). That is, even if the number of viable target cells in a sample is only one, if that cell can grow during the incubation, the estimation method would be successful. As regards

Fig. 7. Linear relationship between the intercept and the initial cell concentration

Closed circles are obtained from the data in Fig. 2. The line is the regression line for the data points.

the rapidity of the method, the incubation period was set at 6 hr in most of our experiments. But, when a target organism grows slowly in a medium, the incubation period can be prolonged to get the slope in the C_T curve.

The estimation algorithm used in this study is a kind of the end point method with a value at a certain period^{8), 9)}. Another estimation algorithm was also developed using the intercept of a regression line for the slope in the C_T curve. As shown in Fig. 2, the slopes between 2 and 6 hr at various initial concentrations were parallel to each other. The regression line for the slope portion intersects the vertical axis at the intercept. For example, point B in Fig. 2 is the intercept at the initial cell concentrations of $10^{5.1}$ CFU/mL. With the lower initial cell concentration, the intercept was higher. There was a highly linear relationship between the intercept and the initial cell concentration with a correlation coefficient of 0.990 (Fig. 7). The regression line was: Intercept $= -0.31 \times \log N_0 + 51$. Using this relationship, we can also estimate the initial cell concentration of a sample from its intercept.

We are now studying the applicability of the estimation method to real food samples, such as raw chicken, which is one of the major causative food materials of *Salmonella* food poisoning outbreaks. Raw chicken inoculated with the test strain at a low cell concentration was used as an example. Here, a 10% chicken homogenate with BPW was inoculated into BPW or EEM at the ratio of 1:9 for incubation, because the chicken itself might include substances that suppress the *Salmonella* growth and/or the PCR reaction. The results of this study will be presented soon. We will also validate our estimation method for other *Salmonella* strains.

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Nomenclature

C_T : threshold cycle

C_{TE} : C_T estimated at T_E

N_0 : initial cell concentration (CFU/mL)

T_E : time for estimation (hour)

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