264 analysis.

265

266 **3.3. *Using M*ultiplex *SG-PCR for Identification of the Causative Pathogens in 35 Foodborne***

267 ***Outbreaks.*** In foodborne outbreaks, stool specimens from patients infected with enteric bacteria

268 with acute severe disease may contain large numbers of causative bacterial species (2 12). In

269 most cases of foodborne outbreak, we found that causative bacteria can be rapidly detected and

270 that a presumptive diagnosis of the causative agent of foodborne outbreak could be made within 3

271 h. We used a combination of the multiplex real-time SG-PCR assay with DNA extraction with

272 the QIAamp DNA Stool Mini kit used for detection. Almost all bacterial pathogens are

273 detectable in stool specimens at a concentration of 10^3 to 10^4 bacteria per g. This is because the

274 concentration of DNA extracted from stool specimens using this DNA extraction kit was finally

275 diluted to 6×10^4-fold in the reaction mixture. The PCR sensitivity for bacteria inoculated in

276 stool samples may be as low as the presence of 10 cells in the reaction mixture, as described in

277 our previous report (2). The real-time SG-PCR assay is a rapid, specific, and sensitive detection

278 technique. The DNA extraction of 7 stool specimens with this DNA extraction kit was carried

279 out within 1 h. Then, the multiplex real-time SG-PCR assay was also carried out within 2 h.

280 The product curves could be monitored in real time without product separation by gel

281 electrophoresis. And we could then specifically identify the products based on a *Tm* curve

282 analysis. For example, Figure 2 shows those of Case 21, in which *C. jejuni* and *astA*-positive *E.*

283 *coli* strains were isolated from 4 and one of 7 stool specimens of symptomatic patients,

284 respectively. Two panels show detection of target genes of foodborne pathogens by primer sets B

285 and G, but it was not detected by the other 6 primer sets. In multiplex PCR analysis, the *C.*

286 *jejuni*-specific gene and the *astA* gene were simultaneously detected by primer sets B and G from
287 the same culture -positive stool specimens.

288 Table 3 shows epidemiological and clinical investigations in 35 foodborne outbreaks
289 (occurred between 2002 and 2009) examined by multiplex SG-PCR analysis in 5 different
290 laboratories on 2009. DNA samples extracted from 2 to 7 feces (2 feces in 3 cases, 3 feces in 1
291 case, 4 feces in 5 cases, 5 feces in 4 case, 6 feces in 4 cases and 7 feces in 18 cases) of
292 symptomatic patients were stored at -20°C until using. In 33 (94.3%) of 35 foodborne outbreak
293 cases, the causative bacteria and/or some sporadic bacteria were comprehensive and
294 simultaneously detected using multiplex SG-PCR from stool specimens. Moreover the same
295 reactions, which IAC-specific low peak was present in reaction tubes added with IAC and IAC
296 primer yersH2 or yers, were observed. This demonstrated the absence of reaction inhibition in
297 DNA specimens extracted from patient stool specimens using this DNA extraction kit. In this
298 study, it was confirmed that using the universal IAC and 2 IAC primers with different *Tm* values
299 was advantageous to allow elimination of false negative results in real-time SG-PCR for the
300 detection of 24 target genes of foodborne pathogens. The results of multiplex real-time SG-PCR
301 assay of 7 foodborne outbreaks were confirmed by the use of universal IAC and 2 IAC primers.
302 The certain amplification of target genes and IAC in each multiplex PCR analysis demonstrated
303 the usefulness of this multiplex real-time SG-PCR as reliable diagnostic PCR.

304 The target genes of 12 species of foodborne bacteria (*C. jejuni, E. coli, C. perfringens, S. aureus,*
305 *Salmonella* spp., *V. parahaemolyticus, V. cholerae* non O1, *B. cereus, P. alcalifaciens, P.*
306 *shigelloides* and *A. hydrophila*), which included 5 groups of *E. coli* (EHEC, EPEC, EAEC, ETEC
307 and *astA*-positive *E. coli*), were detected from 129 (64.8%) of 199 feces in 33 (94.3%) of 35 cases

308 by multiplex SG-PCR; from 1 to 7 samples: 1 (in 2 cases: 20 and 31), 2 (in 8 cases: 7, 9, 13, 15,
309 22, 25, 27 and 34), 3 (in 4 cases: 8, 19, 14 and 35), 4 (in 9 cases: 2, 3, 5, 6, 10, 18, 21, 29 and 30),
310 5 (in 2 cases: 4 and 31), 6 (in 4 cases: 1, 12, 16 and 33) and 7 samples (in 4 cases: 11, 14, 17 and
311 28). Multiplex SG-PCR rapidly and accurately demonstrated that 11 (31.4%) of 35 cases were
312 caused with a single foodborne pathogen such as *C. jejuni* (7 cases), *C. perfringens* (2 cases), *B.*
313 *cereus* (1 case), and *S.* Enteritidis (1 case). There were also 19 (54.2%) cases with plural
314 foodborne bacterial pathogens and 3 (2.9%) cases with foodborne bacterial pathogens
315 (*astA*-positive *E. coli,* EHEC O:26 or *C. perfringens*) and norovirus. The causative pathogens had
316 been isolated from 125 (62.8%) of 199 PCR examined samples and from 216 (56.7%) of 381 total
317 samples in all 35 cases. Although the target genes of EPEC, EAEC, ETEC, *astA*-positive *E. coli,*
318 *P. alcalifaciens* and *A. hydrophila* were detected by SG-PCR, the isolation of these pathogens
319 from the stool samples containing much normal *E. coli* flora was difficult. This analysis may be
320 very useful tool for the detection of these unusual pathogens which are generally difficult to
321 isolate. We previously the presence of any foodborne pathogens at more than 10^3 CFU/g feces
322 was certainly confirmed by melting curve analysis in duplex SG-PCR (2, 4). In this multiplex
323 PCR analysis including IAC, the presence of any foodborne pathogens at more than 10^5 CFU/g
324 feces was certainly confirmed in 40 (97.6%) of 41 samples by melting curve analysis, 10^4 CFU/g
325 feces was confirmed in 7 (63.6%) of 11 samples and 10^4 CFU/g feces in 3 (50%) of 6 samples
326 (Table 4). The sensitivity of this multiplex SG-PCR including IAC might became slightly lower
327 than that of duplex SG-PCR (absent IAC), caused by the interference among 4 primer pairs
328 including IAC primer in the same reaction well. In 2 cases (5.7%), in which *S.* Enteritidis was
329 isolated by direct culture (unknown cfu) from one patient in case 23 and 10^4 cfu/g of feces from 2

330 patients in case 26, the target gene of *Salmonella* was not detected by multiplex SG-PCR, because
331 the sensitivity of invA2 primer may be slightly lower than those of other primers. The choice or
332 design of more sensitive primer for the detection of *Salmonella* spp. is indispensable in future
333 studies.

334 Systematically reviewing clinical implications, public health considerations and cost-effectiveness of
335 rapid diagnostic tests for detection and identification of bacterial intestinal pathogens in feces and food (1),
336 economic modeling suggests that adoption of rapid test methods, especially for PCR, in combination with
337 a routine culture is unlikely to be cost-effective, however, as the cost of rapid technologies decreases, total
338 replacement with rapid technologies may be feasible. Despite the relatively poor quality of reporting of
339 studies evaluating rapid detection methods, the reviewed evidence shows that PCR for *Campylobacter*,
340 *Salmonella* and *E. coli* O157 is potentially very successful in identifying pathogens. It is possibly
341 detecting more than the numbers currently being reported using cultures. Less is known about the
342 benefits of testing for *B. cereus*, *C. perfringens* and *S. aureus*. This review pointed out that further
343 investigation is needed on how clinical outcomes may be altered if test results are available more quickly
344 and at greater precision than the current practice of using bacterial culture (1). In the present study,
345 simple and specific methods were established to detect comprehensive and simultaneously 24 specific
346 genes of foodborne pathogens including main bacterial pathogens such as *Campylobacter*, *Salmonella, E.*
347 *coli* O157, *B. cereus*, *C. perfringens* and *S. aureus* in 7 stool specimens in a real-time SG-PCR assay using
348 a 96-well reaction plate containing a universal noncompetitive IAC. The usefulness of this method for
349 the rapid diagnostic tests was confirmed by the successful detection of causative bacteria in 33 foodborne
350 outbreak cases.

351 In conclusion, the multiplex real-time method described here for simultaneous screening of 24

352 target genes of foodborne pathogens were comprehensive, rapid, inexpensive, highly selective,
353 accurate, and demonstrated detection probability. Due to the use of IAC and 2 IAC primers,
354 the assay is suitable for accurate and rapid diagnosis of almost all foodborne pathogens in stool
355 specimens of foodborne outbreak outbreaks. In future studies, workers should improve the kit
356 of multiplex real-time PCR and select more suitable primers for foodborne pathogens.
357
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361
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481

482 **FIGURE LEGENDS**

483 Fig. 1 Amplification (A) and melting curve analysis (B) of 3 target genes of foodborne pathogens
484 and IAC gene by primer sets A to H in multiplex real-time SG-PCR.
485
486 Fig. 2 Melting curve analysis of multiplex real-time SG-PCR products from 7 stool samples in
487 case 21 of a foodborne outbreak. Two panels show detection of target genes of foodborne
488 pathogens by primer sets B and G, but it was not detected by the other 6 primer sets.
489

TABLE 1. 659 bacterial strains assayed by real-time PCR

Sbacterial strain	Presence of PCR target gene	Control strain	Number of strains	PCR positive result with each primer set[g]																											
				eae	JMS1	JMS2	LT	STa	aggR	EAST	daaD	ipaH	invA	yadA	PSG	PAG	AB	ceuE	AHHl	ompW	tdh	trh	GAP	Lm-hly	FemB	ces	SG	yers	yers H2
Escherichia coli-STEC	*eae, stx1, stx2*	SE02007	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	eae, stx1		15	15	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	eae, stx2		7	7	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	stx1, stx2		2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	stx2		1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli-EPEC	*eae*	EC2736[a]	3	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	eae, astA		5	5	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli-ETEC	*LT*		3	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	ST		9	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	astA, LT		1	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	astA, LT,ST	EC3515[a]	2	-	-	-	2	2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	astA, ST		7	-	-	-	-	3	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli-EAEC	*astA, aggR*	EC4131[a]	8	-	-	-	-	-	8	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	aggR		26	-	-	-	-	-	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	astA		30	-	-	-	-	-	-	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli-DAEC	*daaD, astA*		2	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	daaD	KI2214[b]	2	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli-EIEC	*ipaH*	EA32[c]	5	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Shigella spp.	*ipaH*	I00031	38	-	-	-	-	-	-	-	-	38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella spp.	*invA*	Sal2339	31	-	-	-	-	-	-	-	-	-	31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yersinia enterocolitica	*yadA*	Pa241	28	-	-	-	-	-	-	-	-	-	-	28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	*yadA*	SP988	27	-	-	-	-	-	-	-	-	-	-	27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Plesiomonas shigelloides	*gyrB*	NIID123[d]	4	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Providencia alcalifaciens	*gyrB*	NIID124[d]	8	-	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-
Campylobacter coli	specific gene	SC01	43	-	-	-	-	-	-	-	-	-	-	-	-	-	43	-	-	-	-	-	-	-	-	-	-	-	-
Campylobacter jejuni	*ceuE*	SC009	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13	-	-	-	-	-	-	-	-	-	-	-
Aeromonas hydrophila	*ahh1*	ATCC7966	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45	-	-	-	-	-	-	-	-	-	-
Vibrio cholerae	*ompW*	ATCC14035	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	*tdh*	SVP02	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	48	-	-	-	-	-	-	-	-
	tdh, trh		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-
	trh	NIIDk4[d]	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35	-	-	-	-	-	-	-
Clostridium perfringens	*cpe*	H2[c]	41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	41	-	-	-	-	-	-
Listeria monocytogenes	*hly*	Scott A	46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	46	-	-	-	-	-
Staphylococcus aureus	*FemB*	SS05	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35	-	-	-	-
Emetic *Bacillus cereus*	*ces, nheB*	No.127[f]	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	24	24	-	-
Enterotoxgenic *B. cereus*	*nheB*	No.1[f]	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25	-	-
Yersinia ruckeri	16S rRNA	JCM15110	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1

[a] Strain kindly donated by J. Yatsuyanagi, Akita Prefectural Institute of Public Health (Akita, Japan), [b] K. Ito, Natural Institute of Infectious Disease (Tokyo, Japan)., [c] K. Sugiyama, Shizuoka Prefectural Institute of Public Health (Shizuoka, Japan), [d] E. Arakawa, Natural Institute of Infectious Disease (Tokyo, Japan), [e] S. Kaneko, Tokyo Metropolitan Institute of Public Health. (Tokyo, Japan), [f] S. Ueda, Kagawa Nutrition Univ. (Saitama, Japan), [g] number, positive result; -, negative result. See Table 2 for primer sets..

TABLE 2. Eight sets of real-time multiplex PCR with 4 primer pairs for 3 target genes and an IAC gene prior to comprehensive and rapid analysis of food-borne outbreak.

Primer set		Species	Target gene	Primer name		Gene Bank accession no.	Location	Product size (bp)	*Tm* [a]	*Tm* distance	Ref.
	*[b]	*Clostridium perfringens*	*cpe*	GAP-11	GGTTCATTAATTGAAACTGGTG	X81849	583-604	154	75.8±0.37		11
				GAP-12	AACGCCAATCATATAAATTACAGC		712-736			3.7	
A		*Providencia alcalifaciens*	*gyrB*	PAG38-F	TCTGCACGGTGTGGGTGTT	AJ300547	38-56	73	79.5±0.79		2
				PAG110-R	ACCGTCACGGCGGATTACT		110-92			1	
		EHEC (Stx 2)	*Stx2*	JMS2-F	CGACCCCTCTTGAACATA	EF441616	140-157	108	80.5±0.76		12
				JMS2-R	GATAGACATCAAGCCCTCGT		228-247				
		Campylobacter coli	*ceuE*	ceuE-For	CAAGTACTGCAATAAAAACTAGCACTACG	X88849	2777-2805	72	73.7±0.43		13
				ceuE-Rev	AGCTATCACCCTCATCACTCATACTAATAG		2848-2819			4	
B	*	*Campylobacter jejuni*	specific	AB-F	CTGAATTTGATACCTTAAGTGCAGC	AL111168	381121-	86	77.7±0.96		14
				AB-R	AGGCACGCCTAAACCTATAGCT		381185-			1.9	
		TRH-positive *Vibrio parahaemolyticus*	*trh*	trh250-F	GGCTCAAAATGGTTAAGCG	AY742213	705-687	250	79.6±0.21		15
				trh250-R	CATTTCCGCTCTCATATGC		456-474				
		Listeria monocytogenes	*hly*	Lm-hly-F	GGGAAATCTGTCTCAGGTGATGT	AF253320	973-995	106	77.4±0.78		16
				Lm-hly-R	CGATGATTTGAACTTCATCTTTTGC		1078-1054			1.5	
C	*	Emetic *Bacillus cereus*	*ces*	ces-TM-F	GATGTTTGCGACGATGCAA	DQ360825	8689-8707	65	78.9±0.87		4
				ces-TM-R	CTTTCGGCGTGATACCCATT		8734-8793			1.6	
		ETEC (LT)	*lt*	LT-1	TTACGGCGTTACTATCCTCTCTA	X83966	233-255	275	80.5±0.21		17
				LT-2	GGTCTCGGTCAGATATGTGATTC		507-485				
		ETEC (ST)	*st*	STa-F	GCTAATGTTGGCAATTTTTATTTCTGTA	M25607	294-321	190	77.1±0.55		18
				STa-R	AGGATTACAACAAAGTTCACAGCAGTAA		456-483			1.7	
D	*	EHEC and EPEC	*eaeA*	eae-F2	CATTGATCAGGATTTTTCTGGTGATA	Z11541	899-924	106	78.8±0.54		19
				eae-R	CTCATGCGGAAATAGCCGTTA		979-1000			2.6	
		V. cholerae	ompW	ompW-F	AACATCCGTGGATTTGGCATCTG	X51948	675-692	89	81.4±0.69		20
				ompW-R	GCTGGTTCCTCAACGCTTCTG		741-763				
		EAEC	*aggR*	aggRks1	GTATACACAAAAGAAGGAAGC	Z18751	18-38	254	79.2±0.25		21
				aggRKas2	ACAGAATCGTCAGCATCAGC		170-151			0.8	
E		EHEC (Stx 1)	*Stx1*	JMS1-F	GTCACAGTAACAAACCGTAACA	EF441598	509-488	95	80.0±0.72		12
				JMS1-R	TCGTTGACTACTTCTTATCTGGA		415-437			1.6	
	*	*Staphylococcus aureus*	*femB*	FemB-fw	AATTAACGAAATGGGCAGAAACA	AF106850	277-299	93	81.6±0.62		22
				FemB-rv	TGCGCAACACCCTGAACTT		351-370				
	*	TDH-positive *Vibrio parahaemolyticus*	tdh	tdh-F176	TCCATCTGTCCCTTTTCCTG	X54341	176-195	247	80.1±0.22		This study
				tdh-R422	AGACACCGCTGCCATTGTAT		422-403			1.9	
F		*Y. enterocolitica* and *Y. pseudotuberculosis*	*yadA*	yadA-F1757	ACGAGTTGACAAAGGTTTAGCC	X13882	1757-1778	129	82.0±0.38		This study
				yadA-R1885	GAACCAACCGCTAATGCCTGA		1885-1865			4.3	
		Plesiomonas shigelloides	*gyrB*	PSG-F64	TTAACGCCCTGTCGGATAAG	AJ300545	64-83	250	86.3±0.26		This study
				PSG-R313	TCGAGCAGATGAATCGACAC		313-294				
	*	EAEC	*astA*	EAST-1-S	GCCATCAACACAGTATATCC	L11241	63-82	106	83.7±0.88		23
				EAST-AS	GAGTGACGGCTTTGTAGTCC		148-168			1.5	
G		EIEC and *Shigella* spp.	*ipaH*	ipaH1672-F	CTCTCAGAGGGTGGCTGACC	M32063	1672-1691	90	85.2±0.31		This study
				ipaH1761-R	TCACGCATCACCTGTGCA		1761-1743			3.1	
		Aeromonas hydrophila	*ahh1*	AHH1-F	GCCGAGCGCCCAGAAGGTGAGTT	CP000462	1653360-82	130	88.3±0.48		24
				AHH1-R	GAGCGGCTGGATGCGGTTGT		1653473-92				
		Enterotoxigenic *B. cereus*	*nheB*	SG-F3	GCACTTATGGCAGTATTTGCAGC	DQ153257	2101-2123	152	80.5±0.84		25
				SG-R3	GCATCTTTTAAGCCTTCTGGTC		2231-2252			1.9	
H	*	*Salmonella* spp.	*invA*	invA2-F	GATTCTGGTACTAATGGTGATGATC	M90846	132-156	288	82.4±0.28		26
				invA2-R	GCCAGGCTATCGCCAATAAC		419-400			6.2	
		DAEC	*daaD*	daaD-F31	GTCACCTGCGGGATGTTACT	AF233530	31-50	233	88.6±0.32		This study
				daaD-R263	AGCTCATGACGACCATCCTT		263-244				
IAC for sets A-E		*Yersinia ruckeri*	*16S*	yersH2-F	GGCTCACCTAGGCGACGA	X75275	245-262	211	86.1±0.53		This study
				yersH2-R	TCAGTGCTATTAACACTTAACCCTTCC		455-429				
IAC for sets F,G,H		*Yersinia ruckeri*	*16S*	yers-F	GGAGGAAGGGTTAAGTGTTA	X75275	426-443	68	77.2±0.53		10
				yers-R	GAGTTAGCCGGTGCTTCTT		475-493				

[a] Values represent means±standard deviations of 15 to 60 tests. [b] Eight maine food-borne bacteria

TABLE 3. Epidemiological investigations in 21 food-borne outbreaks examined by SG-PCR and bacteriological cultures in Shimane Prefecture, Japan

Case No.[a]	Date ocurred (day/mo /yr)	Days for examination after occurrence	Infected group	Source of infection (suspected source)	No. of patients / total	No. of examined patients	Real time PCR		Isolation		
							Target genes	No. of positive / examined samples	Causative pathogens	No. of positive / PCR examined samples	No. of positive / total examined samples
1	4-Oct-02	6	School excursion in a mountain area	Stream water	23/33	22	*eaeA* and *astA*	1/7	EPEC	1/7	5/22
							eaeA	3/7	*astA*-positive *E. coli*	1/7	4/22
							astA	2/7			
2	03-Sep-03	3	Protective care school	Catering box lunch	22/46	10	*astA*	2/7	*astA*-positive *E. coli*	2/7	3/10
							eaeA	1/7			
							gyrB of *P.a* and *ahhl*	1/7			
3	01-Oct-03	2	Celebration in a company	Catering box lunch	437/1354	12	*cpe*	5/7	*C. perfringens* O:13, O:16	6/7	10/12
4	11-Jun-04	6	Camping group of high school	Grilled meat (beef, bovine intestinal	4/8	4	Specific gene of *C.j*	5/7	*C. jejuni*	5/7	5/8
5	12,13-Jun-04	6 ~ 7	9 citizen groups in Chophouse	Grilled meat (beef, bovine intestinal	30/UN	12	Specific gene of *C.j*	4/7	*C. jejuni*	5/7	10/12
6	17-Jun-04	5	Cooking practise in a high school	Shelf-cooked lunch (salada mixed	31/41	20	Specific gene of *C.j*	4/7	*C. jejuni*	6/7	17/20
7	07-Jul-04	1	Citizen in Chinese restaurant	Fried rice	6/6	6	*ces* and *nheB*	2/6	*B. cereus*	2/6	2/6
8	11-Oct-04	3	Sport club in a high school	Shelf-cooked lunch	26/47	6	*cpe*	2/6	*C. perfringens* O:16, OUT	3/6	4/6
							astA and *st*	1/6			
9	5~7-Nov-04	5 ~ 7	Restaurant	Unknown	5	5	Specific gene of *C.j*	2/5	*C. jejuni*	2/5	2/5
10	Unknown	Several days (19-Jur-05)	Nursery	Unknown	24/73	22	*eaeA* and *stx1*	4/7	EHEC O26 [Norovirus	5/7	8/22 20/22]
11	28~30-Sep-05	1 ~ 3	Prisoners in a prison	Shelf-cooked meal	113/600	61	*astA and cpe*	1/7	*astA*-positive *E. coli*	7/7	41/46
							astA	6/7	(*C. perfringens : sporadic*	1/7	1/46)
12	2~6-Oct-05	1 ~ 5	Elementary and high school children	Unknown (School lunch)	39/94	39	astA, aggR and Specific gene of *C.j*	1/6	(*C. jejuni :* sporadic case	1/6	1/16)
								1/6			
							astA and aggR	4/6			
13	28~30-May-06	0 ~ 2	Citizens at restaurant	Lunch (pilaf and scrambled agg)	27/34	27	*femB*	1/5	*S. aureus*	2/5	4/8
							astA	1/5			
14	4-Jul-06	0	Boarder of high school	Catering box lunch	34/51	34	*cpe*	7/7	*C. perfringens*	7/7	19/50
15	16-Aug-06	1	Citizens at restaurant	Fried rice	15/34	15	*ces* and *nheB*	1/4	*B. cereus*	2/4	2/4
							aggR	1/4			
16	23~29-Aug-06	2 ~ 8	Boarder of training high school	Supper (contaminated sliced cabbage)	19/43	18	*astA* and Specific gene of *C.j*	5/7	*C. jejuni*	6/7	9/14
							Specific gene of *C.j*	1/7			
17	2-Sep-06	3	Citizens in Buddhist service	Catering box lunch	14/49	6	*tdh*	3/6	*V. parahaemolyticus*	3/6	3/6
							Specific gene of C.j	2/6			
							st	1/6			

TABLE 3. Continue

Case No.[a]	Date ocurred (day/mo /yr)	Days for examination after occurrence	Infected group	Source of infection (suspected source)	No. of patients / total	No. of examined patients	Real time PCR		Isolation		
							Target genes	No. of positive / examined samples	Causative pathogens	No. of positive / PCR examined samples	No. of positive / total examined samples
18	22-Dec-06	5	Citizens at restaurant	Supper (chiken)	12/12	9	Specific gene of *C.j*	4/7	*C. jejuni*	4/7	4/9
19	21-Oct-07	1	Citizens at restaurant	Supper	7/13	7	*gyrB* of *P.s* *eaeA*	2/5 1/5	*P. shigelloides*	2/5	2/5
20	4-Jul-07	6	Citizens at restaurant	Supper (chiken)	7/11	3	Specific gene of *C.j*	1/2	*C. jejuni*	1/2	2/3
21	29-Nov-07	1	Citizens at restaurant	Supper (raw chiken liver)	8/13	6	*astA* and *Specific gene of C.j* *Specific gene of C.j*	1/7 3/7	*C. jejuni* (*astA-positve E. coli*	4/7 1/7)	4/7
22	28-Mar-08	5	Citizens at restaurant	Sushi	2/7	4	Specific gene of *C.j*	2/4	*C. jejuni*	2/4	2/7
23	16-Oct-08	2	workmate	home-made vinegared rice with thin strips of egg	13/15	4	Not detected	0/2	*Salmonella* Enteritidis	1/2	3/4
24	11-Jul-09	6	Citizens at restaurant	Grilled meat (beef, bovine intestinal meat, raw liver)	4/4	4	Specific gene of *C.j*	3/4	*C. jejuni*	3/4	3/4
25	28-Jul-09	2	Citizens at restaurant	Unknown	13/44	5	*eaeA* and *astA* *astA*	1/5 1/5	STEC O63 *(stx2f)*	1/5	1/5
26	25-Aug-09	13	customers of supermarket	bowl of rice topped with deep-fried poak	4/4	2	Not detected	0/2	*Salmonella* Enteritidis	2/2	2/2
27	29-Sep-09	3 ~ 7	Employee of restaurant after EHEC O157 outbreak	Unknown	Not tested	7	*eaeA*, *stx1* and *stx2* *eaeA* and *astA* *astA*	2/7 1/7 3/7	EHEC O157	4/7	4/7
28	1-Jun-08	3	Staff of public services	Catering box lunch	171/296	17	*cpe*	7/7	*C. perfringens*	7/7	16/17
29	20-Aug-09	1	Citizens stayed in a hotel	Box lunch served by the hotel	11/21	9	*femB*	4/4	*S. aureus*	4/4	6/9
30	21-Sep-08	4	Citizens	Unknown	9/16	4	Specific gene of *C.j*	4/4	*C. jejuni*	4/4	4/4
31	14-Jun-09	1~2	Hospital	Supper in hospital	34/148	7	*cpe* and *femB* *cpe*	1/7 4/7	*C. perfringens* [Norovirus	5/7 2/7]	5/7
32	21-Aug-09	5	Citizens at restaurant	Supper	7/10	3	*astA* and Specific gene of *C.j* *astA*	1/3 1/3	*C. jejuni*	1/3	1/3
33	14-Nov-09	5~6	Citizens at restaurant	Supper	9/15	7	*astA* and Specific gene of *C.j* *femB* and Specific gene of *C.j* Specific gene of C.j	2/7 1/7 3/7	*C. jejuni*	6/7	6/7
34	15-Aug-09	2~3	School excursion	Supper (potato salada) in a hotel	32/73		*tdh* *ompW*	1/7 1/7	*V. parahaemolyticus* *V. p* and *V. cholerae* non O1	1/7 1/7	2/7 1/7
35	16-Sep-09	9	Citizens in Buddhist service	Catering box lunch	25/43		*invA*	3/7	*S.* Enteritidis	4/7	4/7
Total							Gne of main pathogen	129/199 64.8%	Main pathogen	125/199 62.8%	216/381 56.7%

[a]Analysis was tested using the ABI 7500 in Shimane (cases 1 to 22), , Fukuoka (cases 23 to 25) and Shizuoka Prefecture (cases 31 to 33); ABI 7500 Fast in Fukuoka Prefecture (cases 26 and 27), Thirmal Cycler Dice Real Time System in Hokkaido (cases 28 to 30) and LightCycler 480 in Kumamoto Prefecture (cases 34 and 35)

TABLE 4: The relationship between PCR detection and CFUb in 15 foodborne outbreak cases by viable cell counting..

Case	Causative foodborne pathogens	Multiplex SG-PCR	Number of samples								
			Total	log 10 cfu/g by viable cell counting.							
				0	2	3	4	5	6	7	8
3	*C. perfringens*	+	**5**					**1**	**3**		**1**
		-	2	2							
6	*C. jejuni*	+	**4**				**1**	**3**			
		-	3	2		1					
8	*C. perfringens*	+	**2**				**1**	**1**			
		-	4	2		1	1				
9	*C. jejuni*	+	**2**				**2**				
		-	3	3							
10	EHEC O26	+	**4**			**1**			**1**	**1**	**1**
		-	3	2		1					
11	*astA* -positive *E. coli*	+	**7**						**1**		**6**
		-	0								
13	*S. aureus*	+	**1**				**1**				
		-	4	3			1				
14	*C. perfringens*	+	**7**					**1**	**3**	**3**	
		-	0								
17	*B. cereus*	+	**1**				**1**				
		-	3	3							
18	*C. jejuni*	+	**5**					**1**		**2**	**2**
		-	1					1			
20	C. jejuni	+	**4**			**1**		**3**			
		-	3	3							
19	*P. shigelloides*	+	**2**					**2**			
		-	3	3							
	astA -positive *E. coli*	+	**1**				**1**				
		-	4	4							
21	*C. jejuni*	+	**4**					**3**	**1**		
		-	3	3							
26	S. Enteritidis	+	**0**								
		-	2				2				
27	EHEC O157	+	**2**		**1**	**1**					
		-	5	5							
	Total	+	**51**	**0**	**1**	**3**	**7**	**15**	**9**	**6**	**10**
		-	43	35	0	3	4	1	0	0	0

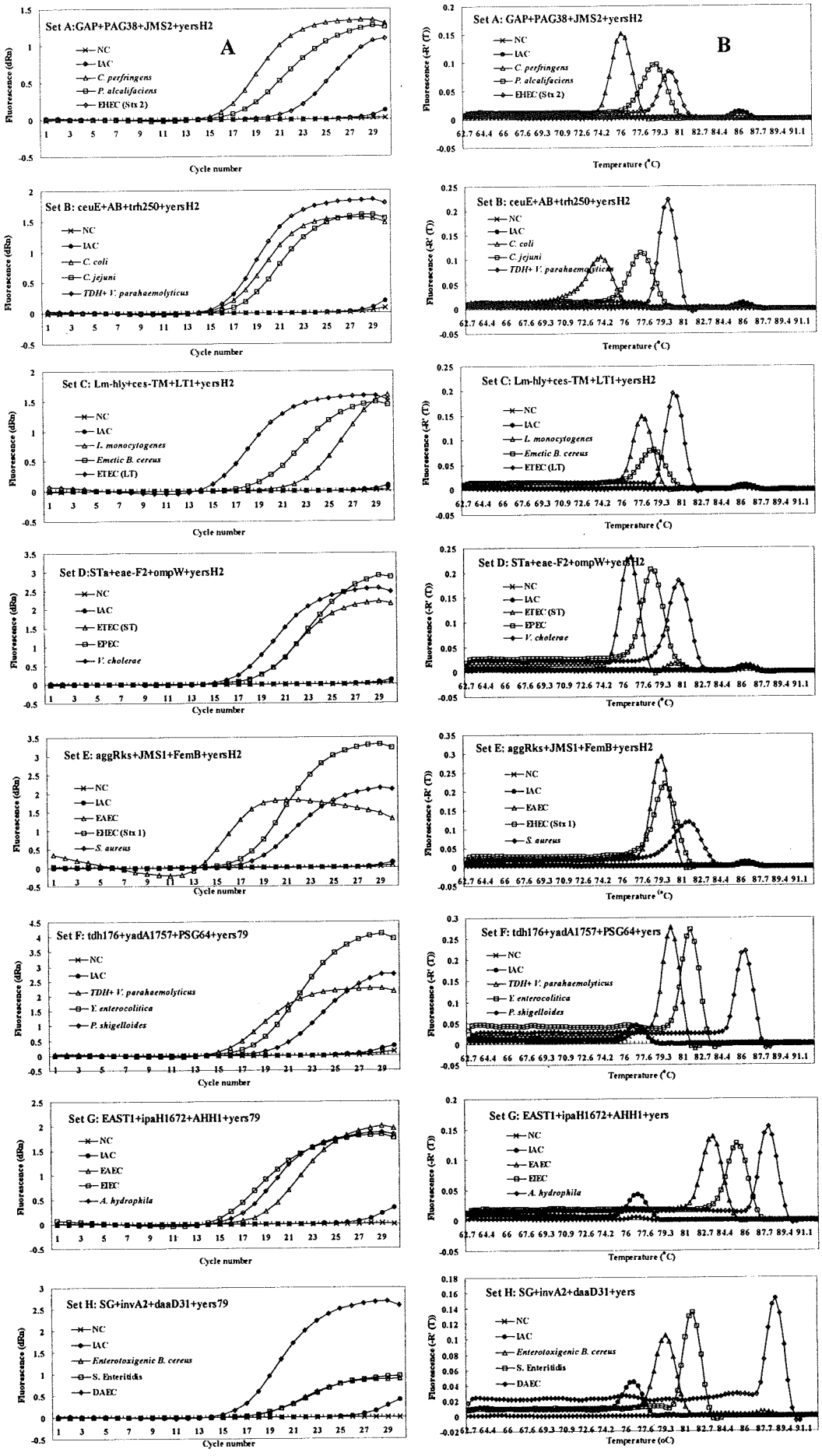
Set A:GAP+PAG38+JMS2+yersH2
A
NC
IAC
C. perfringens
P. alcalifaciens
EHEC (Stx 2)
Fluorescence (dRn)
Cycle number
Set A: GAP+PAG38+JMS2+yersH2
B
Fluorescence (-R' (T))
Temperature (°C)
Set B: ceuE+AB+trh250+yersH2
C. coli
C. jejuni
TDH+ V. parahaemolyticus
Set C: Lm-hly+ces-TM+LT1+yersH2
L. monocytogenes
Emetic B. cereus
ETEC (LT)
Set D:STa+eae-F2+ompW+yersH2
ETEC (ST)
EPEC
V. cholerae
Set E: aggRks+JMS1+FemB+yersH2
EAEC
EHEC (Stx 1)
S. aureus
Set F: tdh176+yadA1757+PSG64+yers79
Y. enterocolitica
P. shigelloides
Set G: EAST1+ipaH1672+AHH1+yers79
EIEC
A. hydrophila
Set H: SG+invA2+daaD31+yers79
Enterotoxigenic B. cereus
S. Enteritidis
DAEC

Fig.1

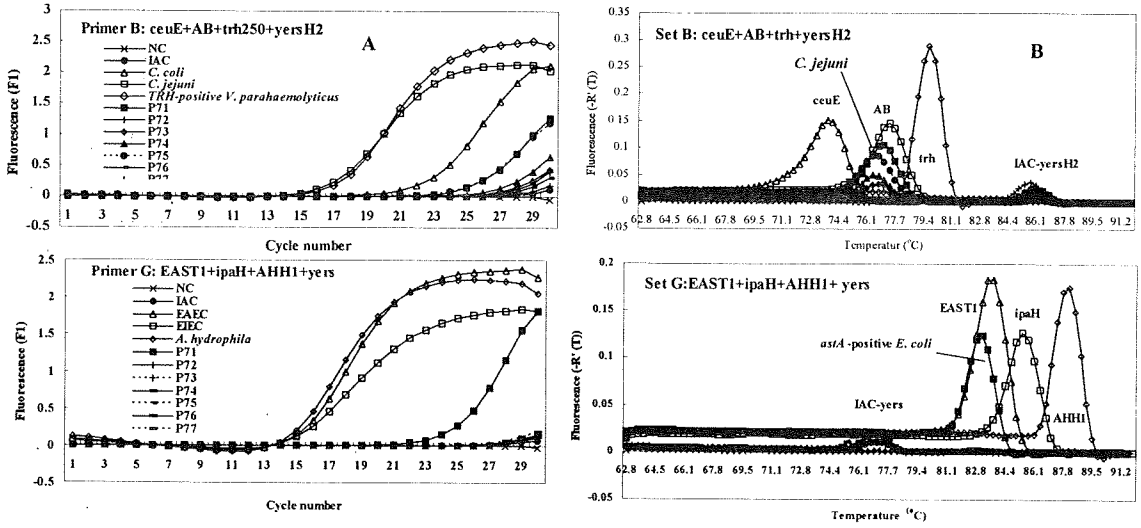
Primer B: ceuE+AB+trh250+yersH2
A
NC
IAC
C. coli
C. jejuni
TRH-positive V. parahaemolyticus
P71
P72
P73
P74
P75
P76
Fluorescence (F1)
Cycle number
Set B: ceuE+AB+trh+yersH2
B
C. jejuni
ceuE
AB
trh
IAC-yersH2
Fluorescence (-R' (T))
Temperatur (°C)
Primer G: EAST1+ipaH+AHH1+yers
NC
IAC
EAEC
EIEC
A. hydrophila
P71
P72
P73
P74
P75
P76
P77
Fluorescence (F1)
Cycle number
Set G:EAST1+ipaH+AHH1+ yers
EAST1
ipaH
astA -positive E. coli
IAC-yers
AHH1
Fluorescence (-R' (T))
Temperature (°C)

Fig 2

厚生労働科学研究費補助金（健康安全・危機管理対策総合研究事業）

地域における健康危機管理に対応するための地方衛生研究所機能強化に関する研究

分担報告書

原因不明感染症に対する迅速な包括的診断法の開発と有効性の評価

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研究概要

本研究では、健康危機発生時に、地方衛生研究所において実施するべきウイルス検査について、特に、呼吸器系、消化器系や中枢神経系症状を主徴とする原因不明感染症の患者検体を対象に、迅速性、網羅性を考慮し、高感度で最適な手法を確立することを目的とした。この目的を達成するために開発したマルチプレックス one-step RT-PCR 法は９属、約２３０種のウイルスを同時に、また高感度で比較的迅速（５時間）に検出することができた。さらに、本方法を用いて、感染症発生動向調査事業で搬入された咽頭拭い液、呼吸器感染症の集団、また個別事例において、その原因と推定されるウイルスを検出でき、健康危機管理上その有効性が認められた。

A. 研究目的

本研究は地域における感染症の健康危機管理事例発生時において、地方衛生研究所の対応能力を向上させるために、原因不明感染症に対して網羅的かつ高感度な迅速鑑別同定法の開発・改良を行い、行政機関に迅速な検査結果の提供を行うことを目的とする。具体的には呼吸器系ウイルスの診断を目的に、マルチプレックス one-step RT-PCR 法による網羅的診断法を確立し、原因不明の集団あるいは個別発生事例を対象に本診断法を応用し、その有効性を評価する。

B. 研究方法

（１）呼吸器系ウイルスのうち、Influenzavirus A および B, RSvirus, human Metapneumovirus, Rhinovirus, Enterovirus, Coronavirus, Influenzavirus C, parainfluenza virus, bocavirus, adenovirus の９属、約２３０種を５本のチューブで検出するマルチプレックス PCR 法を作製した。PCR 法の検出感度の評価はウイルス RNA あるいは PCR 増幅産物をプラスミッドに挿入したものを用いて行った。

PCR 増幅産物の確認はシークエンスを行い塩基配列を決定して確認した。

（２）原因不明感染症の集団発生事例や個別発生事例に対して本開発法を用いて病原体の同定に応用した。

C. 研究結果

(1) 開発したマルチプレックスPCR法の各ウイルスに対する最少検出感度は通常の各感染症で検出同定可能な検出感度であった。

（２）臨床応用

2006 年 12 月に高齢者施設において原因不明の呼吸器疾患の集団発生１事例が発生した。患者１２名の鼻腔吸引液に対して本診断法を応用し、原因ウイルスが Rhinovirus であることが同定できた。

また、ＲＳウイルス感染を疑う個別発生事例１１７例において、約９割の症例で呼吸器ウイルスの遺伝子を同定可能であった。約半数の症例で複数のウイルスが検出された。

D. 考察

本研究により開発したマルチプレックスＰＣＲ法は９属、約２３０種のウイルスを５本のチューブで同時に、また高感度で比較的迅速（５時間）に検出することが可能であった。さらに、呼吸器感染症の集団発生事例で、その原因ウイルスを診断可能であった。また個別発生事例の約半数においても、原因ウイルスの特定は可能であった。さらに、２種類以上のウイルスによる重感染が約半数に認められ、起因ウイルスを完全に同定することは困難であったが、それを推定することは十分可能であった。以上より、本方法は感染症の健康危機管理上、原因ウイルスの同定に有効であると考えられる。

（倫理面への配慮）

（１）対象とした検体の多くは感染症新法に基づく感染症発生動向調査事業により各定点医療機関から搬入されたものである。検体に付随している個票には個人を特定できるような住所、氏名などは記載されておらず、解析に最低限必要な情報として、男女別、年齢、発症年月日、検体搬入日並びに症状のみが記載されている。上記以外の個別発生事例の検体については検体の採取前に、検査の目的及び内容、検査後の成績の取り扱いや説明方法についても十分に説明して、納得、理解を得た後、文書で同意を得ている。本研究については各衛生研究所での倫理委員会で承認を得ている。

E. 結論

１．本研究により開発したマルチプレックス one-step RT-PCR 法は９属、約２３０種のウイルスを同時に、また高感度で比較的迅速（５時間）に検出することができた。さらに、本方法を用いて、呼吸器感染症の集団、また個別事例において、その原因と推定されるウイルスを検出でき、健康危機管理上その有効性が認められた。

F. 研究発表

1.　論文発表

なし。

2.　学会発表

三崎貴子、他

小児感染症学会　2009

G. 知的財産権の出願・登録状況

なし。