Original Article

Comparison of Two Methods of Bacterial DNA Extraction from Human Fecal Samples Contaminated with *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Campylobacter jejuni*

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(Received October 29, 2013. Accepted March 10, 2014)

SUMMARY: In this study, 2 methods of DNA extraction were evaluated for use in conjunction with the screening system Rapid Foodborne Bacterial Screening 24 (RFBS24), which employs multiplex real-time SYBR Green polymerase chain reaction (SG-PCR) and can simultaneously detect 24 target genes of foodborne pathogens in fecal DNA samples. The QIAamp DNA Stool mini kit (Qkit) and Ultra Clean Fecal DNA Isolation Kit (Ukit) were used for bacterial DNA extraction from fecal samples artificially inoculated with *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Campylobacter jejuni*. SG-PCR and simplex real-time quantitative PCR (S-qPCR) analyses revealed higher copy numbers (8–234 times) of DNA in samples obtained using Ukit compared with those obtained using Qkit, resulting in lower cycle threshold values for the Ukit samples of the 4 bacteria on SG-PCR analysis. Fecal DNA samples from patients infected during foodborne outbreaks of *Salmonella* and *Campylobacter* were also prepared by Qkit and Ukit methods and subjected to RFBS24 analyses. Higher numbers of RFBS24 bacterial target genes were detected in DNA samples obtained using Ukit compared with those obtained using Qkit. Thus, the higher DNA extraction efficiency of the Ukit method compared with Qkit renders the former more useful in achieving improved detection rates of these 4 bacteria in fecal samples using SG-PCR.

INTRODUCTION

Foodborne illnesses caused by bacteria occur worldwide and affect countless individuals in every nation each year. The traditional culture methods employed for investigating the causative bacterial pathogens involve the use of multiple reagents and complicated, time-consuming procedures. Fukushima et al. (1) described an expedited screening system that simultaneously analyzes 24 bacterial target genes using multiplex real-time SYBR Green PCR (SG-PCR). This system can be used for efficient culture of bacteria and for the rapid identification of foodborne pathogens, leading to a quicker dissemination of relevant information to public health administrators. We have named this screening system as Rapid Foodborne Bacterial Screening 24 (RFBS24).

RFBS24 includes 8 sets of multiplex real-time SG-PCR assays, with each set comprising 3 primer pairs for

target genes and an additional 1 for the detection of the internal amplification control mixed with each set. Although this process enables the rapid identification of bacterial pathogens from fecal DNA, the entire process is dependent on the quality and quantity of DNA isolated from the contaminated stool sample of patients with foodborne illnesses. The QIAamp DNA Stool Mini kit (Qkit; Qiagen, Hilden, Germany) was used for the extraction of fecal DNA from patients for use in RFBS24 analyses, because of its ability to remove PCR inhibitors such as bile acid and complex polysaccharides that are commonly found in feces (2–4). Qkit uses a surfactant for the lysis of bacterial cell walls; however, gram-positive bacteria have thick cell walls containing peptidoglycans, which cannot be easily disrupted. RFBS24 is designed for the detection of both gram-negative and gram-positive bacterial pathogens; therefore effective DNA extraction from both types of pathogens is crucial. However, an optimal DNA extraction method for use in conjunction with RFBS24 analyses is yet to be evaluated. In this study, we compared 2 methods of DNA extraction from human fecal samples artificially contaminated with *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Campylobacter jejuni*. Furthermore, fecal DNA sam-

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ples from patients infected during foodborne outbreaks of *Salmonella* and *Campylobacter* were examined using RFBS24.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains used in this study include *C. perfringens* (CW-H2 and ATCC 12915), *S. aureus* (FB6001 and ATCC 25923), *S. Typhimurium* (Sal2339), and *C. jejuni* (FB10002 and ATCC 33560). The strain CW-H2 was obtained from the Tokyo Metropolitan Institute of Public Health, and strains ATCC 12915, ATCC 25923, and ATCC 33560 from the American Type Culture Collection (ATCC). FB6001, Sal2339, and FB10002 were isolated from patients with gastroenteritis in Shimane Prefecture, Japan.

DNA extraction from fecal samples inoculated with bacterial pathogens: Fecal samples (1 g) free of pathogenic bacteria were weighed under aseptic conditions, placed into sterile tubes, and homogenized with 9 ml of phosphate-buffered saline (PBS). Cultures of 4 bacterial strains (CW-H2, FB6001, Sal2339, and FB10002) were individually suspended in PBS with the turbidity adjusted to McFarland standard No. 2 and 5 µl of each bacterial suspension was inoculated into 195 µl of fecal samples. Viable counts of these bacteria in the fecal samples were 4.3×10^6 colony-forming units (CFU)/ml (*C. perfringens*), 2.5×10^7 CFU/ml (*S. aureus*), 2.5×10^7 CFU/ml (*S. Typhimurium*), and 3.5×10^6 CFU/ml (*C. jejuni*). DNA was extracted from contaminated fecal samples (200 µl) using either Qkit or Ultra Clean Fecal DNA Isolation Kit (Ukit; MoBio Laboratories, Inc., Carlsbad, CA, USA). For DNA extraction with Qkit, bacteria were lysed under 2 conditions, 70°C and 95°C, and DNA was extraction in triplicate. Ukit, on the other hand, uses a bead-beating method, and bacterial cells in the fecal sample were disrupted using a vortex or Fastprep24 instrument (MP Biomedicals, Santa Ana, CA, USA). Cell disruption by vortexing was performed at maximum speed for 10 min according to the manufacturer's instructions and using the Fastprep24 instrument under 15 conditions of disruption (4.0 m/s for 20, 30, 40, 50, and 60 s; 5.0 m/s for 20, 30, 40, 50, and 60 s; and 6.5 m/s for 20, 30, 40, 50, and 60 s); treatments were performed in triplicate for each condition.

Furthermore, in the Qkit procedure, a modified protocol of DNA extraction involving an additional cell disruption step (Fastprep24 instrument at 4.0 m/s for 60 s following lysis at 70°C) was also included. DNA extrac-

tion for each sample was performed in triplicate.

Comparison of DNA extraction methods using real-time SG-PCR: For the identification of the optimal DNA extraction procedure for RFBS24 analyses, DNA samples extracted by the methods described above were analyzed by SG-PCR for the detection of *C. perfringens* (*cpe*), *S. aureus* (*FemB*), *Salmonella* spp. (*invA*), and *C. jejuni* (species-specific sequence), and mean cycle threshold (Ct) values were compared. Each 20 µl reaction well contained 10 µl of SYBR Premix DimerEraser (TaKaRa, Shiga, Japan), 6.8 µl of PCR-grade H₂O, 0.6 µl of each 10 µM target gene primer, and 2 µl of DNA sample. The cycling conditions in Thermal Cycler Dice Real-Time System (TaKaRa) included initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min; this was followed by a single cycle of dissociation at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. The primer sequences used in this study were those that have been previously used (1).

Preparation of standards for real-time quantitative PCR (qPCR) analysis: Standards for qPCR were chosen according to the method reported by Fey et al. (5). Four primer pairs (Table 1) were designed with the Primer 3 program using sequences submitted to GenBank (6); these primer pairs were located upstream and downstream of the sequences recognized by the qPCR primers and probes. DNA samples were extracted using Qkit from suspensions of bacterial strains (ATCC 12915, ATCC 25923, Sal2339, and ATCC 33560) prepared as described above. The 4 primer pairs were used for the amplification of target bacterial sequences using 20 µl PCR reaction mixtures containing 10 × Ex Taq buffer (TaKaRa), 10 µM of the primers, and 2 µl of genomic DNA, according to the manufacturer's protocol. Amplification conditions included initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min, followed by final extension at 72°C for 10 min. The 4 PCR products obtained were subjected to electrophoresis using 1.0% NuSieve GTG Agarose gel (TaKaRa) prepared in 1 × modified Tris-acetate-ethylenediaminetetraacetic acid buffer containing ethidium bromide (0.5 µg/ml). The band of interest in the agarose gel was visualized using a long-wavelength ultraviolet lamp or an LED trans-illuminator and excised with a razor blade. DNA was extracted from the slice of agarose with the Montage Gel Extraction kit (Millipore, Bedford, MA, USA) or the QIAquick PCR purification kit (Qiagen). The concentration of purified DNA samples was determined through

Table 1. Polymerase chain reaction (PCR) primers for preparing standards

Species	Target gene	Primer name	PCR primer sequences (5' to 3')	Product size	GenBank accession no.
<i>Clostridium perfringens</i>	<i>cpe</i>	cpe413F	CAACTGCTGGTCCAAATGAA	355	X81849
		cpe767R	AGCTTTTGTAGTCCAAGGGTATG		
<i>Staphylococcus aureus</i>	<i>FemB</i>	femB-228F	CAGGAACATGACGCATTTGT	581	AF106850
		femB-808R	CGTTCCCCAGTGATTTTCAT		
<i>Salmonella</i> spp.	<i>invA</i>	invA-349F	ATCTTGATTGAAGCCGATGC	506	M90846
		invA-854R	GTCACGATAAAACCGGCACT		
<i>Campylobacter jejuni</i>	Specific sequence	AB-381012F	ACTATAGGTGGCGGTGCTGT	403	AL111168
		AB-381414R	CCTGATCTCCACCTTAAGCA		

measurements of optical density at 260 nm using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNA samples were stored at -20°C until further use as standards for qPCR.

Comparison of DNA extraction methods using simplex qPCR (S-qPCR): The copy numbers of target genes in *C. perfringens* (*cpe*), *S. aureus* (*FemB*), *Salmonella* spp. (*invA*), and *C. jejuni* (species-specific sequence) was quantified by S-qPCR, as reported previously (7–10). S-qPCR analyses were performed on DNA samples obtained using Qkit (employing 3 conditions for cell lysis: lysis at 70°C or 95°C , and disruption of cells using the Fastprep24 instrument at 4.0 m/s for 60 s subsequent to lysis at 70°C) and Ukit (cell disruption using Fastprep24 instrument at 4.0 m/s for 60 s), as described above. The reaction conditions included initial denaturation at 95°C for 30 s, followed by 40 (for *C. jejuni*) or 45 (for the other bacteria) cycles of 95°C for 5 s and 60°C for 30 s, using Thermal Cycler Dice Real-Time System TP800 (TaKaRa). Each reaction well contained 12.5 μl of Premix Ex Taq (TaKaRa), 5.37 μl of PCR-grade H_2O , 2.25 μl of each 10 μM target gene primer, 0.63 μl of 10 μM probe, and 2 μl of DNA sample in a 25 μl PCR mixture. Moreover, 10-fold serially diluted standard samples (1×10^8 to 1×10^2 copies) were quantified in each S-qPCR cycle.

Comparison of concentrations of DNA samples obtained using Qkit and Ukit: In order to compare concentrations of DNA samples obtained by the 2 procedures, optimal conditions of extraction using Qkit (sample lysis at 70°C) and Ukit (sample disruption using the Fastprep24 instrument at 4.0 m/s for 60 s), as determined by SG-PCR and S-qPCR analyses, were used for DNA extraction from the contaminated fecal samples, as described above. Samples were measured in triplicate using UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan).

Comparison of DNA extraction methods for RFBS24 analyses using patient fecal samples: Bacterial culture method resulted in the isolation of *Salmonella* spp. in 12 of 20 fecal samples from 4 foodborne outbreaks of *Salmonella*, and of *C. jejuni* in 9, *C. coli* in 5, and both *C. jejuni* and *C. coli* in 1 of 23 fecal samples from 7 outbreaks of *Campylobacter*. During these outbreaks, fecal samples (1 g) of patients and cooking or serving staffs at the causative restaurants were diluted 10-fold with PBS, and 200 μl of the fecal suspension was used for DNA extraction using Qkit (cell lysis at 70°C) and Ukit (cell disruption using the Fastprep24 instrument at 4.0 m/s for 60 s) methods. Eight multiplex real-time SG-PCR (RFBS24) assays using Thermal Cycler Dice Real-Time System TP800 (TaKaRa) were performed with these DNA samples (1); the results were interpreted as previously described and indicated as positive or negative.

Ethical considerations: This study was approved by the Ethics Committee of the Shimane Prefectural Institute of Public Health and Environmental Science and follows the ethical guidelines for Epidemiology Research established by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

RESULTS

Comparison of DNA extraction by the Qkit and Ukit methods using SG-PCR: The mean Ct values for target genes from *C. perfringens* (*cpe*), *S. aureus* (*FemB*), *S. Typhimurium* (*invA*), and *C. jejuni* (species-specific sequence) are listed in Figure 1. For *C. perfringens*, mean Ct values obtained by the Ukit method (vortex: 28.17; Fastprep24: 26.23–30.12) were lower than those obtained by the Qkit method (lysis at 70°C : 34.44; 95°C : 35.17); lowest Ct values (26.23–26.98) were obtained by the Ukit method and FastPrep24 conditions of 4.0 m/s for 30 s or 60 s and 6.5 m/s for 60 s.

Similarly, for *S. aureus*, the Ukit method (vortex: 32.04; FastPrep24: 28.55–33.34) yielded lower mean Ct values compared with the Qkit method (lysis at 70°C : 35.67; 95°C : 35.49), and the lowest Ct value (28.55) was obtained by the Ukit method and Fastprep24 condition of 4.0 m/s for 60 s. Thus, for both *C. perfringens* and *S. aureus*, decreasing mean Ct values were obtained with increasing disruption times using the Fastprep24 instrument and Ukit.

Similar trends were also observed with *S. Typhimurium*; the mean Ct values obtained using the Ukit method (vortex: 23.78; Fastprep24: 23.35–26.07) were lower than those obtained using the Qkit method (lysis at 70°C : 28.96; 95°C : 29.66). The lowest Ct values (23.35–23.95) were observed for the Ukit samples prepared using the Fastprep24 conditions of 4.0 m/s for 20–40 s and 60 s and under conditions of vortexing. Similarly, for *C. jejuni*, the Ukit method (vortex: 26.00; Fastprep24: 27.24–30.22) yielded lower mean Ct values compared with the Qkit method (lysis at 70°C : 29.90; 95°C : 31.01). Differences in Ct values between samples obtained by Qkit and Ukit methods were smaller for *C. jejuni* compared with the other bacteria, with the lowest Ct value (26.00) observed for the Ukit samples prepared under conditions of vortexing, and next lowest values (27.24–27.67), under Fastprep24 conditions of 4.0 m/s for 20–60 s.

Comparison of DNA extraction by the Qkit and Ukit methods using S-qPCR: The copy numbers of 4 target genes in *C. perfringens*, *S. aureus*, *S. Typhimurium*, and *C. jejuni* in DNA samples obtained using Qkit and Ukit are shown in Figure 2. For all 4 bacteria, mean copy numbers of the target genes were higher in DNA samples obtained using Ukit compared with those obtained using Qkit method: 234-, 36-, 79-, and 8-times higher for *C. perfringens*, *S. aureus*, *S. Typhimurium*, and *C. jejuni*, respectively. The inclusion of an additional disruption step in the Qkit DNA extraction technique following the lysis step resulted in a slight increase in mean copy numbers of target genes: 6.5-, 1.7-, 2.9-, and 2.4-times higher for *C. perfringens*, *S. aureus*, *S. Typhimurium*, and *C. jejuni*, respectively. The standard curve for all S-qPCR analyses showed good linearity across the range of measurements, with slopes of -2.978 (*C. perfringens*), -3.233 (*S. aureus*), -3.162 (*S. Typhimurium*), and -3.215 (*C. jejuni*), and r^2 values of >0.99 , revealing PCR efficiencies of 1.03–1.16 as calculated using the formula $E = 10^{-1/\text{slope}} - 1$.

Comparison of DNA concentrations of samples obtained by Qkit and Ukit methods: The mean DNA concentration obtained by Qkit (lysis at 70°C) and Ukit

Fig. 1. Mean cycle threshold values (Ct) obtained from real-time SYBR Green polymerase chain reaction (SG-PCR) using DNA samples prepared with the Qkit method (lysis step with surfactant: 70°C and 95°C) and the Ukit method (disrupting fecal samples using vortexing and Fastprep24) from fecal samples inoculated with *C. perfringens* (A), *S. aureus* (B), *S. Typhimurium* (C), and *C. jejuni* (D).

(disruption using Fastprep24 at 4.0 m/s for 60 s) methods are shown in Table 2. For all 4 bacteria, higher values of mean DNA concentration were obtained by the Ukit method compared with those obtained by the Qkit method: 6.9-, 8.3-, 13.6-, and 4.2-times higher for *C. perfringens*, *S. aureus*, *S. Typhimurium*, and *C. jejuni*, respectively.

Comparison of DNA extraction methods in RFBS24 analyses of foodborne outbreaks of *Salmonella* and *Campylobacter*: RFBS24 analyses were performed using DNA extracted from the fecal samples of patients and cooking or serving staffs of causative restaurants during foodborne outbreaks of *Salmonella* and *Campylobacter*. Of the 20 samples from the *Salmonella* outbreak, RFBS24 detected *Salmonella* (*invA*) in 2 and 6 DNA samples obtained by Qkit and Ukit methods, respectively. Thus, 3-times higher rates of positivity were obtained in RFBS24 analyses using DNA extracted using Ukit compared with those using Qkit.

Moreover, of the 23 fecal samples from the *Campylobacter* outbreak, RFBS24 analyses detected *Campylobacter* in 12 (*C. jejuni*: 9; *C. coli*: 3) DNA samples

prepared using Qkit and in 15 (*C. jejuni*: 10; *C. coli*: 4; *C. jejuni* and *C. coli*: 1) DNA samples prepared using Ukit. Thus, the Ukit method resulted in increased rates of detection in RFBS24 analyses.

DISCUSSION

Most methods of bacterial cell wall disruption include cell lysis using enzymes or surfactants and/or bead beating, which disrupts the cell wall through mechanical shearing between the beads and sample by shaking at high speeds (11,12). For RFBS24, fecal DNA samples are typically prepared using Qkit, where the bacterial cell walls is lysed at high temperatures using surfactants and has been used in numerous investigations (13–16). For DNA extraction from gram-positive bacteria using Qkit, because of their thick cell wall which consisting chiefly of peptidoglycan layers (17), high temperatures (95°C) are used at the lysis step as per the manufacturer's instructions; however, the effect of this change has not been sufficiently investigated. Therefore, to optimize the DNA extraction method for use in concert

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Fig. 2. Mean DNA copy numbers obtained from simplex real-time quantitative PCR (S-qPCR) analysis of DNA samples prepared with the Qkit method (lysis step with surfactant: 70°C and 95°C, disrupting fecal samples using Fastprep24 after the lysis step) and the Ukit method (disrupting fecal samples using Fastprep24) from fecal samples inoculated with *C. perfringens* (A), *S. aureus* (B), *S. Typhimurium* (C), and *C. jejuni* (D).

Table 2. DNA concentrations obtained with the Qkit and the Ukit methods

	DNA concentration (ng/PCR reaction well) ¹⁾			
	<i>C. perfringens</i>	<i>S. aureus</i>	<i>S. Typhimurium</i>	<i>C. jejuni</i>
Qkit (lysis step at 70°C)	7.45 ± 2.31	3.18 ± 1.89	3.97 ± 2.18	8.16 ± 2.82
Ukit (Fastprep 24 at 4.0 m/s for 60 s)	51.34 ± 5.08	26.41 ± 5.35	53.86 ± 9.51	34.17 ± 6.31

¹⁾; Results represent the mean ± standard deviation calculated from triplicate assessment.
Qkit, QIAamp DNA Stool mini kit; Ukit, Ultra Clean Fecal DNA Isolation Kit.

with RFBS24 analysis, the Qkit method was compared with Ukit method, which employs bead beating for bacterial cell wall disruption. DNA extraction using enzymes was not investigated in this study as it requires more than an hour for completion, which would eliminate the capacity of the RFBS24 analysis for evaluating 24 target genes of foodborne pathogens in 3 h or less.

The employment of higher lysis temperatures in the Qkit method in conjunction with SG-PCR was anticipated to result in lower Ct values. However, mean Ct values when lysis was performed at 70°C were found to be slightly lower than those at 95°C, and increase in the lysis temperature failed to increase the efficiency of DNA extraction from the 4 test bacteria used.

Bacterial cell wall disruption in the Ukit method could be accomplished via vortexing at the maximum speed for 10 min or using the Fastprep24 instrument. SG-PCR analyses revealed lower mean Ct values by the Ukit method compared with the Qkit method for the 4 test bacteria used, suggesting higher efficiencies of DNA extraction by the Ukit method. Cell disruption using the

Fastprep24 instrument has been considered to be equally good or better compared with vortexing, and mean Ct values for DNA samples from *C. perfringens* and *S. aureus* obtained using Fastprep24 were in fact lower than those obtained by vortexing. However, in the case of *C. jejuni*, the mean Ct value of samples obtained by vortexing was slightly lower than that using Fastprep24 at 4.0 m/s for 60 s, whereas both cell disruption techniques roughly yielded same results in the case of *S. Typhimurium*. Furthermore, the Ct values for *C. perfringens* and *S. aureus* decreased with increasing disruption time using Fastprep24. The values of mean and standard deviation of Ct for the 4 bacteria were low under the Fastprep24 cell disruption condition of 4.0 m/s for 60 s.

Taken together, these results suggest that the use of the Fastprep24 instrument at 4.0 m/s for 60 s represents the most appropriate cell disruption technique for DNA isolation from multiple types of bacteria, although slightly lower Ct values for *C. jejuni* were obtained by the Ukit method and vortexing for cell disruption.

Similar observations were made using S-qPCR, which revealed that the mean copy numbers of DNA obtained for the 4 test bacteria by the Ukit method were 8–234 times higher than those obtained by the Qkit method.

The DNA concentrations of DNA in samples obtained using Ukit were 4.2–13.6 times higher compared with those obtained using Qkit. This is likely one of the primary factors resulting in lower Ct values for samples obtained using Ukit in both SG-PCR and S-qPCR analyses. The difference in DNA extraction efficiency between the Qkit and Ukit methods for *C. jejuni* was small compared with that for the other 3 bacteria, perhaps because of the inefficient lysis of the other 3 bacteria by the surfactant. Similarly, Ariefdjohan et al. (18) reported increased concentrations of extracted fecal DNA using the Fastprep24 bead-beating method compared with the Qkit method, and Klerks et al. (19) that the Ukit method was optimal for DNA extraction from compost. Although the results of the present study agree with these reports, the mean copy numbers of DNA obtained from the 4 test bacteria using Qkit were slightly higher (1.7–6.5 times) with the addition of a disruption step following the lysis step. The reason for this increase was not investigated, but the DNA extraction efficiency of the Qkit method can improve through further cell wall disruption using Fastprep24, beads, or altered reagents.

The practical outcomes of using the 2 DNA extraction kits for RFBS24 analyses in foodborne outbreaks of *Salmonella* and *Campylobacter* were investigated, which revealed a greater number of samples positive for bacterial contamination by the Ukit method compared with the Qkit method in both instances. These results suggest that the Ukit method is better for DNA extraction from patient fecal samples compared with the Qkit method, which is in line with the results obtained using artificially inoculated fecal samples. In addition, the condition (soft, diarrheal, or watery stool) of the patient fecal samples collected during outbreaks of foodborne illnesses often varies, and the amount of fecal sample is often small, particularly in the case of watery stools. A highly efficient DNA extraction method would be useful for the evaluation of various stool conditions, including watery stools.

In conclusion, the DNA extraction efficiency of the Ukit method using bead beating for cell disruption was found to be higher than that of the Qkit method for the 4 test bacteria *C. perfringens*, *S. aureus*, *S. Typhimurium*, and *C. jejuni*. Thus, the Ukit method is the optimal DNA extraction technique for use in conjunction with SG-PCR, S-qPCR, and RFBS24 analyses for the detection of these 4 bacterial species in fecal samples.

Acknowledgments This work was supported in part by a Grant-in-Aid for Research on Health Security Control from the Ministry of Health, Labour and Welfare, Japan (H22-Kiki-Ippan-004).

Conflict of interest None to declare.

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