Original Article

An Improved Multiplex Real-Time SYBR Green PCR Assay for Analysis of 24 Target Genes from 16 Bacterial Species in Fecal DNA Samples from Patients with Foodborne Illnesses

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SUMMARY: Here, we developed a new version of our original screening system (Rapid Foodborne Bacterial Screening 24; RFBS24), which can simultaneously detect 24 genes of foodborne pathogens in fecal DNA samples. This new version (RFBS24 ver. 5) detected all known stx2 subtypes, enterotoxigenic Escherichia coli (STh genotype), and Vibrio parahaemolyticus (thr2), which were not detected by the original RFBS24 assay. The detection limits of RFBS24 ver. 5 were approximately 5.6 × 10⁻⁵ (ng DNA)/reaction, significantly lower (10- to 100-fold) than those of the original RFBS24 for the 22 target genes analyzed here. We also tested the new assay on fecal DNA samples from patients infected with Salmonella, Campylobacter, or enterohemorrhagic E. coli. The number of bacterial target genes detected by RFBS24 ver. 5 was greater than that detected by RFBS24. RFBS24 ver. 5 combined with an Ultra Clean Fecal DNA Isolation Kit showed adequate performance (sensitivity and specificity 89% and 100%, respectively, for Salmonella spp. and 100% and 83%, respectively, for Campylobacter jejuni) in terms of rapid detection of a causative pathogen during foodborne-illness outbreaks. Thus, RFBS24 ver. 5 is more useful than the previous assay system for detection of foodborne pathogens and offers quick simultaneous analysis of many targets and thus facilitates rapid dissemination of information to public health officials.

INTRODUCTION

Estimates from the Ministry of Health, Labour, and Welfare of Japan suggest that there are approximately 1,000 foodborne-illness outbreaks in Japan each year, leading to foodborne illnesses in over 20,000 patients annually (1). Public health officials must investigate the causes of such outbreaks and immediately prevent the spread of foodborne illnesses. On the other hand, traditional culture-based methods that are used for identification of causative bacterial pathogens require multiple reagents and involve complicated time-consuming procedures. Therefore, assays that allow for rapid detection of multiple bacterial species are needed to prevent the spread of foodborne illnesses and to facilitate efficient cultivation of bacteria. Unfortunately, such assays are not known to detect a sufficient number of bacterial species (2–5).

Fukushima et al. (6) described an expedited screening system that can simultaneously analyze 24 bacterial target genes using multiplex real-time SYBR Green polymerase chain reaction (SG-PCR). This screening system, named Rapid Foodborne Bacterial Screening 24 (RFBS24), includes 8 sets of multiplex real-time SG-PCR assays, with each set comprising 4 primer pairs for 3 target genes and an internal amplification control (IAC). RFBS24 can identify foodborne pathogens more quickly than traditional methods can, thus leading to quicker dissemination of relevant information to public health officials. Nonetheless, Fukushima et al. (6) reported that the detection limit of RFBS24 for Salmonella spp. is worse than that for other pathogens and that identification of some bacterial pathogens is difficult due to small differences in melting temperatures (Tm) of PCR products between some target genes. Moreover, RFBS24 does not detect genotype STh of enterotoxigenic Escherichia coli (ETEC) (6). These drawbacks may be attributed to poor design of primers for the detection of Salmonella spp., interactions among primers, and/or suboptimal PCR conditions; however, none of these possible causes have been investigated sufficiently. Furthermore, the stx2 gene of enterohemorrhagic E. coli (EHEC) has recently been classified into 7 subtypes (stx2a, stx2b, stx2c, stx2d, stx2e, stx2f, and stx2g) (7). Although real-time PCR assays have been developed to detect these 7 stx2 subtypes (8–12), detection of these subtypes by RFBS24 has not yet been evaluated.

Therefore, the objective of the present study was to improve the detection limit, to optimize the differences...
in $T_m$ values between appropriate target genes, and to increase the sensitivity of RFBS24 by redesigning the primers and optimizing the IAC. We assessed the performance of the improved RFBS24 assay (RFBS24 ver. 5) by comparing the detection limits of RFBS24 and RFBS24 ver. 5 and by testing fecal DNA samples isolated from cases of EHEC infection and outbreaks of foodborne *Salmonella* and *Campylobacter* infections by means of both assays.

**MATERIALS AND METHODS**

**Bacterial strains:** The bacterial strains that we used in this study included 22 bacterial pathogens (enteroinvasive *E. coli* [EIEC], enteropathogenic *E. coli* [EPEC], *EHEC*, ETDC, enterohaemorrhagic *E. coli* [EHEC], diffusely adhering *E. coli* [DAEC], *Shigella* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Providencia alcalifaciens*, *Plesiomonas shigelloides*, *Campylobacter jejuni*, *Campylobacter coli*, *Vibrio cholerae*, thermostable direct hemolysin (TDH)-positive *V. parahaemolyticus*, thermostable direct hemolysin-related hemolysin-positive *V. parahaemolyticus*, *Aeromonas hydrophila*, *Staphylococcus aureus*, emetic and enterotoxigenic *Bacillus cereus*, *Clostridium perfringens*, and *Listeria monocytogenes*). Some of these bacterial strains were obtained from 3 institutes (Tokyo Metropolitan Institute of Public Health, National Institute of Infectious Diseases in Japan, and Akita Prefectural Institute of Public Health) and 2 universities (Kagawa Nutrition University and the Center for Southeast Asian Studies of Kyoto University), whereas the remaining strains were received from the American Type Culture Collection, Japan Collection of Microorganisms, and Research Institute for Microbial Diseases of Osaka University. Wild strains were isolated from foods and patients admitted to our institutions. The strain numbers and sources are listed in Tables 1 and 2. Regarding DNA extraction from bacterial culture, the bacterial pathogens described above were cultured in an appropriate broth medium, and DNA was isolated from the bacterial suspensions using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA samples were then used for evaluation of the primer specificity and sensitivity of RFBS24 ver. 5 and for comparison of the detection limits between RFBS24 and RFBS24 ver. 5. The concentrations of DNA in the samples were determined by optical density at 260 nm (OD$_{260}$) on a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

**Primers used for RFBS24 ver. 5:** To improve the RFBS24 assay, 13 primer pairs were redesigned, and an appropriate combination of primer pairs was carefully arranged in 8 sets. Primer sequences, $T_m$ values of PCR products (given in °C throughout the text), and references for RFBS24 ver. 5 are listed in Table 3. We designed primer pairs for the cpe gene of *C. perfringens*, stx1 and stx2 genes of *EHEC*, cjo414 of *C. jejuni*, genotypes ST (STp and STh) and LT of ETDC, hly of *Listeria monocytogenes*, aggR of EAE, tdh of *Vibrio parahaemolyticus*, gyrB of *Plesiomonas shigelloides*, ipaH of *Shigella* spp. and EIEC, ahh1 of *Aeromonas hydrophila*, and invA of *Salmonella* spp. using the Primer 3 and AutoDimer software with sequences submitted to GenBank (13-15). The primer combination for each set was chosen to include primer pairs with 3 different $T_m$ values (these pairs were not expected to form primer dimers). Specificity of the primers was tested using the Basic Local Alignment Search Tool of NCBI.

**Preparation of the competitive IAC:** This control was constructed for 8 sets of multiplex real-time SG-PCR. We designed a one-string sequence (Fig. 1) composed of the ahh1 sequence (GenBank Accession No. AB206039, position: 1112–1201) for *A. hydrophila* and primer pairs for cpe of *C. perfringens*, cjo414 of *Campylobacter jejuni*, ces of *B. cereus*, eae of *EHEC* and EPEC, femB of *S. aureus*, tdh of *Vibrio parahaemolyticus*, astA of EAE, and invA of *Salmonella* spp. The sequence shown in Fig. 1 was incorporated into the pUC57 plasmid by BEX Co., Ltd. (Tokyo, Japan). Primers M13F (GTAAAAACGACGGGCAGT) and M13R (CAGGAAAACGCTATGTA) were used to amplify the region (977 bp) containing the sequence shown in Fig. 1 in a 20-μl PCR mixture containing 10 × Ex Taq buffer (Takara Bio, Shiga, Japan), 10 μM primers, and 2 μl of the pUC57 plasmid containing the sequence shown in Fig. 1, according to Takara’s protocol. Amplification was performed as follows: initial denaturation at 95°C for 2 min; 16 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and elongation at 72°C for 30 s; and final

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**Fig. 1. Design of competitive IAC for 8 sets of multiplex real-time SG-PCR, using the RFBS24 ver. 5 system. The competitive IAC was comprised of the ahh1 sequence (Accession No. AB206039, position: 1112–1201) for *Aeromonas hydrophila* and 8 primer pairs for cpe of *Clostridium perfringens*, cjo414 of *Campylobacter jejuni*, ces of *Bacillus cereus*, eae of *EHEC* and EPEC, femB of *Staphylococcus aureus*, tdh of *Vibrio parahaemolyticus*, astA of EAE, and invA of *Salmonella* spp.**
| No. | Bacterial strain | Presence of target gene | Strain | Results of PCR with each primer |
|-----|------------------|-------------------------|--------|--------------------------------|
|     |                  |                         |        | Set A | Set B | Set C | Set D | Set E | STa | STb | tdh | PSG | astA | ipaH | AHH1 | yadA | invA | daaD |
| 1   | *Clostridium perfringens* | *cpe* | H2 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2   | *Providencia alcalifaciens* | *gyrB* | Pa118 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3   | *Escherichia coli*; EHEC | *eae* | SE11061 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx1* | SE9004 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx2* | SE8001 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx1* | 94C | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx1* | 031 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx2* | EC08546 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx2d* | C165-02 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx2e* | S1191 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx2f* | T4/97 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *stx2g* | STp | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 4   | *Campylobacter jejuni* | *cj0414* | FB10002 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 5   | *Vibrio parahaemolyticus* | *trh* | SVP18 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| 6   | *Listeria monocytogenes* | *hly* | JCM7674 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 7   | *Bacillus cereus* | *ces* | No. 1 | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 8   | *Vibrio cholerae* | *ompW* | VC01 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 9   | *E. coli*; ETEC | *LT* | EC2738 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| 10  | *E. coli*; EPEC | *eae* | EC2736 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| 11  | *Campylobacter coli* | *ceuE* | M35 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 12  | *E. coli*; EAEC | *aggR* | EC4131 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *aggR* | EC4196 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| 13  | *Staphylococcus aureus* | *femB* | FB6001 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 14  | *E. coli*; ETEC | *STp* | EC4351 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
|     |                  | *STh* | EC26 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 15  | *Vibrio parahaemolyticus* | *tdh* | SVP03087 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| 16  | *Plesiomonas shigelloides* | *gyrB* | NIID322-73 | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 17  | *Shigella* spp. | *ipaH* | I09001 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 18  | *E. coli*; EIEC | *ipaH* | EC09466 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 19  | *Aeromonas hydrophila* | *ashA* | SAH6 | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 20  | *Yersinia enterocolitica* | *yadA* | HP09008 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 21  | *Y. pseudotuberculosis* | *yadA* | SP1523 | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| 22  | *Salmonella* spp. | *invA* | Sall2339 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 23  | *E. coli*; DAEC | *afaD* | K12214 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |

1): Strain was obtained from Tokyo Metropolitan Institute of Public Health, Japan.
2): Strains were obtained from National Institute of Infectious Disease, Japan.
3): EHEC reference strains in the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* were obtained from the National Institute of Infectious Disease, Japan.
4): Strain was obtained from Kagawa Nutrition University, Japan.
5): Strains were obtained from Akita Prefectural Institute of Public Health, Japan.
RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5; JCM, Japan Collection of Microorganisms.
Table 2. Detection limits of RFBS24 and RFBS24 ver.5 for 28 target genes

| Species (target gene) | Strain no. | Detection limits of RFBS24 and RFBS24 ver.5 in quadruplicate tests1) | DNA concentration (5.6 × 10^-5 ng/PCR tube) | P value |
|-----------------------|------------|---------------------------------------------------------------|-----------------------------------------|--------|
| Clostridium perfringens (cpe) | ATCC12915 | X = 0  | 1 | 2 | 3 | 4 | 5 | 0.0292) |
| Providencia alcalifaciens (gyrB) | Pa113b) | | 1 | 3 | 4 | | | |
| Escherichia coli: EHEC (stx2a) | Sakai O157 | | 1 | 3 | 3 | 1 | | 0.0292) |
| E. coli: EHEC (stx2f) | 07E033 | ND | 1 | 3 | | | |
| E. coli: EHEC (stx1) | Sakai O157 | | 3 | 1 | 2 | 2 | | 0.0292) |
| Campylobacter jejuni (cj0414) | ATCC33560 | | 4 | 1 | 3 | | 0.0292) |
| Vibrio parahaemolyticus (trhl1) | AQ4037d) | 2 | 2 | 1 | 2 | 1 | 0.057 |
| Vibrio parahaemolyticus (trh2) | AT4e) | ND | 1 | 2 | 3 | | |
| E. coli: ETEC (LT) | No. 9 | 2 | 2 | 4 | | | 0.0292) |
| Bacillus cereus (ces) | 22-63 | 2 | 2 | 4 | | | 0.0292) |
| Vibrio cholerae (ompW) | 06-119 | 4 | 1 | 3 | | | 0.0292) |
| Listeria monocytogenes (hlv) | ATCC19114 | 2 | 2 | 4 | | | 0.0292) |
| E. coli: EHEC (eae) | Sakai O157 | 1 | 3 | 2 | 2 | | 0.0292) |
| Bacillus cereus (nheB) | 22-38 | 2 | 1 | 2 | 3 | | 0.686 |
| Campylobacter coli (cve) | JCM2529 | 1 | 3 | 3 | 1 | | 0.0292) |
| E. coli: AEAC (aggP) | 21-1 | 2 | 2 | 1 | 2 | 1 | 0.0292) |
| Staphylococcus aureus (femB) | ATCC25923 | 4 | 4 | 4 | | | 0.0292) |
| E. coli: ETEC (STp) | Kaken9 | 1 | 2 | 2 | 2 | | 0.0292) |
| E. coli: ETEC (STh) | EC2018 | ND | 1 | 1 | 1 | | |
| Vibrio parahaemolyticus (tdh) | RIMD2210633 | 4 | 3 | 1 | | | 0.0292) |
| Plesiomonas shigelloides (gyrB) | ATCC14029 | 2 | 2 | 2 | 1 | | 0.057 |
| E. coli: EHEC (astA) | 07E033 | 4 | 2 | 2 | 2 | | 0.0292) |
| E. coli: EIEC (ipaH) | RIMD05091045 | 4 | 4 | 4 | | | 0.0292) |
| Aeromonas hydrophila (ahh1) | ATCC9796 | 3 | 1 | 4 | | | 0.0292) |
| Yersinia enterocolitica (yadA) | HP9008 | 1 | 3 | 4 | | | 0.0292) |
| Y. pseudotuberculosis (yadA) | SP2536 | 2 | 2 | 3 | 1 | | 0.0292) |
| Salmonella spp. (invA) | 22-62 | 4 | 4 | 4 | | | 0.0292) |
| E. coli: DAEC (afad) | K12124f) | 4 | 4 | 4 | | | 0.0292) |

1) Results of RFBS24 ver.5 are shown as numbers with underlines, and results of RFBS24 are shown as numbers without underlines (e.g., the detection limit of RFBS24 ver.5 for stx2a indicates the number of tests detected until 5.6 × 10^-5 ng/reaction was 1 and the number of tests detected until 5.6 × 10^-4 ng/reaction was 3 in quadruplicate).

2) P < 0.05 is significant by Mann-whitney U test between RFBS24 and RFBS24 ver.5.

3) Strain was obtained from National Institute of Infectious Diseases, Japan.

4) Strain was obtained from Center for Southeast Asian Studies, Kyoto University.

5) ND, not detected; RFBS24, rapid foodborne bacterial screening 24; RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5.

ATCC, American Type Culture Collection; JCM, Japan Collection of Microorganisms; RIMD, Research Institute for Microbial Diseases, Osaka University.

extension at 72°C for 10 min. Purification of the PCR product and determination of the concentration of purified DNA were performed as described previously (24), and the purified DNA samples were stored at −20°C until use as the competitive IAC.

RFBS24 and RFBS24 ver. 5: Multiplex real-time SG-PCR of 8 sets of genes by means of RFBS24 ver. 5 (and RFBS24) was performed on a Thermal Cycler Dice Real-Time System TP860 (Takara Bio). Each reaction was run in a total volume of 20 μL containing 10 μL of SYBR Premix DimerEraser (Takara Bio), 2.4 μL of PCR-grade H2O, 3.6 μL of 10 μM primer pairs for 3 target genes, 2 μL of the competitive IAC, and 2 μL of the DNA sample. PCR mixtures were placed in wells of 96-well plates as described previously (6). The concentration of the IAC (5.6 × 10^2 copies/reaction) was adjusted to attain the cycle threshold (Ct) value between 31 and 34. The cycling program began with an initial denaturation at 95°C for 30 s; 35 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min; and dissociation via one cycle at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. RFBS24 was implemented on a Thermal Cycler Dice Real-Time System TP860 as described previously (6). The results of RFBS24 and RFBS24 ver. 5 were interpreted as described previously (6) and were labeled as either positive or negative.

Measurement of the detection limits of RFBS24 and RFBS24 ver. 5: Ten-fold serial dilutions of 28 DNA samples (2.8 × 10^5–2.8 × 10^-5 ng/μL) extracted from the bacterial strains listed in Table 2 were used to compare the detection limits between RFBS24 and RFBS24 ver. 5. Multiplex real-time SG-PCR by means of RFBS24 and RFBS24 ver. 5 with the 28 DNA samples was performed in quadruplicate for each DNA sample. We defined the lowest concentration DNA that resulted in successful amplification of the target gene in an assay (i.e., RFBS24 and RFBS24 ver. 5) as the detection limit.

Analysis of bacterial DNA isolated from patients’ feces using RFBS24 and RFBS24 ver. 5: In cases of EHEC (5 cases; EHEC O157 harboring eae, stx1, and stx2) in 2 of 9 fecal samples, EHEC O157 harboring eae...
Table 3. Primers used for multiplex real-time SG-PCR, separated into 8 sets

| Primer set | Species | Target gene | Primer name | PCR primer sequence (5′–3′) | GenBank accession no. | Position | Product size (bp) | Tm (°C) | Tm distance | Reference |
|------------|---------|-------------|-------------|-----------------------------|----------------------|----------|------------------|--------|-------------|-----------|
| A | Clostridium perfringens<sup>2</sup> | cpe | CPE-Et-F | TAATAGATAAAAGGGATGGTAG | X81849 | 182–206 | 185 | 75.98 ± 0.15 | 3.3 | This study |
| | | | CPE-Et-R | AATCCATATCTACAGATCTGGT | | 366–343 | 73 | 79.26 ± 0.17 | 16 | (16) |
| B | Providencia alcalifaciens | gyrB | PAG110-R | GAGGCGACTGGTTACGAC | A300547 | 38–56 | 73 | 79.26 ± 0.17 | 16 | (16) |
| | | stx2 | stx2-ET-F | TAATAGATAAAGGAGATGGTTGGAT | M23980 | 351–376 | 68 | 74.63 ± 0.26 | 3.7 | This study |
| | | | stx2-ET-R | AAATCCATATTCTACAGATGCTTG | R-R287 | 674–694 | | | | (18) |
| | EHEC (Stx 1) | cJ014 | AB-F2 | GTAGTCGTAGATGCATGTC | AL11168 | 381129–381151 | 74 | 76.37 ± 0.37 | 3.7 | This study |
| | | | AB-R2 | ACACATGCGAGGCTGTC | X1998 | 675–702 | | | | (19) |
| | Campylobacter jejuni | cj014 | stx2-ET-F | GTAGTCGTAGATGCATGTC | AL11168 | 381129–381151 | 74 | 76.37 ± 0.37 | 3.7 | This study |
| | | | stx2-ET-R | ACACATGCGAGGCTGTC | X1998 | 675–702 | | | | (19) |
| | | | | | | | | | | |
| | ETEC (LT) | LT | LT-F | ATGAGATATTGTTTGTTGTCAGA | X83966 | 456–476 | 52 | 73.91 ± 0.13 | 5.2 | This study |
| | | | LT-R | GCGAGGCTGTC | X1998 | 675–702 | | | | (19) |
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Table 4. Comparison of RFBS24 and RFBS24 ver. 5 in EHEC, Salmonella, and Campylobacter infections

| Causative pathogens | No. of patient samples | No. of positives in culture method | Sensitivity (%) | Specificity (%) |
|---------------------|------------------------|-----------------------------------|----------------|----------------|
| EHEC                | 2                      | 1 (stx1)                          | 50             | 96             |
| Salmonella spp.     | 27                     | 2 (stx1)                          | 74.1           | 100            |
| Campylobacter       | 47                     | 19 (stx1)                         | 40.4           | 100            |

Development of RFBS24 ver. 5: Improving the detection limit and sensitivity of RFBS24 was expected to require attenuation of interactions among primers and changes to the cycling conditions so as not to generate primer dimers. Accordingly, we developed new versions of RFBS24 in stages by redesigning the primers and by optimizing the IAC. Finally, we eliminated one primer pair (which detected the noncompetitive IAC of each set) in RFBS24 ver. 5 and included only 3 primer pairs for detection of foodborne bacterial pathogens and a competitive IAC in each set. In each set used in RFBS24 ver. 5 (Table 3), we included one of the primer pairs designed to detect a target gene from 8 main foodborne pathogens (C. perfringens, Campylobacter jejuni, emetic B. cereus, eae-positive E. coli, S. aureus, TDH-
positive *V. parahaemolyticus*, *astA*-positive *E. coli*, and *Salmonella* spp.), which frequently cause outbreaks of foodborne illnesses in Japan (6). Next, we included 2 primer pairs designed to detect the 3 diatom vesicular gene *E. coli* and other foodborne pathogens that rarely cause foodborne outbreaks in Japan (6). Furthermore, Fukushima et al. (6) reported that multiple target genes could be detected in 26 stool samples corresponding to 15 cases of foodborne Illness outbreaks. We did not test could be detected in 26 stool samples corresponding to Fukushima et al. (6) reported that multiple target genes cause foodborne outbreaks in Japan (6). Furthermore, multiple real-time PCR assays for detection of *stx1* and *stx2* have been developed (8–11), and real-time PCR assays using TaqMan probes, as reported by Derzelle et al. (12), have been shown to detect all known *stx* subtypes. Nonetheless, real-time PCR with TaqMan probes is expensive as compared to that based on SYBR Green I (31). Thus, our method detected all known *stx* subtypes in a cost-effective manner and could distinguish *stx2f* and *stx2g* from *stx2a*, *stx2b*, *stx2c*, *stx2d*, and *stx2e* via determination of the Tm values by melting-curve analysis.

**Comparison of detection limits of RFBS24 and RFBS24 ver. 5:** We assessed the detection limit of RFBS24 ver. 5 and compared it to that of the original version by analyzing 28 DNA samples (Table 2). RFBS24 did not detect *stx2f*, *Sth*, or *trh2* of *V. parahaemolyticus*. The detection limits of all target genes, except *stx2f*, *Sth*, and *trh2*, ranged from 5.6 × 10^3 to 5.6 × 10^4 ng/reaction. The detection limits (5.6 × 10^–5–5.6 × 10^1 ng/reaction) for *femB* of *S. aureus* and *aggR* of EAEc were higher than those for other genes, whereas the detection limits (5.6 × 10^–5–5.6 × 10^4 ng/reaction) for *hly* of *L. monocytogenes* and *nheB* of *B. cereus* were slightly lower than those for other genes. The detection limits of the remaining genes ranged from 5.6 × 10^–4–5.6 × 10^3 ng/reaction. In RFBS24 ver. 5, the detection limit for *stx2f* ranged from 5.6 × 10^–2–5.6 × 10^1 ng/reaction, for *trh1* and *aggR*, it ranged from 5.6 × 10^–2 to 5.6 × 10^4 ng/reaction, and for the remaining genes from 5.6 × 10^–1 to 5.6 × 10^–4 ng/reaction. The detection limits for most target genes ranged from 5.6 × 10^–4 to 5.6 × 10^–5 ng/reaction. The detection limits for all target genes, with the exception of *stx2f*, *trh1*, *trh2*, *nheB*, *Sth*, and *gyrB* of *P. shigelloides*, were significantly lower (10– to 100-fold) than those of RFBS24 (P < 0.05). This result suggested that the changes to the assay, i.e., addition of PCR cycles (30 cycles in RFBS24 and 35 cycles in RFBS24 ver. 5), lowered (i.e., improved) the detection limit for most target genes in RFBS24 ver. 5.

**Testing of RFBS24 ver. 5 on fecal samples of patients with foodborne illnesses:** We performed analyses of fecal DNA samples from patients with foodborne illnesses (EHEC, *Salmonella*, or *Campylobacter* infections) using RFBS24 and RFBS24 ver. 5 (Table 4). Among the 9 samples from patients with EHEC infections, the numbers of samples positive for *eae*, *stx1*, or *stx2* genes according to RFBS24 combined with Qkit were 1, 0, and 2, respectively, or 5, 1, and 3, respectively, when RFBS24 was combined with Ukit, whereas those numbers for RFBS24 ver. 5 were 5, 1, and 5, respectively, when combined with Qkit, or 9, 4, and 7, respectively, when combined with Ukit. Among the 27 samples from patients with *Salmonella* infections, *Salmonella* (invA) was detected in 3 samples by RFBS24 combined with Qkit, 8 samples by RFBS24 combined with Ukit, 13 samples by RFBS24 ver. 5 combined with Qkit, and 17 samples by RFBS24 ver. 5 combined with Ukit. Among the 47 samples from patients with *Campylobacter* infections, the numbers of samples positive for *C. jejuni* or
Fig. 2. Melting curve of 3 target genes of foodborne pathogens and the IAC by multiplex real-time SG-PCR in sets A–H. In sets A–F, the Tm values were 73.65–82.53 for the target genes and 86.63–87.18 for competitive IAC. In sets G and H, the Tm values were 81.87–88.62 for the target genes and 77.17–77.21 for competitive IAC. The differences between Tm values among the target genes in the 8 sets ranged from 1.6 to 5.4.
Fig. 3. Melting curve of the EHEC stx2 subtype by multiplex real-time SG-PCR in set A. The Tm values of stx2a, stx2b, stx2c, and stx2e were almost the same (82.74–82.91). The Tm value of stx2f (81.72) was lower than those of stx2a, stx2b, stx2c, stx2d, and stx2e, and the Tm value of stx2g (83.64) was slightly higher than those of stx2a, stx2b, stx2c, stx2d, and stx2e.

C. coli according to RFBS24 combined with Qkit were 20 and 4, respectively; according to RFBS24 combined with Ukit: 22 and 4, respectively; according to RFBS24 ver. 5 combined with Qkit: 25 and 7, respectively; and according to RFBS24 ver. 5 combined with Ukit: 28 and 7, respectively. The sensitivity in terms of detection of Salmonella spp., C. jejuni, or C. coli was 16%, 83%, and 57%, respectively, for RFBS24 combined with Qkit: 42%, 92%, and 57%, respectively, for RFBS24 combined with Ukit: 68%, 96%, and 100%, respectively, for RFBS24 ver. 5 combined with Qkit: and 89%, 100%, and 100%, respectively, for RFBS24 ver. 5 combined with Ukit. Fukushima et al. (6) reported that the detection limit of RFBS24 for Salmonella spp. is higher than that for other bacterial pathogens. In the present study, however, the number of samples designated as Salmonella positive and sensitivity for Salmonella was 16%, 83%, and 57%, respectively, for RFBS24 and both of these parameters were further improved in RFBS24 ver. 5 combined with Ukit. Similar results were obtained regarding detection of EHEC and Campylobacter. These results suggested that the detection limits of RFBS24 ver. 5 for EHEC, Salmonella, and Campylobacter in real-life patients’ samples were improved as compared to RFBS24. The improvement of analytical characteristics of RFBS24 ver. 5 by Ukit may be attributed to highly efficient DNA extraction by disruption of bacterial cells via mechanical shearing, as reported previously (24).

For Salmonella and Campylobacter infections, the results of the culture method and of our PCR assay for bacterial pathogens, except for the causative pathogens detected with RFBS24 ver. 5, are listed in Table 5. The numbers of test-positive samples obtained with RFBS24 ver. 5 were greater than those of the traditional culture method for EAEC (astA), EPEC (eae), DAEC (afaD), C. perfringens, P. alcalifaciens, and A. hydrophila (Table 5). At the same time, the PCR products of those target genes in RFBS24 ver. 5 were confirmed to have the correct molecular weight by agarose gel electrophoresis; therefore, it is likely that these additional test-positive samples were not false positive results. With the culture methods, screening for EAEC (astA), EPEC (eae), and DAEC (afaD) by the colony-sweep PCR method yielded some positive results (Campylobacter infections: astA and eae were detected in 2 and 2 samples, respectively; Salmonella infections: astA and afaD were detected in 7 and 2 samples, respectively). Nevertheless, EAEC (astA) and EPEC (eae) were successfully isolated by the culture method from 1 and 2 samples, respectively, for Campylobacter infections; whereas EAEC (astA) was successfully isolated from 3 samples, and DAEC (afaD) was not isolated by the culture method from samples corresponding to Salmonella infections. These discrepancies between RFBS24 ver. 5 and culture methods may be attributed to the lack of appropriate culture methods for some diarrheagenic E. coli or to the detection of dead bacteria by RFBS24 ver. 5. To resolve such inconsistencies, Ziegler et al. (32) tested whether the level of the C value obtained with real-time PCR could be used for diagnosis of severe sepsis versus nonsevere sepsis. Similarly, in future studies, we may have to determine whether appropriate diagnoses can be made on the basis of the C value.

Furthermore, RFBS24 ver. 5 often detected EAEC (astA) and EPEC (eae) in samples from patients with Salmonella and Campylobacter infections (astA was detected in 12 samples corresponding to Campylobacter
infections and in 10 samples corresponding to *Salmonella* infections; *eae* was detected in 4 samples corresponding to *Campylobacter* infections and 8 samples corresponding to *Salmonella* infections). In outbreaks of foodborne illnesses caused by diarrheagenic *E. coli*, the detection rate for diarrheagenic *E. coli* among patients is usually high (33–35). On the other hand, Fujihara et al. (36) reported that EAEC, DAEC, and EPEC can be isolated from 0.9–2.4% of healthy individuals. Therefore, to facilitate detection of the corresponding target genes by RFBS24 ver. 5, it is better to first determine the suspected causative pathogen on the basis of the number of samples that would be expected to be positive for EAEC, EPEC, or other pathogens among patients infected with foodborne illnesses.

For detection of *S. aureus*, the numbers of test-positive samples obtained by the traditional culture method were greater than those obtained with RFBS24 ver. 5 (Table 5). It is likely that *S. aureus* was not the cause of either outbreak because the symptoms of patients during both outbreaks were different from those generally observed during *S. aureus* infection. Nonetheless, it will be necessary to redesign the primer pair used for detection of *S. aureus (femB)* to further improve the detection limit in the future.

**Conclusion:** In contrast to RFBS24, RFBS24 ver. 5 detected all known *stx*2 subtypes, *V. parahaemolyticus* (*trh2*), and ETEC (STh) as well as an increased variety of target genes. The detection limit of RFBS24 ver. 5 is lower than that of RFBS24. Moreover, RFBS24 ver. 5 can detect the causative pathogens in patients' fecal samples collected during actual outbreaks of foodborne illnesses and infections caused by EHEC, *Salmonella*, or *Campylobacter*. RFBS24 ver. 5 when combined with Ukit shows adequate performance in terms of rapid detection of these causative pathogens. Therefore, RFBS24 ver. 5 is a more useful assay (than RFBS24) for simultaneous detection of multiple foodborne pathogens and may enable more rapid dissemination of information to public health officials during outbreaks of foodborne illnesses. Further studies are required to evaluate applicability of this system to outbreaks of foodborne illnesses caused by other bacterial pathogens.

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**Conflict of interest** None to declare.

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