表 7 共通試料を用いた菌種別・機種別試験結果 -換算菌数と Ct 値-

group	対象菌種	換算菌数	Ct値				group	対象菌種	換算菌数	Ct値			
			7500	7500FAST	Mx3000P	TP800				7500	7500FAST	Mx3000P	TP800
		cfu/mL	ABI	ABI	Stratagene	TaKaRa			cfu/mL	ABI	ABI	Stratagene	TaKaRa
A	*Cl. Perfringens*	5.0E+08	15.2	13.0	12.6	14.6	E	*S. aureus*	1.4E+09	16.8	15.0	14.2	17.0
		5.0E+07	19.0	17.1	16.4	17.6			1.4E+08	20.4	19.5	18.4	20.1
		5.0E+06	23.0	21.7	20.8	21.8			1.4E+07	23.6	23.7	22.4	23.7
		5.0E+05	24.2	25.5	24.1	25.2			1.4E+06	27.4	26.8	25.4	26.5
		5.0E+04	26.0	–	28.2	27.9			1.4E+05	30.0	–	–	29.1
		5.0E+03	28.2	–	–	–			1.4E+04	–	–	–	–
B	*C. jejuni*	4.9E+08	15.8	14.0	13.1	15.8	F	*tdh*陽性	4.1E+07	16.1	13.8	13.4	19.8
		4.9E+07	18.8	18.1	16.8	19.1		*V. parahaemolyticus*	4.1E+07	19.5	18.1	16.8	22.7
		4.9E+06	22.4	22.8	21.7	22.5			4.1E+06	21.9	21.7	20.6	26.1
		4.9E+05	24.4	26.4	25.0	26.0			4.1E+05	24.2	26.0	25.2	28.3
		4.9E+04	25.7	–	28.8	28.2			4.1E+04	26.9	–	29.0	–
		4.9E+03	27.1	–	–	–			4.1E+03	28.6	–	–	–
C	Emetic toxin producing	3.3E+07	21.0	19.7	18.8	20.6	G	*astA*陽性*E. coli*	1.1E+09	14.6	11.5	11.1	17.3
	B. cereus	3.3E+06	25.2	23.7	22.3	23.9			1.1E+08	16.5	15.2	14.1	20.6
		3.3E+05	29.2	–	26.9	27.0			1.1E+07	19.7	19.5	18.7	24.0
		3.3E+04	–	–	–	29.2			1.1E+06	22.8	22.7	21.6	27.1
		3.3E+03	–	–	–	–			1.1E+05	25.2	26.8	25.4	29.0
		3.3E+02	–	–	–	–			1.1E+04	28.7	–	–	–
D	*eae*陽性*E. coli*	1.5E+09	16.5	15.2	14.5	17.5	H	*Salmonella* spp.	6.3E+08	19.9	17.3	16.2	21.9
		1.5E+08	19.8	19.4	17.9	20.3			6.3E+07	23.2	21.1	20.2	24.5
		1.5E+07	23.5	23.3	22.1	24.1			6.3E+06	26.1	25.8	–	27.0
		1.5E+06	25.7	–	26.4	26.9			6.3E+05	–	–	–	28.2
		1.5E+05	29.3	–	–	–			6.3E+04	–	–	–	–
		1.5E+04	29.7	–	–	–			6.3E+03	–	–	–	–

表 8 SYBR Green 法に供した食中毒事例の便試料と検査結果（FP/08-12 事例）

番号	分類	事例区分	症状		ウェルシュ菌						セレウス菌					
			嘔吐	下痢	菌	菌	RPLA	PCR		SYBR	菌	RPLA	PCR		SYBR	
					直接塗抹	増菌	便*	便増菌	菌株		増菌	下痢毒	下痢毒	嘔吐毒	下痢毒	嘔吐毒
1	有症者便	A-1	×	○	+	+	+			+	−				−	−
2	有症者便	A-1	×	○	+	+	+	+		+	−				−	−
3	有症者便	A-2	×	○	−	+	NT	+	+	−	+	+			−	−
4	有症者便	A-3	×	○	+	+	+	+	+	+	−				−	−
5	有症者便	A-3	×	○	+	+	+	+	+	+	+	−	−	−	−	−
6	有症者便	A-3	×	○	+	+	NT	+	+	+	+	−	−	−	−	−
7	有症者便	A-4	×	○	+	+	+	+		+	−				−	−
8	有症者便	A-5	×	○	+	+	+	+		+	+	−	−	−	−	−
9	有症者便	A-1	×	○	+	+	NT	+		−	+	−	−	+	−	−
10	有症者便	A-3	×	○	+	+	+	+		+	−				−	−
11	有症者便	A-3	×	○	+	+	+	+		+	−				−	−
12	有症者便	A-2	×	○	−	+	NT	+		+	+	+	+		−	−
13	有症者便	A-6	×	○	+	+	NT	+		+	+	+	+		−	−
14	有症者便	A-6	×	○	+	+	NT	+		+	+	+	+		−	−
15	有症者便	A-6	×	○	−	+	NT	+	+	−	+	−	−	+	−	−
16	有症者便	A-5	○	○	−	−	NT	+	−	−	+	−	−	+	−	−
17	有症者便	A-5	○	×	−	+	NT	+		+	+	+		−	−	−
18	従事者便	A-7	−	−	−	−	−	−	−	+	+	+	+	−	−	−
19	従事者便	A-7	−	−	+	−	−	−	−	+	+	+	+	−	−	−
20	従事者便	A-7	−	−	+	−	−	−	−	−	+	+	+	−	−	−

NT: not tested

表 9　SYBR Green 法に供した食中毒事例の食品試料と検査結果(FP/08-12 事例)

	Clostridium perfringens												*Bacillus cereus*											
	菌培養				PCR				SYBR Green				菌培養				PCR				SYBR Green			
	原材料	検食	残品A	残品B	原材料	検食	残品A	残品B	原材料	検食	残品A	残品B	原材料	検食	残品A	残品B	原材料	検食	残品A	残品B	原材料	検食	残品A	残品B
煮物 鶏肉		×	○	×		×	○	○		○	○	○		×	○	○		×	○	○		×	○	○
煮物 こんにゃく			×				×				×				○				○				○	
煮物 ゆで卵		×	×	×		×	×	×		×	○	×		×	○	○		×	×	○		×	○	×
ひじき煮		×	×	×		×	×	×		×	×	×		×	○	○		×	×	×		×	×	×
白菜おひたし		×		×		×		×		×		×		×		×								
カニ甲羅揚げ		×	×			×	×			×	○			×	○			×	○			×	○	
サツマイモ天ぷら				×				×				×				×				×				×
細切りキャベツ																								
焼き魚		×		×		×		×		×		×		×		×		×		×		×		×
エビフライ	×	×	×	×	×	×	×	×	×	×	○	○	×	×	×	×	×	×	×	×	×	×	○	×
サニーレタス	×				×				×				×				×				×			
スパゲティケチャップ和え		×				×				×				×				×				○		
卵焼き																								
メンチカツ	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	○	×	×	×	○	×	×	×	×
スパゲティサラダ		×				×				×				×				×				×		
大根漬け物		×	×	×		×	×	×		×	×	×		×	×	×		×	×	×		×	×	×
ご飯			×	×			×	×			×	×			×	×			×	×			○	×

実勢枠は食品が単品に分離できなかったために1種類として検査したもの。

×で示した枠はそれぞれの方法で対象菌もしくは対象遺伝子が検出されなかったもの。○で示した枠はそれぞれの方法で対象菌もしくは対象遺伝子が検出されたもの。

表 10　平成 19 年度島根県における食中毒事例の検査

事例番号	発生日時	検査日時	発生施設	原因食品	患者数/喫食者	原因菌	糞便検査（陽性検体数/検体数）			
							Duplex SYBR Green PCR			分離培養
							初回	2回目	最終	
1	7/4	7/10	飲食店	食事（生牛レバー）	6/11	*C. jejuni*	2/3	-		2/3
2	10/21	10/22	飲食店	食事	7/13	EPEC	2/5			0/5
						P. shigelloides		2/5		2/5
3	11/26	11/30	飲食店	食事（生鶏レバー）	8/8	*C. jejuni*	3/5	-	4/7	4/8
						astA 陽性*E.coli*	1/5		1/7	1/8

表 11　RFBS24 を用いたリアルタイム PCR 法と従来法との比較

No。	検査時の日数経過	摂食者	原因食	患者数/総数	Real time PCR 陽性数/検査数	培養法 病原体	培養法 PCR 陽性数/検査数	培養法 陽性数/検査数	備考
1	2	仕事仲間	ちらし寿司	13/15	0/2	*Salmonella* Enteritidis	1/2	3/4	
2	6	市民	焼き肉	4/4	3/4	*C. jejuni*	3/4	3/4	
3	2	市民	不明	13/44	2/5	STEC O63 *(stx2f)*	1/5	1/5	*eaeA* and *astA* 1/5
4	13	市民	おにぎり	4/4	0/2	*Salmonella* Enteritidis	2/2	2/2	
5	3 〜 7	従業員	不明	-	3/7	EHEC O157	4/7	4/7	
6	3	グループ	弁当	171/296	7/7	*C. perfringens*	7/7	16/17	
7	1	投宿者	弁当	11/21	4/4	*S. aureus*	4/4	6/9	
8	4	市民	不明	9/16	4/4	*C. jejuni*	4/4	4/4	

表 12　食中毒事例の原因と考えられた菌種別のリアルタイム PCR と培養法の比較

	菌種・菌群	事例数	SYBR		培養		SYBR が陽性多い			培養が陽性多い		
			陽性	合計	陽性	合計	事例数	SYBR 陽性/計	培養陽性/計	事例数	SYBR 陽性/計	培養陽性/計
1	*Campylobacter jejuni*	14	51	77	50	77	1	5/6	1/6	2	4/7	5/7
											4/7	6/7
2	*Clostridium perfringens*	5	25	34	28	34	0			2	4/7	6/7
											2/6	3/6
3	EPEC, EHEC, *astA* 陽性	6	18	40	20	40	0			2	4/7	5/7
											3/7	4/7
4	*Salmonella* spp.	3	3	11	7	11	0			3	0/2	1/2
											0/2	2/2
											3/7	4/7
5	*Staphylococcus aureus, Bacillus cereus*	3	6	13	8	13	0			2	1/5	2/5
											1/4	2/4
6	*Vibrio parahaemolyticus Plesiomonas shigelloides*	3	6	18	7	18	0			1	1/7	2/7
	計	34	109	193	120	193	1			12		

Fukushima *et al*[13]ならびに表 11 について本研究事業の結果のみを合算した

図 1　Rapid Foodborne Bacteria Screening 24 の各ウェル

	1	2	3	4	5	6	7	8	9	10	11	12
反応系	陰性コントロール	IACコントロール	陽性コントロール	陽性コントロール	陽性コントロール	検体	検体	検体	検体	検体	検体	検体
A: GAP+PAG +JMS2+yersH2	試薬A: 16μl	IAC 2.5 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	PC-A1 ウエルシュ菌 2μl	PC-A2 P. a 2μl	PC-A3 EHEC 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl
B: ceuE+AB +trh250+yersH2	試薬B: 16μl	IAC 2.5 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	PC-B1 C.coli 2μl	PC-B2 C. jejuni 2μl	PC-B3 TRH V.p 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl
C:+yersH2 hly+ces+LT	試薬C: 16μl	IAC 2.5 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	PC-C1 リステリア菌 2μl	PC-C2 嘔吐型B.c 2μl	PC-C3 ETEC 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl
D: STA+eae+virA +yersH2	試薬D: 16μl	IAC 2.5 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	PC-D1 ETEC 2μl	PC-D2 EHEC 2μl	PC-D3 赤痢菌 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl
E: aggR+JMS1+FemB +yersH2	試薬E: 16μl	IAC 2.5 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	PC-E1 EAEC 2μl	PC-E2 EHEC 2μl	PC-E3 ブドウ球菌 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl
F: tdh176+yadA+PSG +yers79	試薬F: 16μl	IAC 1 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	PC-F1 TDH V. p 2μl	PC-F2 エルシニア菌 2μl	PC-F3 P. s 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl
G: ompW+EAST +AHH1+yers79	試薬G: 16μl	IAC 1 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	19: コレラ菌 2μl	20: EAEC 2μl	21: エロモナス菌 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl
H: SG+invA+daaD +yers79	試薬H: 16μl	IAC 1 加試薬Aを各wellに18μl										
	水: 4μl	水: 2μl	22: 下痢型B. c 2μl	23: サルモネラ 2μl	24: DAEC 2μl	検体1: 2μl	検体2: 2μl	検体3: 2μl	検体4: 2μl	検体5: 2μl	検体6: 2μl	検体7: 2μl

①各反応系において反応試薬の反応液の調合方法に従い、添付の混合プライマーとSYBR Premix 、ROX Reference Dye II 、水を12.5well分調製し、軽く混和後その16μlを第1wellに分注。

②残りの試薬にIAC液23μlを入れ、軽く混和後その18μlずつを第2～12wellに分注。

③各行の第1wellにPCRグレード水4μlを分注（陰性コントロール）

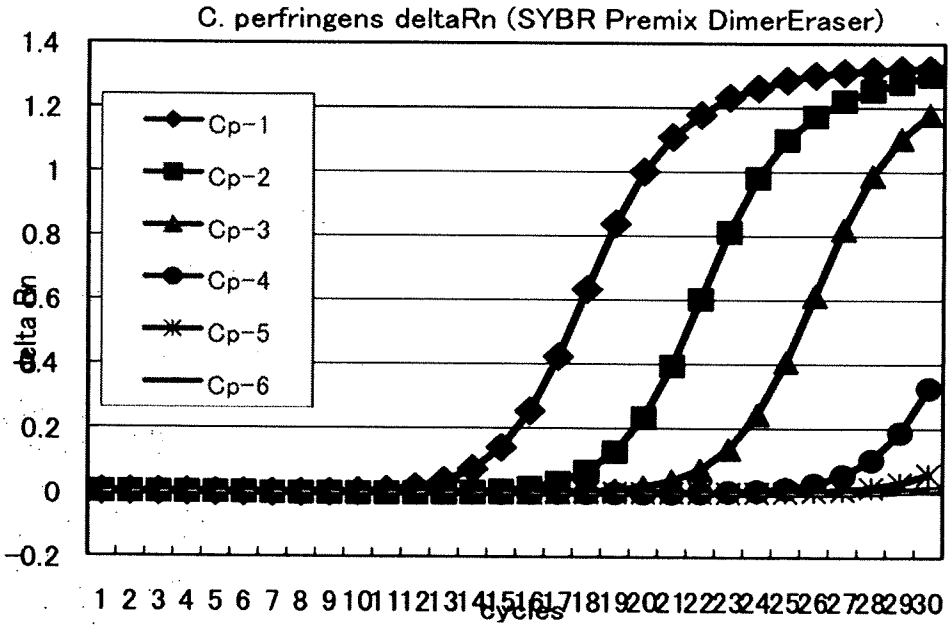
④各行の第2wellにPCRグレード水2μlを分注 （IACコントロール）

⑤各行の第3～5wellに各食中毒原因菌の鋳型DNAを2μlずつ分注 （陽性コントロール）

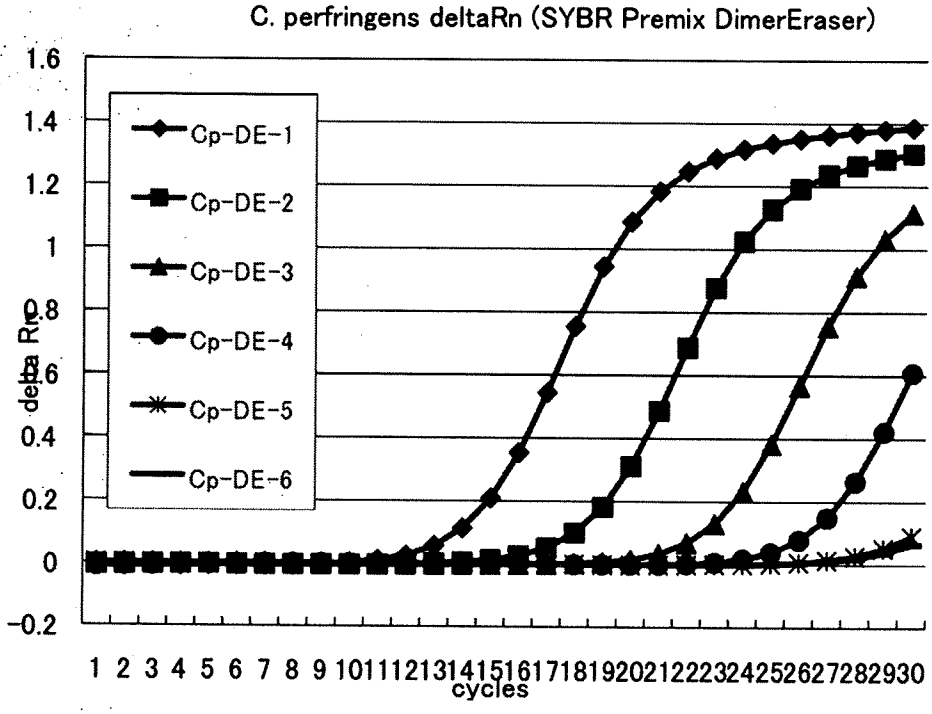
⑥各行の第6～12wellに患者糞便から抽出した鋳型DNAを2μlずつ分注

図 2　　ABI7500FAST を用いた RFBS24 結果(*C. perfringens*)

施設 A



施設 B



Simultaneous Screening of 24 Target Genes of Foodborne Pathogens in 35 Foodborne Outbreaks Using Multiplex Real-time SYBR Green PCR Analysis

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26

27 Key words: Multiplex real-time SYBR green PCR, Internal amplification control, Foodborne

28 pathogens, Foodborne outbreak

29 Running Head: Multiplex Real-time PCR Analysis of foodborne outbreak

30

31 Abstract:

32 A set of 8 multiplex real-time SYBR Green PCR (SG-PCR) assays including 3 target primers and

33 an internal amplification control (IAC) primer was simultaneously evaluated in 3 h or less with

34 regard to detection of 24 target genes of 23 foodborne pathogens in 7 stool specimens of

35 foodborne outbreak using a 96-well reaction plate. This assay, combined with DNA extraction

36 (QIAamp DNA Stool Mini kit), offered detection of greater than 10^3-10^4 foodborne pathogens per

37 g in stool specimens. The products formed were identified using melting point temperature (*Tm*)

38 curve analysis. This assay was evaluated for the detection of foodborne pathogens from 33 of 35

39 foodborne outbreaks, using different 4 PCR instruments in different 5 laboratories. No

40 interference from the multiplex real-time SG-PCR assay, including IAC, was observed in stool

41 specimens in any analysis. We found multiplex real-time SG-PCR assay for simultaneous

42 detection of 24 target genes of foodborne pathogens to be comprehensive, rapid, inexpensive,

43 accurate, of high selectivity, and good for screening probability.

1. Introduction

Technological advances in the past 2 decades have substantially increased the possibility of rapid diagnostic testing for many diseases. However, for bacterial pathogens which cause foodborne infections or foodborne outbreak, traditional culture methods, which can take up to 1 week, are still the only method many microbiology laboratories routinely use for diagnosis (1). Real-time PCR is one of the principle methodologies emerging for rapid diagnosis of foodborne outbreak. We previously reported a duplex real-time SYBR Green PCR (SG-PCR) screening system of 8 specific genes of foodborne pathogens in 5 fecal samples. (2, 3, 4) The introduction of this screening system in foodborne outbreak investigations provides an opportunity for comprehensive and rapid detection of pathogens in fecal samples. The results can quickly inform a public health administrator about the causative pathogens of foodborne outbreak, allowing a more accurate, effective and timely response. The real-time SG-PCR assay combined with DNA extraction using a QIAamp DNA Stool Mini kit offered detection of greater than 10^3-10^4 foodborne pathogens per g in fecal samples. For diagnosis and management of foodborne outbreaks, this could distinguish patients infected with foodborne pathogens from healthy carriers. In this real-time PCR assay using the LightCycler, a set of 4 duplex PCR should be evaluated from a first run within 2 h for detection of 8 main foodborne pathogens in 5 stool specimens. Other specific genes of approximately 10 foodborne pathogens should be analyzed by the 2nd and 3rd runs within a further 90 (45 and 45 min) min. If it is possible to test for multiple foodborne pathogens including enteric and toxin-producing bacteria at a time, real time PCR tests will certainly be useful for multiplex screening of foodborne pathogens. With multiplex PCR tests, if plural bacteria could be simultaneously detected in the same reaction tube or during the same run, molecular diagnosis may prove very cost-effective. However, at present, published evaluations of these assays are insufficient.

66 One of the risks associated with testing samples by PCR is the occurrence of a false negative resulting
67 from PCR inhibition (5, 6). While positive and negative controls are normally run with every PCR master
68 mix to ensure integrity of the reagents, PCR inhibition by the sample matrix can prevent amplification of
69 the target template, resulting in false-negative reporting (5, 6). Therefore, it is necessary to include an
70 internal amplification control (IAC) in each individual reaction mixture to prevent reporting of false
71 negatives (5). Previous works have utilized various methods for developing and using an IAC for detection
72 of a single target gene, except in the case of 4-target TaqMan multiplex PCR to detect *V. parahaemolyticus*
73 (7).

74 The objective of the present study was to establish simple and specific methods to simultaneously
75 detect 24 specific genes of foodborne pathogens in 7 stool specimens in a real-time SG-PCR assay using a
76 96-well reaction plate containing a universal, noncompetitive IAC.

77

78 **2. MATERIAL AND METHODS**

79 **2.1. *Bacterial Strains.*** The 659 foodborne pathogens used in this study are shown in Table 1.
80 The 23 foodborne pathogens (enteroinvasive *Escherichia coli*, enteropathogenic *E. coli*,
81 enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli,* diffusively adherent
82 *E. coli*, *Shigella* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Y. pseudotuberculosis*,
83 *Providencia alcalifaciens*, *Plesiomonas shigelloides*, *Campylobacter jejuni*, *C. coli*, *Vibrio*
84 *cholerae*, TDH-positive *V. parahaemolyticus*, TRH-positive *V. parahaemolyticus*, *Aeromonas*
85 *hydrophila*, *Staphylococcus aureus*, emetic *Bacillus cereus,* enterotoxigenic *B. cereus,*
86 *Clostridium perfringens* and *Listeria monocytogenes*) described as control strain in Table 1 are
87 used as control for PCR analysis. DNA was isolated from cultured bacteria to test the specificity

88 of the primers used in this study. Bacterial cultures and viable-cell counting were as previously
89 described (8).
90
91 **2.2. *Internal Amplification Control (IAC) and IAC Primers for PCR.*** An IAC was included
92 in the assay by adding a small amount of PCR products using IAC primers from the bacterium
93 *Yersinia ruckeri* (JCM15110), which is the causative agent of enteric red-mouth disease in
94 salmonid fish species (9) and is not found naturally in human fecal samples or food samples.
95 Bacterium used for DNA extraction was grown on brain heart infusion broth (Difco) at 30°C for 2
96 days. For the IAC primers for amplifying 16S rRNA gene (GenBank accession no. X75275) of
97 *Y. ruckeri*, two primer sets with different *Tm* of PCR products were used. One IAC primer was
98 yers described by Lund et al. (10) and the *Tm* value of PCR product used for this primer was
99 77.3±0.15°C. Another IAC primer sequence of yersH2-F and yersH2-R were chosen by
100 alignment of 16S rRNA gene sequence from foodborne pathogens shown in Table 2 using the
101 BLAST program within GenBank and was designed by Biosearch Technologies Inc. The *Tm*
102 value of PCR product used for this primer was 86.0±1.5°C.
103
104 **2.3. *DNA Extraction*** One milliliter of broth culture was centrifuged at 12,000× *g* for 3 min.
105 The pellet was then washed in 1 ml of distilled water, centrifuged, and suspended into 1 ml of
106 distilled water. Each 200 microliters of suspension, containing 10^8 foodborne bacterial cells, was
107 treated with the QIAamp DNA Stool Mini kit (Qiagen) according to manufacturer instructions.
108 DNA preparations were used immediately for PCR amplification and stored at -20 °C. Four μl
109 of DNA sample were used for PCR assay. Stool samples (1 g) were weighed aseptically, placed

110 into sterile tubes, and homogenized with 9 ml of distilled water. The 200 μl of stool suspension
111 was then treated with the QIAamp DNA Stool Mini kit in 1 h or less.
112
113 **2.4. *Target Primers*** Primers were used for 24 specific genes of 23 foodborne pathogens which
114 belonged to 16 species: *Escherichia coli* (enteroinvasive *E. coli*, enteropathogenic *E. coli*,
115 enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli* and diffusively
116 adherent *E. coli*), *Shigella* spp., *Salmonella* spp., *Yersinia enterocolitica*, *Y. pseudotuberculosis*,
117 *Providencia alcalifaciens*, *Plesiomonas shigelloides*, *Campylobacter jejuni*, *C. coli*, *Vibrio*
118 *cholerae*, *V. parahaemolyticus* (TDH-positive and TRH-positive types), *Aeromonas hydrophila*,
119 *Staphylococcus aureus*, *Bacillus cereus* (emetic and enterotoxigenic types), *Clostridium*
120 *perfringens* and *Listeria monocytogenes*, and the 2 IAC primers are listed in Table 2. The size and
121 melting point temperature (*Tm*) values of PCR products are also listed in Table 2. The
122 specificity and sensitivity of PCR assay using each primer were confirmed in each referred report.
123 The primer pairs of tdh-F176 and tdh-R422 for the detection of *tdh*-positive *V. parahaemolyticus*,
124 yadA-F1757 and yadA-R1885 for the detection of *Y. enterocolitica* and *Y. pseudotuberculosis*,
125 PSG-F64 and PSG-R313 for the detection of *P. shigelloides*, ipaH1672-F and ipaH1761-R for the
126 detection of *Shigella* spp. and EIEC and daaD-F31 and daaD-R263 for the detection of DAEC
127 were chosen by alignment of virulent or specific gene sequences from foodborne pathogens
128 shown in Table 2 using the BLAST (Basic Local Alignment Search Tool) program within
129 GenBank and was designed by Biosearch Technologies Inc. (Tokyo). The *Tm* values of these
130 primers varied from 74.5 to 88.7.
131

2.5. *Real-time Multiplex SG-PCR* Real-time multiplex SG-PCR and data analysis were performed for a total volume of 20 μl using 96-well reaction plates and an ABI7500 or ABI7500 Fast Real Time PCR system (Applied Biosystems), LightCycler 480 (Roche) or Thirmal Cycler Dice^{R} Real Time System (Takara, Japan). Each reaction tube contained 10 μl of SYBR *Premix DimerEraser* (Takara, Japan), 0.4 μl of ROX Reference Dye II (50×) (for ABI 7500 and ABI7500 Fast), 0.8 μl (for ABI 7500 and ABI 7500 Fast) or 1.2μl (for LightCycler 480 and Thirmal Cycler Dice) of PCR-grade H_2O, each 1.2 μl of a 10 μM primer set for 3 target genes, 1.2 μl of a 10 μM IAC primer set, 2 μl of IAC DNA and 2 μl of sample DNA in a 20-μl PCR mixture. In each of 8 lines (12 wells per line) on a 96-well reaction plate, the samples were set as: negative control (4 μl of dH_2O) in the 1st well, each 2 μl of IAC and dH_2O in the 2nd well, each 2 μl of IAC and 1 out of 3 target positive controls in the 3rd to 5th wells, and 2 μl of IAC and each 7 stool DNA samples in the 6th to 12th wells. The concentrations of positive control and IAC were adjusted to become the C_t values of the amplicons resulting from foodborne pathogens to 17 to 21 and those from IAC to 27 to 29 by dilution of 10^3 to 10^4-fold and 10^6 to 10^7-fold, respectively, with Easy Dilution (Takara, Japan). The assay cycling profile was one cycle of 95°C for 30 s followed by 30 cycles of denaturation at 95°C for 5 s (3 s for ABI 7500 Fast), annealing at 55°C for 30 s (34 s for ABI 7500) and then 72°C for 30 s (34 s for ABI 7500) and a dissociation stage of 1 cycle at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. The specificity of the reaction was found by the detection of the *Tm*s of the amplification products immediately after the last reaction cycle. These reactions were finished in 2 h or less. Results were analyzed with SDS software provided with each real time PCR system.

2.6. *Multiplex Real-time SG-PCR Analysis in 35 foodborne Outbreaks.* Multiplex real-time SG-PCR analysis of foodborne outbreak was experimentally tested using the ABI 7500 in Shimane (22 cases between 2002 and 2009), Fukuoka (3 cases between 2006 and 2009) and Shizuoka Prefecture 3cases on 2009), using ABI 7500 Fast in Fukuoka Prefecture (2 cases on 2009), using Thirmal Cycler Dice^{R} Real Time System in Hokkaido (3 cases between 2008 and 2009) and using LightCycler 480 in Kumamoto Prefecture (2 cases on 2009) (Table 3). The DNA samples were extracted with the QIAamp DNA Stool Mini kit from patient fecal samples (within 1 h) and were set on a 96-well reaction plate as described above. The samples before 2008 were used after 1 to 3 years store at -20°C. The multiplex PCR assay was evaluated with regard to detection (in 2 h or less) of 24 specific genes of foodborne pathogens in 7 stool specimens. Each PCR product was generated with a different *Tm* curve among 4 *Tm* curves of PCR target gene products. These could all be resolved using each software and *Tm* curve analysis whenever target bacteria were present in the reaction well.

3. Results and Discussion

3.1. *Universal Noncompetitive IAC and IAC Primers.* In this study, the *Y. ruckeri* bacterium was successfully used as a universal noncompetitive IAC and for 2 pairs of IAC primer for routine detection of 24 target genes of foodborne pathogens. Using an IAC with real-time PCR detection is important to identify false negative results and to control for the presence of amplification inhibitors (27). It is important to take into account that components of the sample or the competing micro flora may influence the effectiveness of the PCR, especially by reducing the detection limit and producing false negative results. The consequences of false negative

results in the detection of a pathogenic micro organism may potentially be life threatening (28). The European Standardization Committee, in collaboration with International Standard Organization (ISO) has proposed a general guideline for PCR testing of foodborne pathogens that requires presence of IAC in the reaction mixture (29).

While some design approaches such as cloning require substantial technical skills, others can be done using basic PCR methodology. There are two main strategies for use of an IAC in a diagnostic real-time PCR assay. Their difference lies in whether the IAC is to be used competitively or noncompetitively (5). By using the composite primer technique, the target and the competitive IAC are amplified with one common set of primers and under the same conditions and in the same real-time PCR tube. The competitive IAC method was used for TaqMan PCR to detect *S. enterica* (27, 30, 31), *E. coli* O157 (32) and *C. botulinum* (33) and real-time SG-PCR to detect *C. botulinum* (34). However, these competitive IAC methods can lower the amplification efficiency, which results in a lower detection limit (5). In the noncompetitive IAC method, the target and IAC are amplified using a different primer set for each. The disadvantage is that amplification of the IAC may not accurately reflect amplification of the target. This method was used for TaqMan PCR to detect *Campylobacter* spp. (10, 35), *B. cereus* (36), *C. botulinum* (37), and *V. parahaemolyticus* (7). These assays were used for the detection of single target gene except for the four-target TaqMan multiplex PCR to detect *V. parahaemolyticus* (7). Although the main advantage of the noncompetitive IAC method is that it can be used for many different assays in the same laboratory (5), we do not have a unique real-time PCR assay for the detection of almost all foodborne pathogens using universal IAC.

Each previously described method for introduction of an IAC is limited due to primer

198 competition or because it requires the presence of a specific substrate or organism. The new
199 approach presented in this paper comprises a separate amplification of target DNA and
200 noncompetitive IAC-DNA using each specific target primer set and two different IAC primer sets
201 on the detection of each foodborne pathogens. The latter is based on 16S rRNA of *Y. ruckeri,*
202 which is not found naturally in human stool and food samples.

203 The IAC primer yersH2 was used for detection of 15 target genes of foodborne pathogens
204 which *Tm* values of amplicons were lower than 83°C and shown as primer sets A to E (described
205 in the next section), and the IAC primer yers was used for detection of 9 target genes of
206 foodborne pathogens which *Tm* values of amplicons were over than 80°C and shown as primer
207 sets F to H (described in the next section). The IAC-specific low peak on a *Tm* curve analysis
208 was present in all reaction tubes added with IAC and IAC primers and in all the negative results
209 of target PCR in reaction tubes added with IAC primers (Figures 1 and 2).

210

211 **3.2. *Development of PCR Procedures for a Set of 8 Multiplex Assays.*** We developed the
212 ultimately new PCR screening system for foodborne pathogens in stool specimens. One can
213 simultaneously analyze 24 pathogenic or specific genes of foodborne pathogens in 7 stool
214 specimens by using multiplex real-time SG-PCR containing IAC and 96-well reaction plate.
215 Single or multiple real-time PCR assays were reported for detection of one species among
216 foodborne pathogens, such as *E. coli* (12, 18, 19,23), *Salmonella* (27, 29, 30), *C. jejuni* (14, 38), *V.*
217 *cholerae* (20), *V. parahaemolyticus* (398), and *S. aureus* (22). Comprehensive, rapid real-time
218 SG-PCR procedures, which used 24 primer pairs for detection of 15 bacterial species including: 6
219 groups of *E. coli,* 2 subgroups each of *B. cereus* and *V. parahaemolyticus* and 2 primer pairs for

220 an IAC, were developed using a set of 8 multiplex PCR assays with 3 primer pairs for foodborne
221 pathogens and an IAC primer pair. Nineteen pairs of primers for foodborne pathogens were
222 selected from earlier publications (Table 1), and 5 pairs of primers for *tdh* gene of TDH-positive
223 *V. parahaemolyticus, yadA* gene of *Y. enterocolitica* and *Y. pseudotuberculosis, gyrB* gene of *P.*
224 *shigelloides, ipaH* gene of EIEC and *Shigella* spp., and *daaD* gene of DAEC were constructed.
225 This was done to make all 24 SG-PCR methods suitable for the same PCR conditions (an
226 annealing temperature of 60 °C). The sequence, target, PCR product size, threshold cycle (C_t)
227 values and *Tm* values of 24 primer pairs for target genes and 2 primer pairs for IAC are listed in
228 Table 2. The specificity of the PCR assay was tested on 659 strains listed in Table 1. The
229 STa-F and STa-R primer pair could not detect *st* gene from 5 of 18 *st*-positive ETEC strains.
230 The ipaH1672-F and ipaH1761-R primer pair cross-reacts with *Shigella* spp. and EIEC, the
231 eae-F2 and eae-R primer pair cross-reacts with EPEC and EHEC, and the EAST-1S and
232 EAST-1AS primer pair cross-reacts with EAEC and some strains of EPEC, ETEC and DAEC.
233 The yadA667-F and yadA851-R2 for *Yersinia* adhesion reacts with virulent *Y. enterocolitica* and
234 *Y. pseudotuberculosis,* but not with nonpathogenic strains of *Yersinia* spp. (4). The SG-F and
235 SG-R primer pair cross-reacts with enterotoxigenic and emetic *B. cereus.*
236 A Foodborne Outbreak Investigation Report (http://www.mhlw.go.jp/topics/syokuchu/), by
237 the Ministry of Health, Labor and Welfare, Japan during 2005 to 2008 shows that 97% of
238 foodborne outbreaks were caused by the following 7 species of foodborne pathogens: *C. jejuni*
239 (56.5%), *S. enterica* (16.0%), TDH-positive *V. parahaemolyticus* (10.0%), *S. aureus* (6.8%), *C.*
240 *perfringens* (3.4%), emetic *B. cereus* (2.0%), and EHEC (2.4%); and other virulent *E. coli* (2.1%)
241 which include *astA*-positive *E. coli* which is a strain of *E. coli* that does not possess any

242 diarrheagenic characteristics except the EAEC heat-stable toxin 1 (EAST1) gene and is frequently
243 isolated in diarrhea outbreaks (40). Each primer set was combined with 4 primer pairs designed
244 for 1 of 8 main foodborne pathogens (TDH-positive *V. parahaemolyticus, C. perfringens, S.*
245 *enterica, C. jejuni, S. aureus,* emetic *B. cereus,* EHEC and *astA*-positive *E. coli*) and were also
246 designed for IAC and 2 of 16 target genes of other foodborne pathogens (Table 2). In the
247 SG-PCR assay, each PCR product, with 8 primer sets including set A (GAP, PAG, JSM2 and
248 yersH2), set B (ceuE, AB, trh and yersH2), set C (Lm-hly, ces, LT and yersH2), set D (STa, eae,
249 ompW and yersH2), set E (aggR, JMS1, FemB and yersH2), set F (tdh, yadA, PSG and yers), set
250 G (EAST, ipaH, AHH1 and yers) and set H (SG, invA, daaD and yers) could be resolved in the
251 ABI 7500 by using T_m curve analysis (Table 2, Figures 1 and 2). IAC primer yersH2 (*Tm* value
252 is 86) was used for primer sets A to E (*Tm* values are under 83) and IAC primer yers (*Tm* value is
253 77.3) was used for primer sets F, G and H (*Tm* values are over 80). The fluorescent
254 amplification curves and *Tm* curves of the multiplex SG-PCR products of the DNA of foodborne
255 pathogens and IAC were shown in Figure 1. The C_t values of the amplicons resulting from
256 foodborne pathogens were 17 to 21 and those of IAC were 27 to 29. In each analysis, the T_m
257 distance was from 0.8 to 6.2°C [illegible]. The IAC-specific low peak
258 was present in all samples with added IAC (C_t: 27 to 29) using real-time SG-PCR analysis of the
259 24 target genes of primer sets A to E including primer yersH2 and of primer sets F, G and H
260 including primer yers. IAC was certainly amplified in the IAC-only samples. These could be
261 resolved in the ABI 7500 by using *Tm* curve analysis when a target bacterium was present in the
262 reaction tube. The *Tm* values of PCR products of stool samples, including each foodborne
263 pathogens, could be identified with that of control bacteria in the same run based on a *Tm* curve