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Determination and quantification of *Escherichia coli* by capillary electrophoresis

Zhenqing Li,*[a] De Li,[a] Dawei Zhang[a] and Yoshinori Yamaguchi*[bc]

Capillary electrophoresis (CE) is widely employed for the separation of nucleic acids or protein, but it is rarely applied in the quantification of *Escherichia coli* (*E. coli*). Here, we have analysed *E. coli* by CE with mercury lamp induced fluorescence, and demonstrated the relationship between its fluorescence intensity with the concentration of *E. coli* for the first time. The gradient concentration of *E. coli* was obtained by polymerase chain reaction (PCR) with different amplification cycles and dilution certain PCR products of *E. coli*, respectively. Results show that the peak area was linearly related to the logarithm of the concentration of *E. coli* and the logarithm of PCR replication numbers. The correlation coefficients R^2 are 0.957 and 0.966, respectively. The limit of detection (LOD) was found to be about 8.913×10^{-15} mol μl^{-1}. The reproducibility of capillary electrophoresis may make this technique possible for quantitative measurement of bacteria in bio-analytical science.

Introduction

Escherichia coli (*E. coli*), which forms a part of the intestinal microflora, is an important pathogen causing intestinal and systemic illness of humans and other animals.[1,2] Its presence in food or water indicates fecal contamination, and some research studies suggest that analysis of *E. coli* specifically may be a better indicator.[3–6] Therefore, quantification of *E. coli* will be of great value in daily life.

Traditional analysis of *E. coli* relied mostly on light and electron microscopy and cultural techniques.[7–9] However, few microorganisms have sufficiently distinctive morphology to be recognized by microscopy. Culture-dependent methods are restricted, because a microorganism can be cultivated only after its physiological niche is perceived and duplicated experimentally.[10] Furthermore, it is time-consuming as bacterial growth requires more than a day, so it is said that about 80% or more of microbes remain undiscovered.[11] Polymerase chain reaction (PCR) is a common method for the microbiological diagnosis. The gene coding the small subunit of 16S ribosomal RNA (16S rRNA) has been frequently used as a target of the PCR examination because of its structural characteristics.[12] The nucleotide sequences of some portions of the 16S rRNA are highly conserved through evolution, while other regions contain more variable sequences.[13,14] Thus real-time PCR was widely employed in the quantification of *E. coli* during recent years. For example, Mark Ibekwe's group has performed detection and quantification of *E. coli* O157:H7 in soil, manure, cow and calf feces, and dairy wastewater by real-time PCR.[15] John Penders and coworkers have monitored the prevalence and counts of *E. coli* in breast and formula-fed infants by real-time PCR assay.[16] Although real-time PCR shows probably the best performance in terms of sensitivity, specificity and rapidity, the major disadvantage of the real-time PCR assay is that it requires expensive equipment and reagents. Tamiya's group has developed a method for the detection of *E. coli* based on linear sweep voltammetry.[5] The setup they developed is very portable, but the sensitivity may be lower than that of the fluorescence-based detection method.

Capillary electrophoresis (CE) has many advantages, such as high resolution, fast speed, and excellent reproducibility.[17] PCR coupled with CE has been developed as a specific molecular technique for detecting the target genes. Most research studies so far about CE were mainly focused on the size determination of nucleic acids, but only a few research studies were about the quantitative measurement of *E. coli*. To enhance the sensitivity, a laser induced fluorescence detection method was also introduced into electrophoresis. For example, Timo Hardiman *et al.* performed the quantification of rRNA in *E. coli* using capillary gel electrophoresis coupled with laser induced fluorescence detection (CGE-LIF).[18] Fang's group carried out the quantification of

[a]*Engineering Research Center of Optical Instrument and System, Ministry of Education, Shanghai Key Lab of Modern Optical System, University of Shanghai for Science and Technology, No. 516 Jungong Road, Shanghai 200093, China. E-mail: zhenqingli@163.com*

[b]*Photonics and Bio-medical Research Institute, Department of Physics Faculty of Science, East China University of Science and Technology, No. 130 Meilong Road, Shanghai 200237, China. E-mail: yoshi.yamaguchi@ap.eng.osaka-u.ac.jp*

[c]*Department of Applied Physics, Graduate School of Engineering, Osaka University, Yamadaoka Suita-city, Osaka 565-0871, Japan*

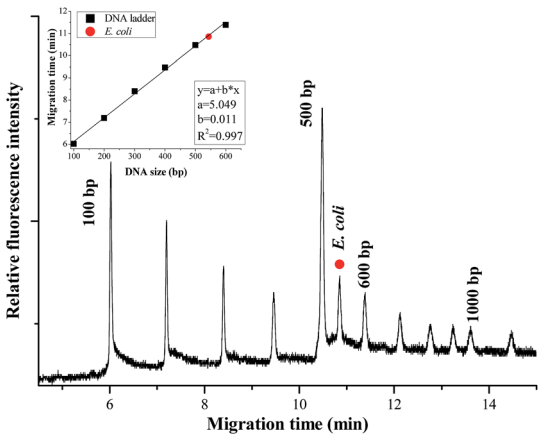

Fig. 1 Separation of the mixture of PCR products of *E. coli* and 100 bp DNA ladder markers by CE. Electrophoretic conditions, polymer: 0.5% HEC (1300k); sample loadings: 67 V cm^{-1} (1.0 s); total length and effective length of the capillary: 12 cm/8 cm; electric field strength: 80 V cm^{-1}.

E. coli in surface water with microchip electrophoresis (MCE-LIF).[19] Park's group has performed quantification of mRNA in recombinant *E. coli* using CE based on single-strand conformation polymorphism coupled with reverse transcription.[20] However, the apparatus for LIF is not only complicated, but also expensive. Based on the self-build CE system in our lab,[21,22] herein we report CE as a tool for quantification of *E. coli* by investigating the relationship between the fluorescence intensity and the concentration of *E. coli*.

Methods and materials

CE with mercury lamp induced fluorescence

Briefly, the CE system consisted of a high-voltage power supply (MODEL 610E, TREK, Medina, NY, USA) and a microscope with epi-illumination (IX71, Olympus, Tokyo, Japan). The excitation wavelength from a mercury lamp was filtered to be 460–495 nm using an optical filter (U-MWIB-3, Olympus, Tokyo, Japan), which was the wavelength of the excitation maximum of the conjugate of SYBR Green I and the nucleic acid. Fluorescence was collected with a 60× objective (PlanApo/IR, Olympus). A certain length fused-silica capillary (id/od = 75 μm/365 μm) was covalently coated with polyacrylamide.[23,24] A transparent window in the capillary with a length of 2.0 mm was made by a lighter for fluorescence detection. The fluorescence signal was detected using a photomultiplier (R928, Hamamatsu Photonics, Hamamatsu, Japan). All experiments were performed at 26 °C in a clean room controlled using an air-conditioner.

PCR protocols

A primer pair for selective amplification of a 16S rRNA gene region (544 bp) in *E. coli* (Takara, Shiga, Japan) (100 ng μl^{-1}) ECA75F (forward, targeting bases 75 to 99, 5′-GGAA-GAAGCTTGCTTC TTTGCTGAC-3′) and ECA619R (reverse, targeting bases 594 to 619; 5′-AGCCCGGGGATTTCACATCTGACTTA-3′) were used.[5] The reaction was performed with a 1.0 μl sample (2 ng μl^{-1}) and 49 μl reaction volumes containing a 5.0 μl 10× Fast Buffer I and 4.0 μl dNTP mixture (2.5 μM), 200 nM primers (FASMAC Co., Ltd., Kanagawa, Japan), and 0.25 μl SpeedSTAR HS DNA Polymerase (Takara, Shiga, Japan). The thermo-cycling program was cycles of 95 °C for 10 s (denaturation) and 64 °C for 30 s (annealing and extension) with an initial cycle of 95 °C for 2 min. The thermo-cycling was performed with 15×, 20×, 25×, 30×, 35×, and 40× on a T100 thermal cycler (Bio-Rad, USA), respectively.

Results and discussion

Evaluation of PCR primer selectivity and specificity

For quantitative analysis, we carried out CE of 100 bp DNA (Takara, Shiga, Japan) ladder and PCR products of an *E. coli* mixture to check out the size of PCR amplification products. The DNA ladder markers were sized from 100 to 1500 bp. PCR amplification products of *E. coli* were diluted to 5% of their original concentration before application to CE, and then they were introduced into 0.5% hydroxyethylcellulose (HEC) polymer for separation. HEC polymer solution containing 1× SYBR Green I (Invitrogen, Carlsbad, CA, USA) was prepared by dissolving in the 0.5× Tris-broate-EDTA buffer (Bio-Rad, Hercules, CA, USA). CE was carried out at 80 V cm^{-1} and the results are demonstrated in Fig. 1. It shows that they were baseline resolved within 15 min. Theoretically, the migration time of nucleic acid was linearly related to its size in CE for the short DNA fragment,[25] and thus the PCR product size was determined by the calibration plot of DNA ladder size *versus* its migration time, which is shown in the inset of Fig. 1. The linear regression fit for migration time and DNA size (100–600 bp) was achieved with correlation coefficient $R^2 = 0.997$. Thus PCR products of *E. coli* were determined and are marked with red solid circles in Fig. 1. We also performed CE of PCR negative control

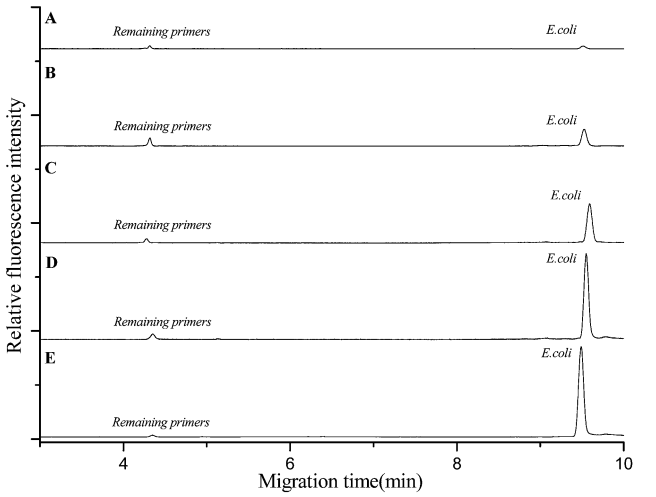

Fig. 2 Separation of PCR products of *E. coli* with different amplification cycles: (A) 15×, (B) 20×, (C) 25×, (D) 30× and (E) 35×. CE was carried out at 100 V cm^{-1}. Other electrophoretic conditions were the same as those in Fig. 1.

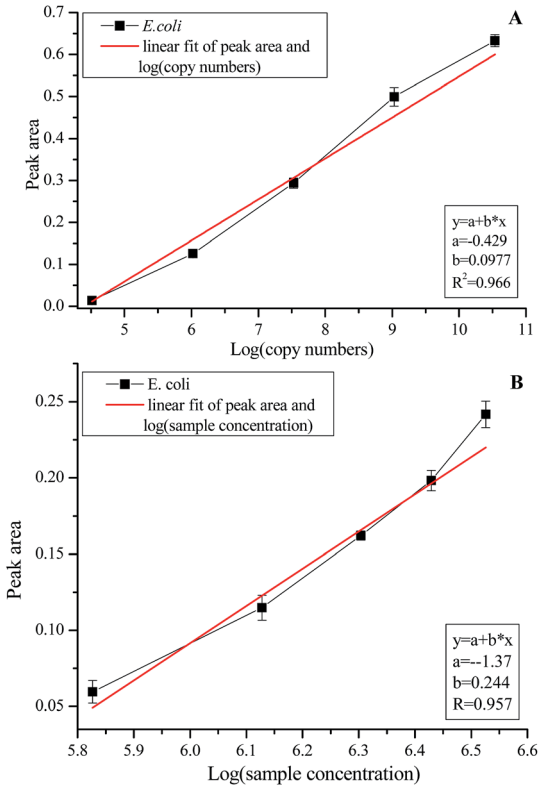

Fig. 3 Peak area *versus* (A) log(replication numbers) in PCR and (B) gradient concentration of *E. coli* in CE.

(containing no DNA template) (data not shown), and only peaks of the primers existed in the electropherogram.

Relationship between fluorescence intensity and PCR amplification cycles

In PCR, the final concentration of PCR products was determined by the amplification cycles. The fluorescence intensity of the PCR products was related to the peak area in the electropherogram. We have also carried out CE of *E. coli* with different PCR amplification cycles in 0.5% HEC (1300k) at 100 V cm^{-1}. In CE, the electrophoretic conditions (*e.g.* sample loadings, total and effective capillary length, and separation voltage) were the same. Each sample was carried out 5 times in CE. Because when *E. coli* was amplified more than 40 cycles, the fluorescence intensity of PCR products in CE was beyond the maximum detection limit. Therefore, we demonstrate the CE results of *E. coli* with amplification cycles of 15×, 20×, 25×, 30×, and 35× in Fig. 2. It shows that the migration time of *E. coli* was 9.53 min with a variation of 3.8%. Furthermore, Fig. 2 shows that the peak of *E. coli* increased with the increase of PCR amplification cycles, whereas the peak of the remaining primers decreased, which was in accordance with the fact that the amount of the PCR product increases with the decrease of the primer during the amplification process.

Relationship between fluorescence intensity and concentration of *E. coli* for certain PCR amplification cycles

Then we evaluated the sample fluorescence intensity by calculating the peak area of *E. coli* in Fig. 2. The regression results are shown in Fig. 3A. It shows that the correlation coefficient R^2 was 0.966. Furthermore, the fluorescence intensity (F) and PCR amplification cycles (N) could be expressed as the following equation: $F = a + b \log(2^N)$, where a and b are constants, respectively. This was possibly because the concentration of PCR products exponentially increased with the initial concentration of sample when the PCR efficiency was high. A detailed description of data in Fig. 3A is given in Table .1. The imprecision was expressed as the coefficient of variation (CV%).

Moreover, we obtained the gradient concentration of *E. coli* by diluting its PCR products (25×), and then performed CE in 0.2% HEC (1300k) at 150 V cm^{-1} (total capillary length: 11 cm, effective capillary length: 6 cm). The fluorescence intensity was calculated by the same method mentioned above, and then we estimated the relationship between the fluorescence intensity and the logarithm of the concentration of *E. coli*. The regression results are demonstrated in Fig. 3B. It shows that the correlation coefficient R^2 was 0.957. The relationship between the fluorescence intensity and concentration (C) of *E. coli* can be expressed as the following equations: $F = a + b \log(C)$, where a and b are constants, respectively. A detailed description of the data in Fig. 3B is summarized in Table 2.

Limit of detection

In order to determine the limit of detection (LOD, signal/noise = 3) of CE with mercury lamp fluorescence, we have further performed the PCR of *E. coli* with different amplification cycles from 3× to 10×, and then analysed the PCR products with concentrations from 10% to 100%. Results show that the lowest fluorescence signal was from 5× PCR amplification of *E. coli* (Fig. 4A) and its dilution PCR products by 10%, and thus the LOD was deduced to be 3.2 ng μl^{-1}, which is about 8.913 × 10^{-15} mol μl^{-1}.

Table 1 Peak area of *E. coli* in CE with different replication numbers

Log (replication number)	Number of repetitions	Peak area (mean ± S.D.)	CV (%)
4.515	5	0.014 ± 0.001	7.1
6.021	5	0.126 ± 0.006	4.8
7.526	5	0.294 ± 0.012	4.1
9.031	5	0.500 ± 0.022	4.4
10.536	5	0.633 ± 0.014	2.2

Table 2 Peak area of *E. coli* with gradient concentration in CE

Log (*E. coli* concentration)	Number of repetitions	Peak area (mean ± S.D.)	CV (%)
5.827	5	0.060 ± 0.007	11.7
6.128	5	0.115 ± 0.008	7.0
6.304	5	0.162 ± 0.003	1.9
6.429	5	0.198 ± 0.007	3.5
6.526	5	0.242 ± 0.009	3.7

Detection of *E. coli* from real samples

In the end, we have extracted plasmid DNA from the *E. coli* strain DH5α, and then carried out 40× PCR amplification with the primers of ECA75F and ECA619R. The PCR products were diluted 20 times for CE (Fig. 4B). Thus the DNA size of the PCR product was found to be 544 bp, and the concentration of the DNA in *E. coli* was calculated to be about 200 ng μl^{-1} by the method proposed in this work.

Conclusions

In summary, we have investigated the relationship between the fluorescence intensity in the electropherogram and the concentration of *E. coli*. The gradient concentration of the sample was obtained by two ways: (1) PCR amplification of *E. coli* with different amplification cycles; (2) diluting PCR products of *E. coli* with certain amplification cycles. Results show that the fluorescence intensity, which was related to the peak area in the electropherogram, was linearly related to the logarithm of DNA replication numbers ($R^2 = 0.966$) and the logarithm of the concentration of *E. coli* ($R^2 = 0.957$). Furthermore, the LOD was found to be about 8.913×10^{-15} mol μl^{-1}.

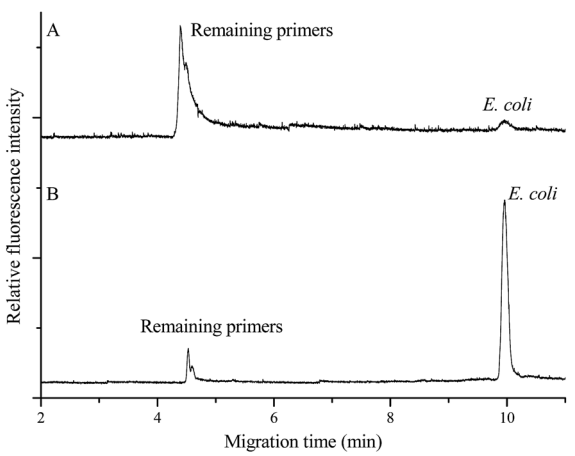

Fig. 4 The electropherogram of (A) limit of detection and (B) PCR products of *E. coli* from real samples by CE. Electrophoretic conditions: polymer: 0.5% HEC (1300k); sample loadings: 100 V cm^{-1} (1.0 s); total length and effective length of the capillary: 14 cm/8 cm; electric field strength: 100 V cm^{-1}.

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