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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* (*trh2*), which were not detected by the original RFBS24 assay. The detection limits of RFBS24 ver. 5 were approximately 5.6×10^{-2}–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 (T_m) 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

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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, ETEC, enteroaggregative *E. coli* [EAEC], 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, *cj0414* of *C. jejuni*, genotypes ST (STp and STh) and LT of ETEC, *hly* of *Listeria monocytogenes*, *agg*R of EAEC, *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 *Aeromonas hydrophila* and 8 primer pairs for *cpe* of *C. perfringens*, *cj0414* of *Campylobacter jejuni*, *ces* of *Bacillus cereus*, *eae* of EHEC and EPEC, *femB* of *S. aureus*, *tdh* of *Vibrio parahaemolyticus*, *astA* of EAEC, 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 (GTAAAACGACGGCCAGT) and M13R (CAGGAAACAGCTATGA) 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

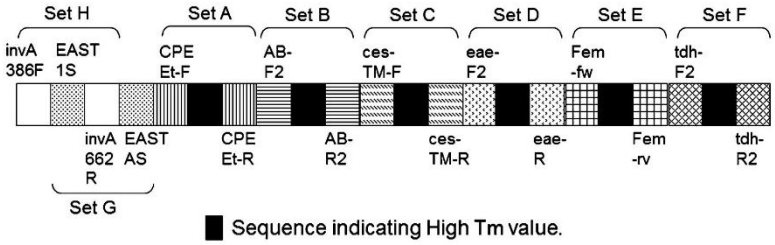

Fig. 1. Design of competitive IAC for 8 sets of multiplex real-time SG-PCR, using the RFBS24 ver.5 system. The competitive IAC was comprised of the *ahh1* sequence (Accession No. AB206039, position: 1112–1201) for *Aeromonas hydrophila* and 8 primer pairs for *cpe* of *Clostridium perfringens*, *cj0414* of *Campylobacter jejuni*, *ces* of *Bacillus cereus*, *eae* of EHEC and EPEC, *femB* of *Staphylococcus aureus*, *tdh* of *Vibrio parahaemolyticus*, *astA* of EAEC, and *invA* of *Salmonella* spp.

Table 1. Primer sensitivity and specificity of RFBS24 ver.5

No.	Bacterial strain	Presence of target gene	Strain	Results of PCR with each primer																					
				Set A			Set B			Set C			Set D			Set E			Set F			Set G			Set H		
				CPE	PAG	stx2	stx1	AB	trh	LT	ces	ompW	Lmhly	eae	nheB	ceuE	aggR	FemB	STa and STb	tdh	PSG	astA	ipaH	AHH1	yadA	invA	daaD
1	*Clostridium perfringens*	*cpe*	H21[1]	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	*Providencia alcalifaciens*	*gyrB*	Pa118[2]	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	*Escherichia coli*: EHEC	*eae stx1 stx2*	SE11061	–	–	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
		eae stx1	SE9004	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
		eae stx2	SE8001	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
		stx1 stx2a	94C[3]	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		stx2b astA	EH250[3]	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
		stx2b stx2c	031[3]	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		stx2c	EC08546	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		stx2d	C165-02[3]	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		stx2e astA	S1191[3]	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
		eae stx2f astA	T4/97[3]	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–
		stx2g STp *astA*	7v[3]	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–
4	*Campylobacter jejuni*	*cj0414*	FB10002	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	*Vibrio parahaemolyticus*	*trh*	SVP18	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	*Listeria monocytogenes*	*hly*	JCM7674	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	*Bacillus cereus*	*ces nheB*	No. 1[4]	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8	*Vibrio cholerae*	*ompW*	YC01	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	*E. coli*: ETEC	LT	EC2738[5]	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	*E. coli*: EPEC	*eae*	EC2736[5]	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11	*Campylobacter coli*	*ceuE*	M35	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
12	*E. coli*: EAEC	*aggR astA*	EC4131[5]	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–
		aggR	EC4196[5]	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
13	*Staphylococcus aureus*	*femB*	FB6001	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
14	*E. coli*: ETEC	STp *astA*	EC4351[5]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–
		STh	EC26[5]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
15	*Vibrio parahaemolyticus*	*tdh*	SVP03087	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
16	*Plesiomonas shigelloides*	*gyrB*	N1ID322-73[3]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
17	*Shigella* spp.	*ipaH*	I09001	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
18	*E. coli*: EIEC	*ipaH*	EC09466	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
19	*Aeromonas hydrophila*	*ahh1*	SAH6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
20	*Yersinia enterocolitica*	*yadA*	HP09008	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
21	*Y. pseudotuberculosis*	*yadA*	SP1523	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
22	*Salmonella* spp.	*invA*	Sal2339	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
23	*E. coli*: DAEC	*afaD*	K12214[5]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+

[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 quadruple tests[1] DNA concentration (5.6×10^{-x} ng/PCR tube) X = 0	1	2	3	4	5	*P* value
Clostridium perfringens (*cpe*)	ATCC12915			3	1 1	2	1	0.029[2]
Providencia alcalifaciens (*gyrB*)	Pa118[3]			1	3	4		0.029[2]
Escherichia coli: EHEC (*stx2a*)	Sakai O157			1	3	3	1	0.029[2]
E. coli: EHEC (*stx2f*)	07E033	ND		1	3			
E. coli: EHEC (*stx1*)	Sakai O157			3	1	2	2	0.029[2]
Campylobacter jejuni (*cj0414*)	ATCC33560				4	1	3	0.029[2]
Vibrio parahaemolyticus (*trh1*)	AQ4037[4]		2	2 1	2	1		0.057
Vibrio parahaemolyticus (*trh2*)	AT4[4]	ND			1	3		
E. coli: ETEC (LT)	No. 9			2	2	4		0.029[2]
Bacillus cereus (*ces*)	22–63			2	2	4		0.029[2]
Vibrio cholerae (*ompW*)	06–119			4	1	3		0.029[2]
Listeria monocytogenes (*hly*)	ATCC19114				2	2	4	0.029[2]
E. coli: EHEC (*eae*)	Sakai O157			1	3	2	2	0.029[2]
Bacillus cereus (*nheB*)	22–38				2 1	2 3		0.686
Campylobacter coli (*ceuE*)	JCM2529			1	3	3	1	0.029[2]
E. coli: EAEC (*aggR*)	21–1	2	2	1	2	1		0.029[2]
Staphylococcus aureus (*femB*)	ATCC25923		4		4			0.029[2]
E. coli: ETEC (STp)	Kaken9		1	2	1	2	2	0.029[2]
E. coli: ETEC (STh)	EC2018	ND				3	1	
Vibrio parahaemolyticus (*tdh*)	RIMD2210633				4	3	1	0.029[2]
Plesiomonas shigelloides (*gyrB*)	ATCC14029			2	2 1	2	1	0.057
E. coli: EHEC (*astA*)	07E033				4	2	2	0.029[2]
E. coli: EIEC (*ipaH*)	RIMD05091045				4	1	3	0.029[2]
Aeromonas hydrophila (*ahh1*)	ATCC7976			3	1	4		0.029[2]
Yersinia enterocolitica (*yadA*)	HP9008		1	3	4			0.029[2]
Y. pseudotuberculosis (*yadA*)	SP2536		2	2	3	1		0.029[2]
Salmonella spp. (*invA*)	22-62			4		4		0.029[2]
E. coli: DAEC (*afaD*)	KI2214[3]				4	4		0.029[2]

[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.
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, ResearchInstitute 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 H_2O, 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 (C_t) 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 (2.8×10^0–2.8×10^{-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[1]	Tm *distance*	Reference
A	*Clostridium perfringens*[2]	*cpe*	CPE-Et-F	TAATAGATAAAGGAGATGGTTGGAT	X81849	182–206	185	75.98 ± 0.15		This study
			CPE-Et-R	AAATCCATATTCTACAGATGCTTG		366–343			3.3	
	Providencia alcalifaciens	*gyrB*	PAG38-F	TCTGCACGGTGTGGGTGTT	AJ300547	38–56	73	79.26 ± 0.17		(16)
			PAG110-R	ACCGTCACGGCGGATTACT		110–92			3.3	
	EHEC (Stx 2)	*stx2*	stx2-ET-F	CATGACAACGGACAGCAGTTAT	X07865	625–646	114	82.53 ± 0.21		This study
			stx2-ET-R	AACTCCATTAACGCCAGATATGA		738–716		(81.11 ± 0.13[3])		
B	EHEC (Stx 1)	*stx1*	Stx1-ET-F	CATTACAGACTATTTCATCAGGAGGT	M23980	351–376	68	74.63 ± 0.26		This study
			stx1-ET-R	CAAATTATCCCCTGAGCCACTA		418–397			1.7	
	Campylobacter jejuni[2]	*cj0414*	AB-F2	GATACCTTAAGTGCAGCCTGTGA	AL111168	381129–381151	74	76.37 ± 0.37		This study
			AB-R2	ACGCCTAAACCTATAGCTCCTTC		381202–381180			3.7	
	TRH-positive *Vibrio parahaemolyticus*	*trh*	F-trh82	CCATCMATACCTTTTCCTTCTCC	AY742213	879–857	206	80.04 ± 0.38		(17)
			R-trh287	ACYGTCATATAGGCGCTTAAC		674–694				
C	ETEC (LT)	LT	LT-F2	AGCCATTGAAAGGATGAAGGA	X83966	456–476	52	73.91 ± 0.13		This study
			LT-2	GGTCTCGGTCAGATATGTGATTC		507–485			5.2	
	Emetic *Bacillus cereus*[2]	*ces*	ces-TM-F	GATGTTTGCGACGATGCAA	DQ360825	8689–8707	65	79.12 ± 0.26		(18)
			ces-TM-R	CTTTCGGCGTGATACCCATT		8753–8734			1.7	
	Vibrio cholerae	ompW	ompW-F	AACATCCGTGGATTTGGCATCTG	X51948	763–741	89	80.78 ± 0.07		(19)
			ompW-R	GCTGGTTCCTCAACGCTTCTG		675–692				
D	*Listeria monocytogenes*	*hly*	Lm-hly-F	GGGAAATCTGTCTCAGGTGATGT	AF253320	973–995	72	73.65 ± 0.26		This study
			Lm-hly-R-kai1	GTAAATTACGGCTTTGAAGGAAGA		1044–1021			5.4	
	EHEC and EPEC[2]	*eae*	eae-F2	CATTGATCAGGATTTTTCTGGTGATA	Z11541	899–924	102	79.01 ± 0.26		(18)
			eae-R	CTCATGCGGAAATAGCCGTTA		1000–980			1.6	
	Enterotoxigenic *Bacillus cereus*	*nheB*	SG-F3	GCACTTATGGCAGTATTTGCAGC	DQ153257	2101–2123	152	80.61 ± 0.27		(20)
			SG-R3	GCATCTTTTAAGCCTTCTGGTC		2252–2231				
E	*Campylobacter coli*	*ceuE*	ceuE-For	CAAGTACTGCAATAAAAACTAGCACTACG	X88849	2777–2805	72	74.11 ± 0.14		(21)
			ceuE-Rev	AGCTATCACCCTCATCACTCATACTAATAG		2848–2819			4.4	
	EAEC (aggR)	*aggR*	aggRks1-kai1	GTATACACAAAGAAGGAAGCAATA	Z18751	202–226	221	78.47 ± 0.22		This study
			aggRKas2-kai1	TTTGACCAATTCGGACAAC		422–404			2.8	
	Staphylococcus aureus[2]	*femB*	FemB-fw	AATTAACGAAATGGGCAGAAACA	AF106850	277–299	93	81.23 ± 0.26		(22)
			FemB-rv	TGCGCAACACCCTGAACTT		370–352				
F	ETEC (STp and STh)	STp and ST h	STa-Et-F2	CTGTATTATCTTTCCCCTCTTTTAGTC	M25607	317–343	167	76.64 ± 0.09		This study
			Sta-Et-R	AGGATTACAACACAGTTCACAGCAG		483–459				
			STb-Et-F	CGCTCAGGATGCTAAACCAG	M29255	78–97	145		2.6	
	TDH-positive *Vibrio parahaemolyticus*[2]	tdh	tdh-F2	ATGAGATATTGTTTGTTGTTCGAGA	X54341	208–232	177	79.26 ± 0.16		This study
			tdh-R2	TCACAGTCATGTAGGATGTCA		384–364			3.2	
	Plesiomonas shigelloides	*gyrB*	PSG-F64	TTAACGCCCTGTCGGATAAG	AJ300545	64–83	86	82.42 ± 0.22		This study
			PSG-R149	TACCGGCTCACCCAGATG		149–132				
G	EAEC (astA)[2]	*astA*	EAST-1-S	GCCATCAACACAGTATATCC	L11241	63–82	106	83.64 ± 0.27		(23)
			EAST-AS	GAGTGACGGCTTTGTAGTCC		168–149			1.9	
	EIEC and Shigella spp.	*ipaH*	ipaH1631-F	CAGGCAGAAGAGCAGAAGTATGA	M32063	1631–1653	141	85.58 ± 0.19		This study
			ipaH1771-R	CTGTTCAGTCTCACGCATCAC		1771–1751			3.0	
	Aeromonas hydrophila	*ahh1*	AHH1-1062-F	CCACGATTACCGGGTCCIAAC	AB206039	1062–1081	180	88.62 ± 0.33		This study
			AHH1-1241-R	GGCACGAACCCCTTGTAGC		1241–1223				
H	*Yersinia enterocolitica and Y. pseudotuberculosis*	*yadA*	yadA-F1757	ACGAGTTGACAAAGGTTTAGCC	X13882	1757–1778	203	82.38 ± 0.17[4]		(6)
			yadA-R1885X	GAACCAACCGCTAATGCCTGA		1885–1865		(81.87 ± 0.12[5])	3.3	
	Salmonella spp.[2]	*invA*	invA386-F	CGTTCGGGCAATTCGTTAT	M90846	386–404	277	85.68 ± 0.09		This study
			invA662-R	ATAAACTTCATCGCACCGTCA		662–642			2.9	
	DAEC	*afaD*	daaD-F31	GTCACCTGCGGGATGTTACT	AF233530	31–50	233	88.54 ± 0.33		(6)
			daaD-R263	AGCTCATGACGACCATCCTT		263–244				

[1]: Tm values represent means ± standard deviations calculated from 6 to 22 tests.
[2]: Eight main foodborne bacteria.
[3]: Tm value of stx2f.
[4]: Tm value of *Y. enterocolitica*.
[5]: Tm value of *Y. pseudotuberculosis*.
Tm, melting point temperature by melting curve analysis.

Table 4. Comparison of RFBS24 and RFBS24 ver.5 in EHEC, *Salmonella*, and *Campylobacter* infections

Causative pathogen	No. of patient samples	No. of positives with culture method	Results of RFBS24 and RFBS24 ver.5									
			RFBS24 combined with Qkit			RFBS24 combined with Ukit			RFBS24 ver.5 combined with Qkit		RFBS24 ver.5 combined with Ukit	
			No. of positives	Sensitivity (%)	Specificity (%)	No. of positives	Sensitivity (%)	Specificity (%)	No. of positives	Sensitivity (%)	No. of positives	Sensitivity (%)	Specificity (%)
EHEC	9	2 (*eae*/*stx1*/*stx2*) 2 (*eae*/*stx1*) 5 (*eae*/*stx2*)	1/0/2[1)]	—	—	5/1/3[1)]	—	—	5/1/5[1)]	—	—	9/4/7[1)]	—	—
Salmonella spp.	27	19	3	16	100	8	42	100	13	68	100	17	89	100
Campylobacter (*C. jejuni*/*C. coli*)	47	24/7[2)]	20/4[2)]	83/57[2)]	100/100[2)]	22/4[2)]	92/57[2)]	100/100[2)]	25/7[2)]	96/100[2)]	91/100[2)]	28/7[2)]	100/100[2)]	83/100[2)]

[1)]: The results of *eae*, *stx1*, and *stx2* are listed.
[2)]: The results of *C. jejuni* and *C. coli* are listed.
RFBS24, rapid foodborne bacterial screening 24; RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5; Qkit, QIAamp DNA stool mini kit; Ukit, ultra clean fecal DNA isolation kit.

and *stx2* in 2 of 9 fecal samples, EHEC O26 harboring *eae* and *stx1* in 2 of 9 fecal samples, and EHEC O121 harboring *eae* and *stx2* in 3 of 9 fecal samples, as determined by the bacterial culture method), *Salmonella* (5 outbreaks; *Salmonella* spp. were isolated from 19 of 27 fecal samples, as determined by the bacterial culture method), and *Campylobacter* (8 outbreaks; *C. jejuni* in 23 of 47 fecal samples, *C. coli* in 6 of 47 fecal samples, and *C. jejuni* and *C. coli* in 1 of 47 fecal samples, as determined by the bacterial culture method) foodborne outbreaks, fecal samples (1 g) from patients and from cooking or serving staff at causative restaurants were diluted 10-fold with phosphate-buffered saline. Next, 200 μL of the fecal suspension was used for isolation of DNA by means of the QIAamp DNA Stool Mini Kit (Qkit; Qiagen) or Ultraclean Fecal DNA Isolation Kit (Ukit; MoBio Laboratories, Inc., Carlsbad, CA, USA), according to modified methods described previously (24). These DNA samples were then subjected to analysis by RFBS24 and RFBS24 ver. 5. The results were interpreted as previously described and labeled as either positive or negative (6). Furthermore, when a bacterial pathogen was not isolated from a patient's fecal sample by the culture method but was detected by RFBS24 ver. 5, we analyzed the molecular weight of the PCR product obtained with RFBS24 ver. 5 by agarose gel electrophoresis and confirmed whether the PCR product had the expected molecular weight.

Statistics: Statistical analysis was performed in the PASW Statistics 18.0 software (SPSS, Chicago, IL, USA). Comparisons of the detection limits of RFBS24 and RFBS24 ver. 5 were conducted by nonparametric Mann-Whitney U tests. Differences with P values of less than 0.05 were considered statistically significant.

Ethical considerations: Because in this study, we tested patients' fecal samples according to the Food Sanitation Law and the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections, informed consent was waived. The Ethics Committee of the Shimane Prefectural Institute of Public Health and Environmental Science approved this study's protocol, including the waiver of informed consent, and the study was performed according to the Ethical Guidelines for Epidemiology Research of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

RESULTS AND DISCUSSION

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 16 genes of diarrheagenic *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 simultaneous amplification of multiple target genes in the same reaction well. Thus, the appropriate combination of primer pairs was carefully arranged in 8 sets, and we reduced the risk for combinations of primer pairs to amplify multiple target genes simultaneously in the same reaction well (e.g., combinations of primer pairs for *aggR* and *astA* of EAEC; for *eae*, *stx1*, and *stx2* of EHEC; for genotypes LT and ST of ETEC; for *astA* of EAEC and *afaD* of DAEC; for *trh* and *tdh* of *V. parahaemolyticus*; for *ces* and *nheB* of *B. cereus*; and for *C. jejuni* and *C. coli*).

We designed a competitive IAC (Fig. 1) for amplification with a primer pair from the 8 main foodborne pathogens in each set, rather than using a noncompetitive IAC. The use of an IAC in real-time PCR for detection of foodborne pathogens is necessary for identification of false negative results arising from the presence of DNA polymerase inhibitors or poor quality of DNA (25). Of the 2 types of IACs that can amplify target sequences (i.e., competitive IACs and noncompetitive IACs) (26–30), a competitive IAC was used for RFBS24 ver. 5. However, amplification of the target gene and the IAC with a common primer pair in the same reaction lowers the amplification efficiency for the target gene (26). Therefore, the concentration of the competitive IAC was adjusted to attain a C_t value between 31 and 34 to allow for amplification of the target gene.

We designed primers such that the T_m values of PCR products varied among the 3 target genes in each set (Table 3 and Fig. 2). In sets A–F, the T_m values ranged from 73.65°C to 82.53°C for the target gene and from 86.63°C to 87.18°C for the competitive IAC. In sets G and H, the T_m values were 81.87–88.62°C for the target gene and 77.17–77.21°C for the competitive IAC. The differences in T_m values among the target genes in the 8 sets ranged from 1.6°C to 5.4°C. For RFBS24, some target genes in set E showed only slight differences in T_m values (e.g., 0.8°C between *stx1* and *aggR*), and it was therefore difficult to distinguish between *stx1* and *aggR* in set E (6). Such small differences in T_m values among target genes were not observed in RFBS24 ver. 5, and discrimination of a target gene was therefore easier in the new RFBS24 ver. 5 assay than in the original assay.

Primer sensitivity and specificity of RFBS24 ver. 5: We evaluated the specificity and sensitivity of RFBS24 ver. 5 for multiplex real-time SG-PCR with sets A–H using 35 DNA samples (Table 1). False positive and false negative results were not observed in multiplex real-time SG-PCR for sets A–H. Although RFBS24 analyses did not detect genotype STh (6), multiplex real-time SG-PCR for set F in RFBS24 ver. 5 did detect both STp and STh. Furthermore, multiplex real-time SG-PCR for set A, which contained the *stx2* primer pair, detected all known *stx2* subtypes of EHEC (Table 1 and Fig. 3). The T_m values of *stx2a*, *stx2b*, *stx2c*, *stx2d*, and *stx2e* were highly similar (ranging from 82.74°C to 82.91°C). In contrast, the T_m value of *stx2f* (81.72°C) was lower than those of *stx2a*, *stx2b*, *stx2c*, *stx2d*, and *stx2e*, and the T_m value of *stx2g* (83.64°C) was slightly higher than those of *stx2a*, *stx2b*, *stx2c*, *stx2d*, and *stx2e*. Several 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 *stx2* 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 *stx2* subtypes in a cost-effective manner and could distinguish *stx2f* and *stx2g* from *stx2a*, *stx2b*, *stx2c*, *stx2d*, and *stx2e* via determination of the T_m 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^{0} to 5.6×10^{-4} ng/reaction. The detection limits (5.6×10^{0}–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^{-3}–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^{-1}–5.6×10^{-3} ng/reaction. In RFBS24 ver. 5, the detection limit for *stx2f* ranged from 5.6×10^{-2}–5.6×10^{-3} 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^{-3} to 5.6×10^{-5} 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

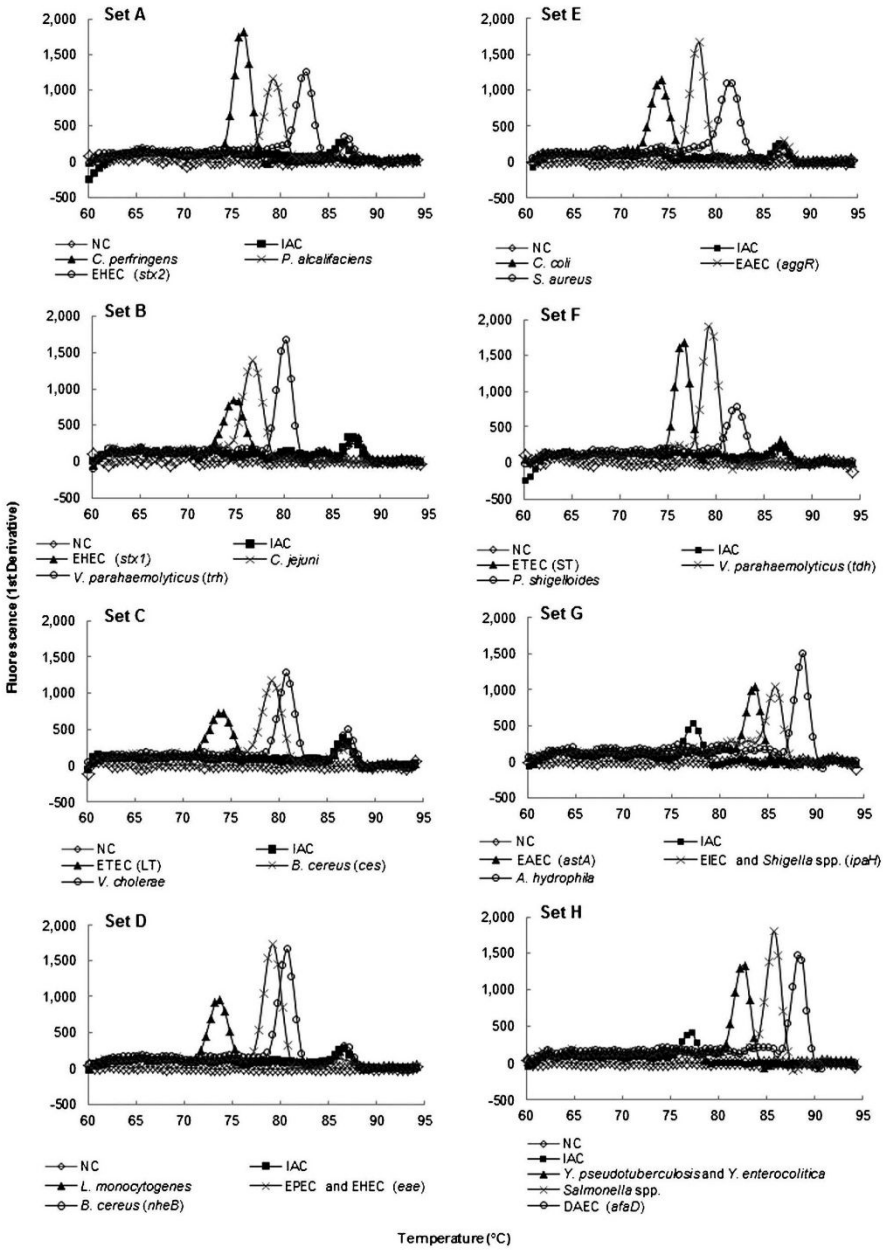

Fig. 2. Melting curve of 3 target genes of foodborne pathogens and the IAC by multiplex real-time SG-PCR in sets A–H. In sets A–F, the Tm values were 73.65–82.53 for the target genes and 86.63–87.18 for competitive IAC. In sets G and H, the Tm values were 81.87–88.62 for the target genes and 77.17–77.21 for competitive IAC. The differences between Tm values among the target genes in the 8 sets ranged from 1.6 to 5.4.

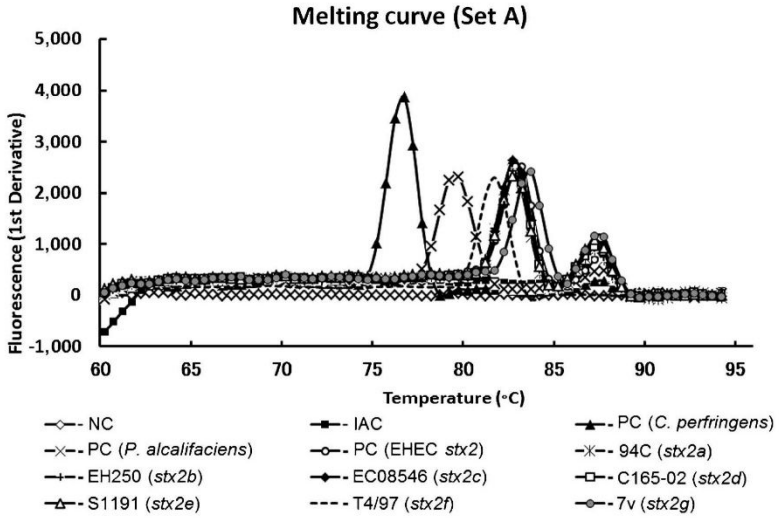

Fig. 3. Melting curve of the EHEC *stx2* subtype by multiplex real-time SG-PCR in set A. The Tm values of *stx2a*, *stx2b*, *stx2c*, *stx2d*, and *stx2e* were almost the same (82.74–82.91). The Tm value of *stx2f* (81.72) was lower than those of *stx2a*, *stx2b*, *stx2c*, *stx2d*, and *stx2e*, and the 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* were higher for RFBS24 ver. 5 than 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_t 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_t 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*

Table 5. Number of bacterial pathogens, excluding causative pathogens, detected using RFBS24 ver.5 and culture methods in *Salmonella* and *Campylobacter* infections

Bacterial pathogen (Target gene)	No. of positives			
	Campylobacter		*Salmonella*	
	RFBS24 ver.5 combined with Ukit	Culture	RFBS24 ver.5 combined with Ukit	Culture
EAEC (*astA*)	12	1	10	3
EPEC (*eae*)	4	2	8	
S. aureus (*femB*)	1	9	1	5
DAEC (*afaD*)	2		3	
P. alcalifaciens (*gyrB*)	1			
A. hydrophila (*ahh1*)			1	
C. perfringens (*cpe*)			2	
No. of patient samples	47		27	

RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5; Ukit, ultra clean fecal DNA isolation kit.

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 *stx2* 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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