表2 リアルタイムPCR法で検査した食中毒事例

| 事例番号 | 発生年月日 | | 検査までの日数 | 原因施設 | 原因食品 | 患者数/喫食者数 | 原因物質 | 検出および分離状況（陽性株数/検体数） | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | リアルタイムPCR法 | | | | | 培養法 |
| | | | | | | | | 1回 | 2回 | 3回 | 最終 | 合計 | |
| 1 | 平成14年 | 10月4日 | 6 | 野外・遠足 | 山水 | 23/33 | 腸管病原性大腸菌 O:125, O:166, O:UT | 1/7 | − | − | 4/22 | 7/22 | 5/22 |
| | | | | | | | *astA*陽性大腸菌 O:1, O:UT | | | | 3/22 | | 4/22 |
| 2 | | 9月3日 | 3 | 飲食店 | 委託給食 | 22/46 | *astA*陽性大腸菌 O:18, O:20, O:114, O:159, O:UT | 1/5 | − | − | 6/10 | 6/10 | 3/10 |
| | | | | | | | ノロウイルス | | | | 6/7 | | |
| 3 | 平成15年 | 10月1日 | 2 | 式典 | 昼食弁当 | 437/1354 | ウエルシュ菌 O:13, O:16 | 5/5 | − | − | 7/12 | 7/12 | 10/12 |
| 4 | 平成16年 | 6月11日 | 6 | キャンプ | 焼肉 | 4/8 | カンピロバクター・ジェジュニ | 1/4 | − | − | 1/8 | 1/8 | 5/8 |
| 5 | | 6月12、13日 | 6〜7 | 飲食店 | 焼肉 | 30/UN | カンピロバクター・ジェジュニ | 4/5 | − | − | 8/12 | 8/12 | 10/12 |
| 6 | | 6月17日 | 5 | 学校調理室 | 調理実習 | 31/41 | カンピロバクター・ジェジュニ | 4/5 | − | − | 12/14 | 12/14 | 17/20 |
| 7 | | 7月8日 | 1 | 飲食店 | チャーハン | 6/6 | セレウス菌（嘔吐型） | 1/1 | − | − | 2/6 | 2/6 | 2/6 |
| 8 | | 10月11日 | 3 | 高等学校 | 昼食 | 26/47 | ウエルシュ菌 O:16, OUT | 1/5 | − | − | 3/6 | 3/6 | 4/6 |
| 9 | | 11月5-7日 | 5〜7 | 一般食堂 | 食事 | 5 | カンピロバクター・ジェジュニ | 2/5 | − | − | | 2/5 | 2/5 |
| 10 | 平成17年 | 1月8-21日 | 数日 | 保育園 | 不明 | 24/73 | 腸管病原性大腸菌 O26 | | | | 8/22 | 8/22 | 8/22 |
| | | | | | | | ノロウイルス | | | | 20/22 | | |
| 11 | | 9月28-30日 | 1〜3 | 刑務所 | 給食 | 113/600 | *astA*陽性大腸菌 | − | − | 14/14 | | 14/14 | 41/46 |
| | | | | | | | ウエルシュ菌 | 1/5 | | | | | 1/46 |
| 12 | | 10月2-6日 | 1〜5 | 小中学校 | 不明 | 39/94 | *astA*陽性大腸菌 | 5/5 | − | − | | 5/5 | 不能 |
| | | | | | | | 腸管病原性大腸菌 | 2/5 | | | | | 不能 |
| | | | | | | | カンピロバクター・ジェジュニ | 1/5 | | | | | 1/16 |
| | | | | | | | エロモナス・ヒドロフィラ | 2/5 | | | | | 不能 |
| 13 | 平成18年 | 5月28-30日 | 0〜2 | 一般食堂 | 食事 | 27/34 | 黄色ブドウ球菌 | 2/5 | − | − | | 2/5 | 4/8 |
| | | | | | | | *astA*陽性大腸菌 | 1/5 | | | | | |
| 14 | | 7月4日 | 0 | 寮の給食施設 | 弁当 | 34/51 | ウエルシュ菌 | 5/5 | − | − | 8/8 | 8/8 | 19/50 |
| 15 | | 8月16日 | 1 | 飲食店 | チャーハン | 15/34 | セレウス菌（嘔吐型） | 1/4 | − | − | | 1/4 | 2/4 |
| 16 | | 8月23-29日 | 2〜8 | 寮の給食施設 | 給食 | 19/43 | カンピロバクター・ジェジュニ | 3/5 | − | − | 6/9 | 8/9 | 9/14 |
| | | | | | | | *astA*陽性大腸菌 | 4/5 | | | 5/9 | | 不能 |
| 17 | | 9月2日 | 3 | 仕出店 | 仕出し | 14/49 | TDH産生腸炎ビブリオ | 4/5 | − | − | 4/6 | 4/6 | 3/6 |
| 18 | | 12月22日 | 5 | 一般食堂 | 食事 | 12/12 | カンピロバクター・ジェジュニ | 3/5 | − | − | 4/9 | 4/9 | 4/10 |
| 19 | 平成19年 | 7月4日 | 6 | 一般食堂 | 食事 | 7/11 | カンピロバクター・ジェジュニ | 1/2 | − | − | | 1/2 | 2/3 |
| 20 | | 10月21日 | 1 | 宿泊施設 | 食事 | 7/13 | 腸管病原性大腸菌 | 2/5 | | − | | 3/5 | 不能 |
| | | | | | | | プレシオモナス・シゲロイデス | | 2/5 | | | | 2/5 |
| 21 | | 11月29日 | 1 | 一般食堂 | 食事 | 8/13 | カンピロバクター・ジェジュニ | 3/5 | − | − | 4/7 | 4/7 | 4/7 |
| | | | | | | | *astA*陽性大腸菌 | 1/5 | | | | | 1/7 |
| 合計 | | | | | | | | 54/93 | | | | 110/191 | 162/292 |
| | | | | | | | | 58.1% | | | | 57.6% | 55.4% |

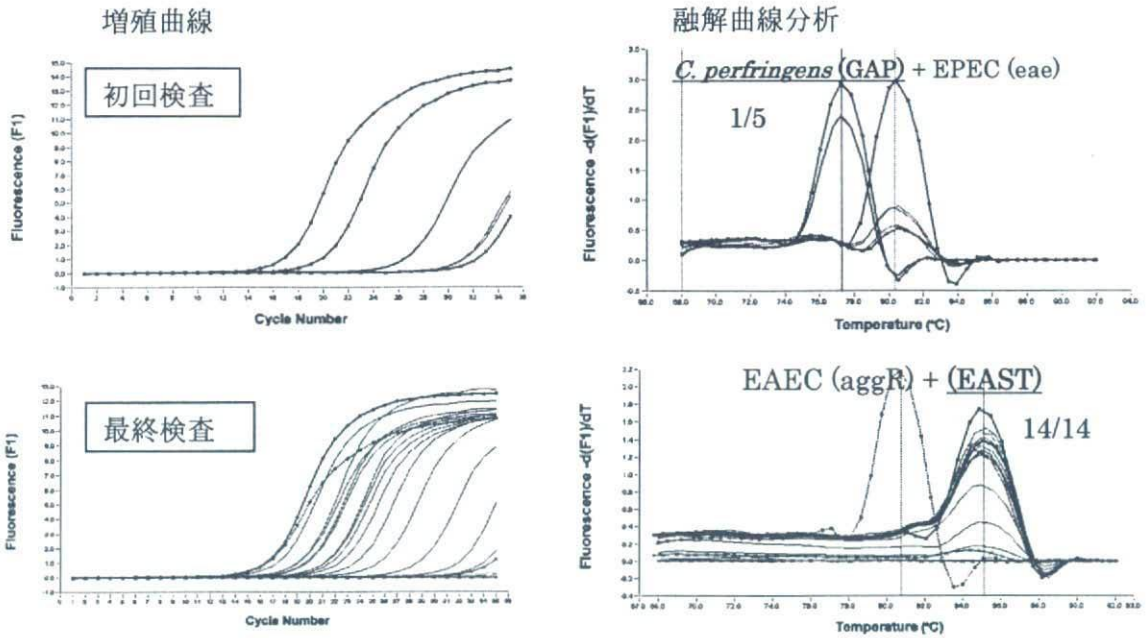


図１　事例１１　刑務所における *astA* 陽性大腸菌による食中毒

Duplex PCR における標的遺伝子を保有する菌種名と括弧内のプライマー名を融解曲線の Tm 値が低い順に示した。 PCR 陽性を示した菌種とプライマー名をアンダーライン付き太字で示し、その陽性コントロールを—●—、陽性例が少ないか陰性であった菌の陽性コントロールを- -○ - -で示す。

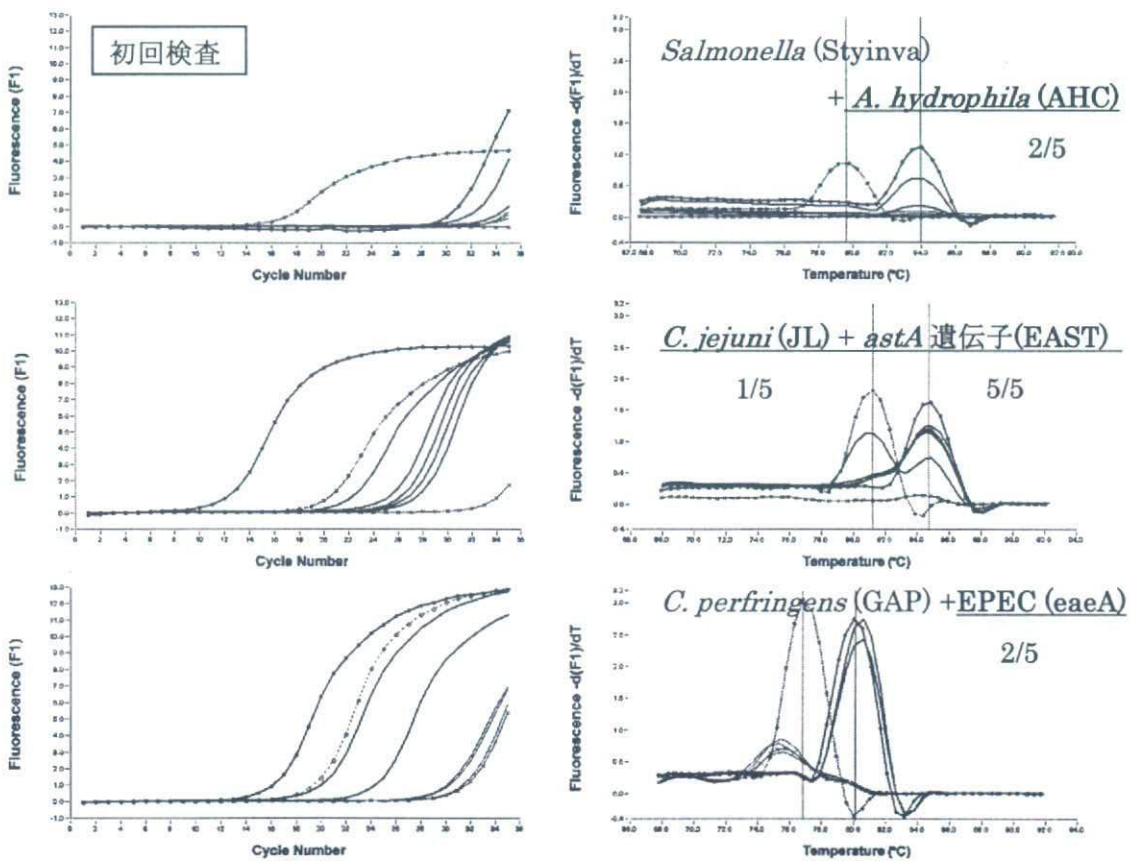


図２　事例 12　小中学校における原因不明の集団腸管感染症

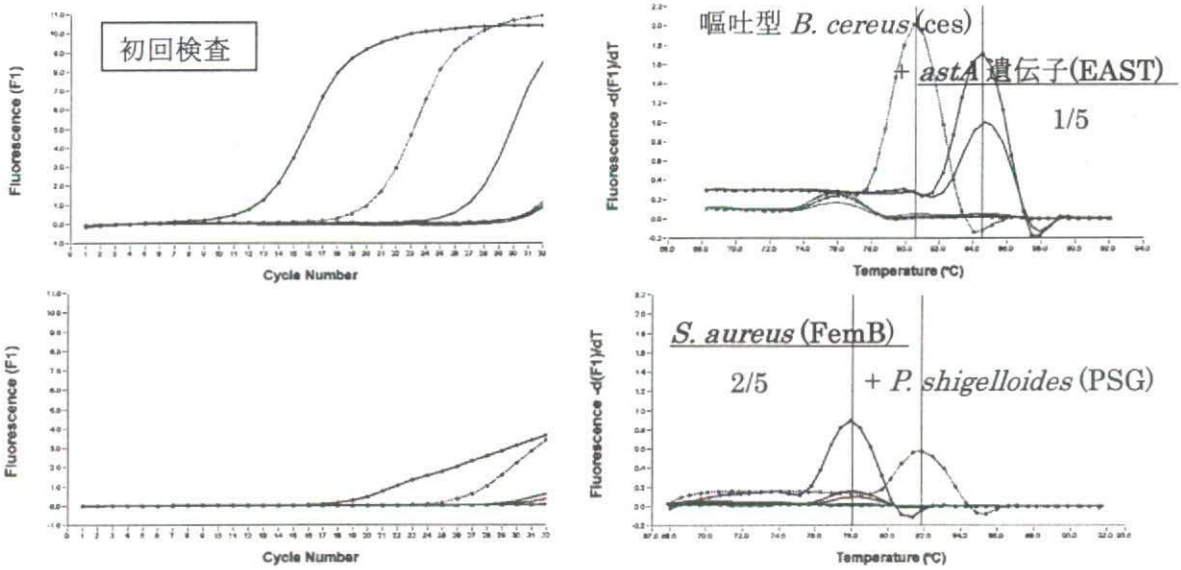


図 3　事例 13　バイキング料理による黄色ブドウ球菌食中毒事例

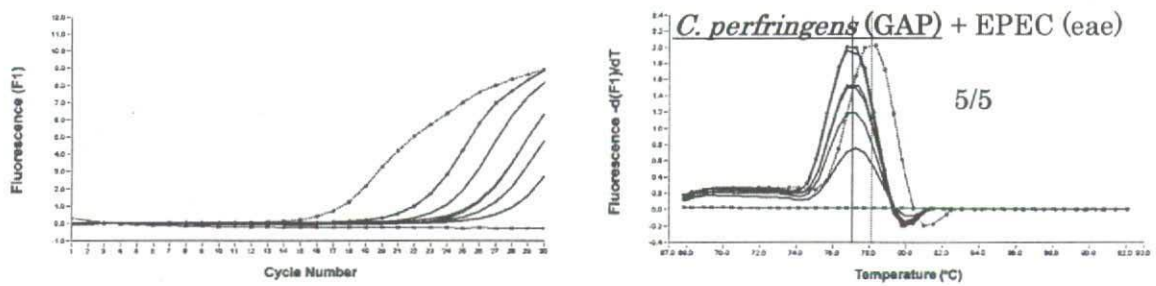


図 4　事例 14　学生寮における給食（仕出し弁当）によるウエルシュ菌食中毒

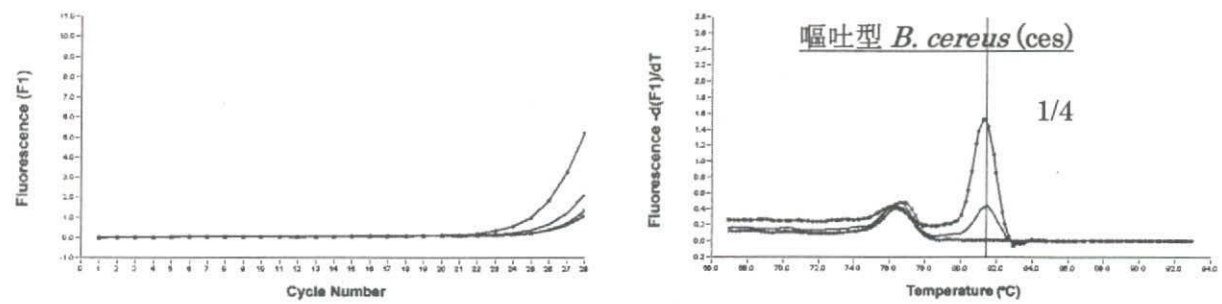


図 5　事例 15　チャーハンによる *B. cereus* 食中毒事例

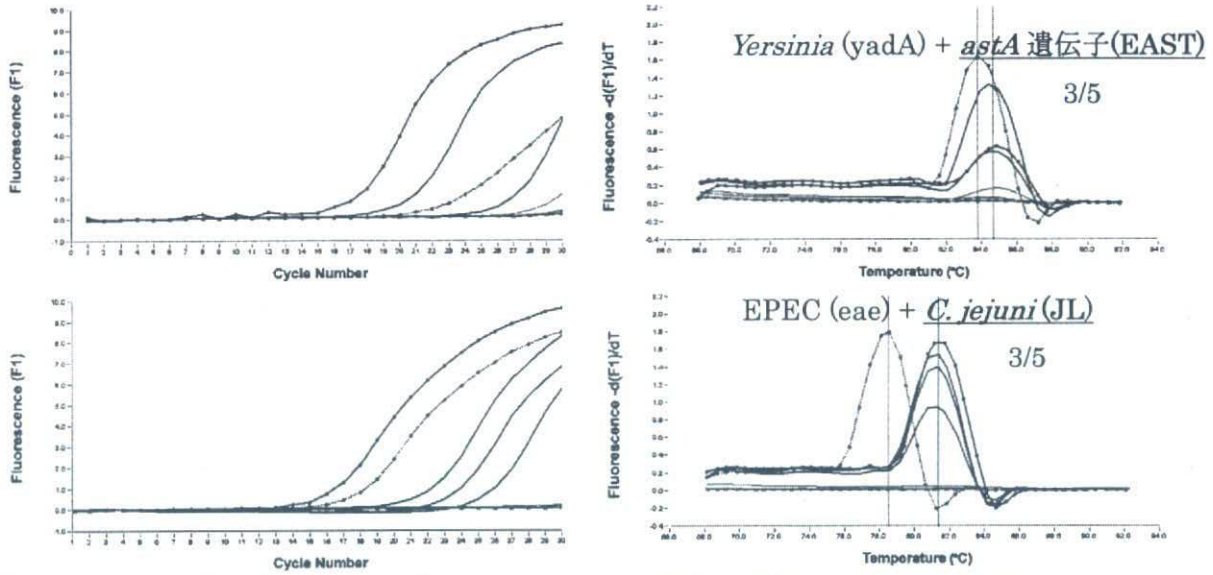


図 6　事例 16　学生寮の給食による *C. jejuni* と *astA* 陽性大腸菌による食中毒事例

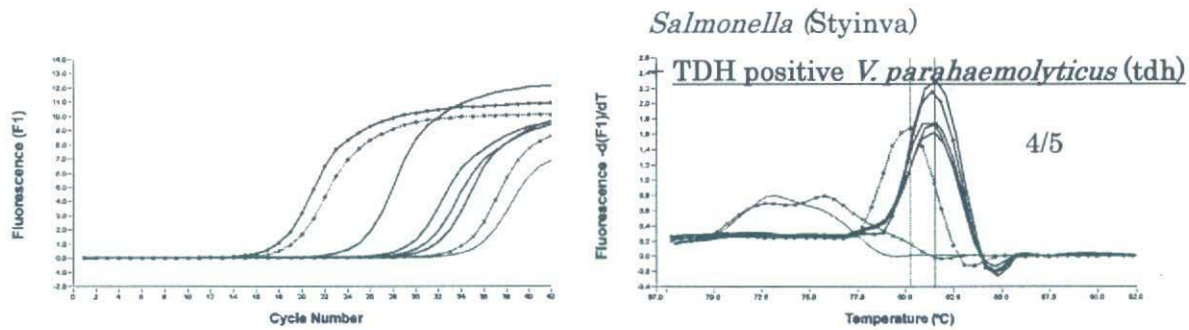


図 7　事例 17　法事の仕出し料理による腸炎ビブリオ食中毒事例

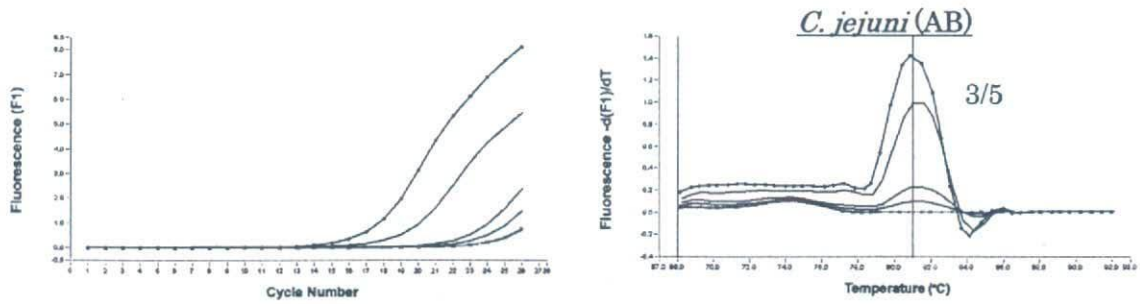


図 8　事例 18　焼き鳥による *C. jejuni* 食中毒事例

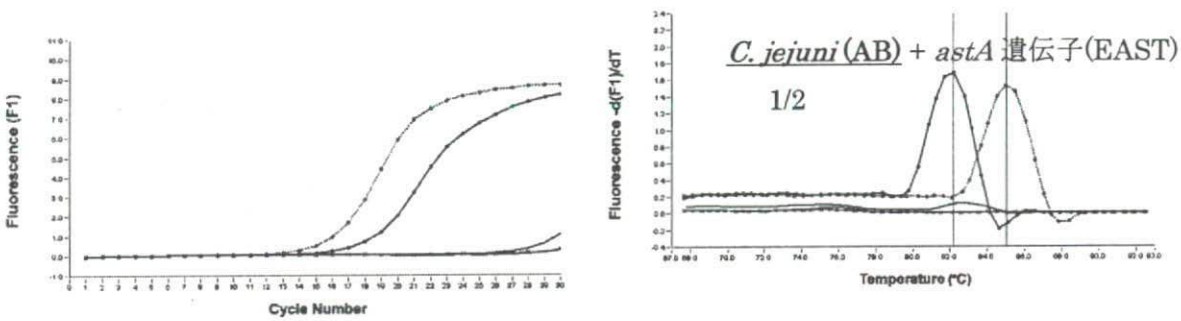


図 9　事例 19　焼き肉による *C. jejuni* 食中毒事例

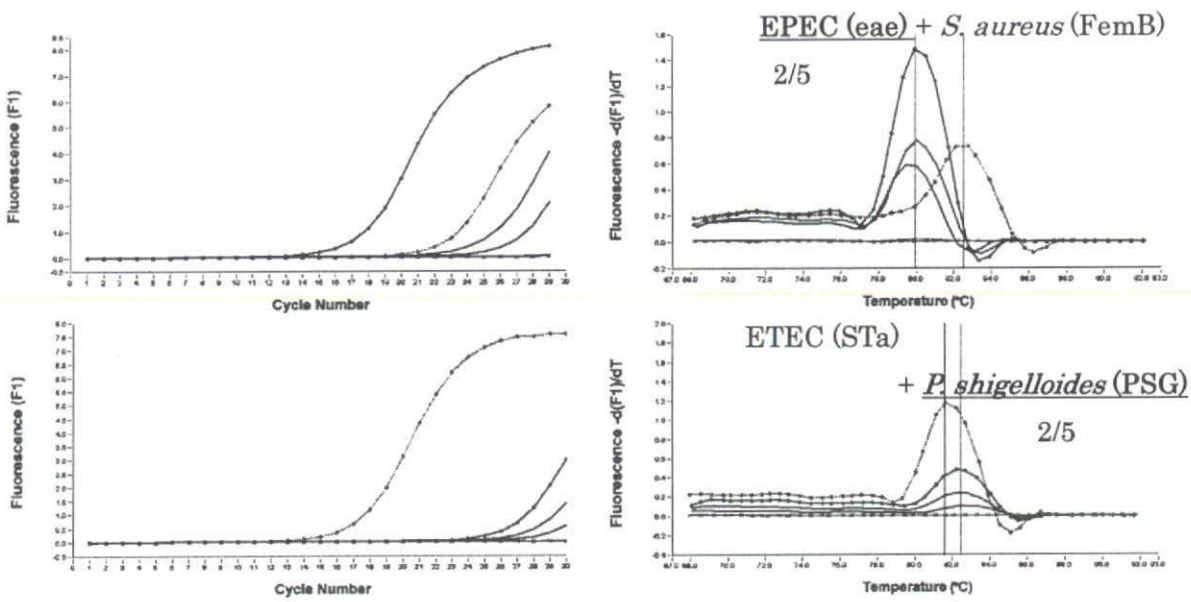


図 10　事例 20　会食による病原性大腸菌と *P. shigelloides* による食中毒事例

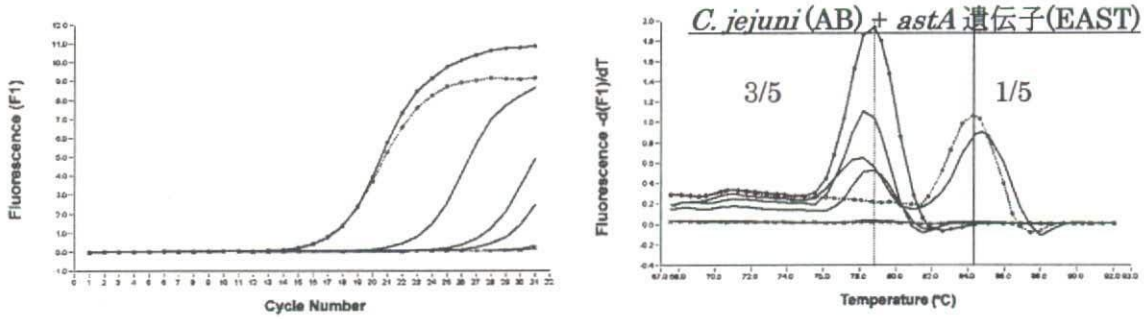


図 11　事例 21　焼肉による *C. jejuni* による食中毒事例

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# Comprehensive and Rapid Real-Time PCR Analysis of 21 Food-borne Outbreaks

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**SUMMARY**

A set of four duplex SYBR Green I PCR (SG-PCR) assay combined with DNA extraction using QIAamp DNA Stool Mini kit was evaluated for the detection of food-borne bacteria from 21 food-borne outbreaks. The causative pathogens were detected in almost all cases in 2 h or less. The first run was for the detection of 8 main food-borne pathogens in 5 stool specimens within 2h and the second run was for the detection of other unusual suspect pathogens within a further 45 min. After 2 to 4 days, the causative agents were isolated and identified. The results proved that for comprehensive and rapid molecular diagnosis in food-borne outbreaks, Duplex SG-PCR assay is not only very useful, but is also economically viable for one-step differentiation of causative pathogens in fecal specimens obtained from symptomatic patients. This then allows for effective diagnosis and management of food-borne outbreaks.

Key words: Real-Time SYBR Green I PCR, Food-borne Pathogens, Food-borne outbreak, Feces, *Salmonella, Campylobacter jejuni, Clostridium perfringens, Bacillus cereus*, EHEC, *astA*-positive *E. coli, Staphylococcus aureus,* TDH-producing *Vibrio parahaemolyticus, Plesiomonas shigelloides*

## INTRODUCTION

The introduction of real-time PCR in food-borne outbreak investigations provides an opportunity for rapid detection of pathogens in food and clinical settings [5]. The benefits to public health administration from rapid real-time PCR assays are most notable after comprehensive and rapid detection of bacteria. The results can quickly inform a public health administrator about the causative pathogens of food-borne outbreak, allowing a more accurate, effective and timely response. Abubakar et al.[2] implied in the Health Technology Assessment program (now part of the National Institute for Health Research, UK), that the feasibility of conversion to rapid methods such as multiplex PCR and DNA microarrays is dependent on localized considerations, including the community prevalence rates for specific pathogens, the skill base and subsequent training costs for laboratory staff and spare capacity available to ensure adequate laboratory space for new equipment. Although these tests look promising, further studies are necessary to assess their usefulness [2].

Apart from saving time, real time PCR is sensitive, highly specific and offers the potential for quantification [19]. The risk of cross-contamination is significantly reduced, and high-throughput performance and automation are possible since no post-PCR manipulations are required [7]. In

principle, two different chemistries are available for real-time detection of PCR products: fluorescent probes that bind specifically to certain DNA sequences and fluorescent dyes that intercalate into any double-stranded DNA. Fluorescent-probe based real-time PCR (TaqMan PCR) studies to detect causative pathogens from food-borne outbreaks in feces using TaqMan probes have been carried out [7, 13, 14, 19]. TaqMan PCR assays require the availability of primers and probes that must be selected according to very rigid criteria. Use of simple, cheaper double-stranded DNA-binding dye SYBR green I for detection of PCR amplicons (SG-PCR) overcomes this limitation. Therefore, real time PCR could be applied without the need for fluorescent probes [1]. In the absence of probes, the specificity of the reaction is determined on the basis of the melting temperature (*Tm*). The advantages of SG-PCR over TaqMan PCR include the relative simplicity and reduced cost of SYBR Green I compared to TaqMan probes [24]. Recently, the application of SG-PCR for the detection of food-borne bacteria in different samples has been increased [4, 5, 10, 11, 16]. Duplex SG-PCR assays have been carried out to detect causative bacteria in feces from food-borne outbreaks [7, 9, 10].

We previously reported a set of four duplex SG-PCR assays for one-step differentiation of 8 genes of food-borne pathogens in DNA extracted from 5 feces using 32 capillary tubes of LightCycler (Roche). The first run was for the detection of 8 main food-borne pathogens and the second run was for the other pathogens. We reported here that improved diagnostic duplex SG-PCR assays were upgraded with new highly sensitive primer pairs for 11 food-borne pathogens. These assays successfully identified the causative pathogens of food-borne outbreaks caused by enteropathogenic *Escherichia coli*, enterohemorrhagic *E. coli*, *astA*-positive *E. coli*, *Plesiomonas shigelloides*, *A. hydrophila*, *Vibrio parahaemolyticus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Bacillus cereus* or *Staphylococcus aureus* in 21 cases from 2002 to 2007. This assay is simple, rapid, inexpensive, reliable as well as suitable for comprehensive, rapid detection of causative pathogens in food-borne outbreaks.

## MATERIAL AND METHODS

**Bacterial strains**. The 27 food-borne bacteria used in this study are *E. coli* (enteroinvasive *E. coli* [EIEC], enteropathogenic *E. coli* [EPEC], enterohemorrhagic *E. coli* [EHEC], enterotoxigenic *E. coli* [ETEC], and enteroaggregative *E. coli* [EAEC]) , *Shigella sonnei*, *Salmonella* Enteritidis, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Providencia alcalifaciens*, *Plesiomonas shigelloides*, *Campylobacter jejuni*, *C. coli*, *Vibrio cholerae*, TDH-positive *V. parahaemolyticus*, TRH-positive *V. parahaemolyticus*, *Aeromonas hydrophila*, *Staphylococcus aureus*, emetic *Bacillus cereus*, enterotoxigenic *B. cereus* and *Clostridium perfringens* (Table 1). Bacterial cultures and viable-cell counting were described in a previous report [10]. For template DNA of each food-borne pathogen as a PCR control, 200μl of each bacterial culture ($10^8$ CFU/ml) was treated with a QIAamp DNA Stool Mini kit (Qiagen) in the same procedure as the following stool

treatments.

**Primer design.** The 22 primer pairs used in this study for the detection of *E. coli* (EIEC, EPEC, EHEC, ETEC, and EAEC), *Salmonella enterica*, *Shigella* spp., *Y. enterocolitica, Y. pseudotuberculosis, P. alcalifaciens, C. jejuni, C. coli, V. cholerae, V. parahaemolyticus, A. hydrophila, P. shigelloides, S. aureus, C. perfringens*, and *B. cereus*) were described in our previous reports [9,10] for cases 1 to 19. The newly designed 22 primer pairs listed in Table 2 were used for cases 19 to 21. In this study, 10 primer pairs (marked with * in Table 2) were newly designed or selected from earlier publications (see Table 2 references). The 4 primer pairs (ces, yadA-X, CCceuE and aggR-Z) were newly designed. The ces primer was constructed from cereulide synthetase gene of emetic *B. cereus* [7], the yadA-X primer from *yadA* gene on the plasmid present in virulent *Yersinia* spp. [26], the CCceuE primer from *ceuE* gene encoding of a lipoprotein component of a binding-protein-dependent transport system for the siderophore enterochelin of *C. coli* [12] and the aggR-Z primer from *aggR* gene encoding of a transcriptional activator for EAEC aggregative adherence fimbria I expression [20]. To determine the specific primers ces, yadA-X, CCceuE and aggR-Z, the genes of *ces, yadA, ceuE* and *aggR* that were expected to be unique were selected with the BLAST (Basic Local Alignment Search Tool) program within GenBank and were designed by Biosearch Technologies Inc. (USA). Other primer pairs were those used in earlier publications (see Table 2 references). All oligonucleotide primers were synthesized by Invitrogen (Yokohama, Japan) or Biosearch Technologies Inc. (USA).

**Duplex SG-PCR with feces.** Feces (1 g) from 5 patients were weighed aseptically from the mass sample collected for virological inspection, placed into sterile tubes, and homogenized with 9 ml of distilled water. Then, 200-μl of stool suspension was treated with a QIAamp DNA Stool Mini kit. For real time PCR, we used SYBR *Premix EX Taq* (Takara, Japan), 32 glass capillary tubes, and a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer. Duplex SG-PCR was performed using 32 glass capillary tubes with 4 groups of 2 primer sets on the LC instrument for each run. Analysis of each group of primer pairs was made in 8 glass capillary tubes, each of which included 1 negative DNA control consisting of PCR-grade water, 2 positive controls, and template DNA from 5 feces. The first run of duplex SG-PCR was analyzed using 4 primer sets selected from 11 primer sets described in our previous reports [9, 10]. The newly first run primer set including eae plus FemB, AB plus EAST1, Tdh plus Ces-TM and Styinva plus GAP (see Table 2) was used for analysis of cases 19 to 21. The second run was analyzed using 4 primer sets selected from the following primer sets: LT plus AHH1, STa plus PSG, aggR-Z plus virA, and SG plus PAG and the third run using yadA-X plus CCceuE, and hlyA plus Trh. The *eaeA*-positive samples were analyzed by simple PCR using primers JMS1 and JMS2. Each reaction tube contained 10 μl of SYBR *Premix EX Taq*, 6.8 μl of PCR-grade $H_2O$, 0.4 μl of both forward and reverse

primers (10 μM) for the target gene of two food-borne pathogens and 2 μl of template DNA in a 20-μl PCR mixture. The assay cycling profile was 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 5 s and then annealing at 60°C for 20 s. Fluorescence signals were measured once per cycle at the end of the extension step. After PCR amplification, a melting temperature curve analysis was done. Next, the LightCycler PCR products were cooled to 65°C and then heated to 95°C at a rate of 0.1°C per s. The fluorescence signals obtained were continuously monitored to confirm amplification specificity during 1 h of analysis. The products' melting temperature peaks were calculated by performing 10 or more assays per sample and were based on the initial fluorescence curve found by plotting the negative derivative of fluorescence over temperature versus temperature. To quantify target bacteria in feces, DNA samples extracted with the QIAamp DNA Stool Mini kit from target bacteria were used to form a standard curve. Two microliters of a serial 10-fold dilution of DNA (Easy Dilution from Takara, Japan) were prepared and analyzed under the conditions specified above.

**Duplex SG-PCR analysis in 21 food-borne outbreaks.** 21 food-borne outbreak cases examined by duplex SG-PCR in Shimane Prefecture, Japan from 2002 to 2007 are shown in Table 3.

## RESULTS AND DISCUSSION

**Duplex SG-PCR procedures.**

We previously reported duplex SG-PCR assays for detection of 19 species of food-borne pathogens using 22 primer pairs [9, 10]. After that, more accurate duplex SG-PCR assays were designed by 10 more sensitive and specific primers including 6 primers (FemB, AB, ces-TM, Styinva, SG and AHH1) selected from earlier publications (see references in Table 2) and 5 new primers (eae, aggR-Z, yadA-X, and CCceuE) constructed in this study. The new primer set was used for cases 19 to 21. Real-time SG-PCR procedures using 22 primer pairs for the detection of 15 bacterial species, including 5 *E coli* subgroups, were developed for the duplex assay. The primer sequence, target, SG-PCR product size, $T_m$ values (mean plus standard deviation from a range of 10 assays), specificities, and references are summarized and listed in Tables 1 and 2. The primer virA detects *virA* gene of *Shigella* spp. and EIEC, the primer eae detects *eaeA* gene of EPEC and EHEC, and the primer EAST-1 detects *astA* gene of EAEC and ETEC. Primer hlyA detected *hlyA* gene of *V. cholerae* strains O1 and O139 as well as non-O1 strains. The primer SG for the detection of *nheB* (nonhemolytic enterotoxin B) gene of *B. cereus* cross-reacts with enterotoxigenic and emetic strains and the primer ces-TM detects cereulide synthetase gene of emetic strain of *B. cereus*. The *nheB* and *ces* gene positive strains were identified with emetic strains and the *nheB* gene positive and *ces* gene negative strains with enterotoxigenic strains. A new primer yadA-X for *Yersinia* adhesion reacts with virulent *Y. enterocolitica* and *Y. pseudotuberculosis*, but not with nonpathogenic strains of *Yersinia* spp. (data not shown). Other primers, including new primers aggR-Z and CCceuE, specifically detect each gene of EAEC and *C. coli*. Food-borne outbreak

Investigation Report (http://www.mhlw.go.jp/topics/syokuchu/), Ministry of Health, Labor and Welfare, Japan during 2005 to 2007 shows that 97% of food-borne outbreaks were caused by the following 7 species of food-borne pathogens: *S. enterica* (58.3%), *C. jejuni* (15.2%), TDH-producing *V. parahaemolyticus* (8.3%), *S. aureus* (7.2%), *C. perfringens* (3.6%), emetic *B. cereus* (1.6%), and EHEC (2.9%); and other virulent *E. coli* (2.1% ) which include *astA*-positive *E. coli* which is a strain of *E. coli* that does not possess any diarrheagenic characteristics except the EAEC heat-stable toxin 1 (EAST1) gene and is frequently isolated in diarrhea outbreaks [23]. Using of 4 primer sets of 2 primer pairs, including newly selected or designed 6 primer pairs, for the detection of 7 main food-borne pathogens and *astA*-positive *E. coli* in the first run of duplex SG-PCR brought out the comprehensive, rapid and sensitive detection of causative pathogens in food-borne pathogens to cases 19 to 21 (Table 2 and Figures 1 and 2). The second run of duplex SG-PCR used 4 primer sets and the final run utilized 2 primer sets selected from the remaining 4 primer pairs. The primers JMS1 and JMS2 were used for the single PCR detection of *stx1* and/or *stx2* genes from the *eaeA* gene-positive samples for the confirmation of EHEC. Figures 1 and 2 show the $T_m$ curves of the duplex SG-PCR products of the template DNA samples in each run. In duplex SG-PCR assay with two primer pairs, each PCR product was generated with a different $T_m$ curve. These could be resolved in a LightCycler by using $T_m$ curve analysis when a target bacterium was present in the reaction tube.

**Using duplex SG-PCR for identification of the causative agent in 21 food-borne outbreaks.**

Table 3 shows epidemiological and clinical investigations in 21 food-borne outbreaks examined by duplex SG-PCR analysis in Shimane Prefecture, Japan from 2002 to 2007. From samples of feces, we used a combination of duplex SG-PCR assay with DNA extraction using a QIAamp DNA Stool Mini kit. The SG-PCR assay is rapid, specific, and sensitive as a detection technique. The DNA extraction of 5 stool specimens with the QIAamp DNA Stool Mini kit was carried out within 1 h and it effectively removed inhibitors present in feces. The duplex SG-PCR assay was also carried out within 1 h. The 8 species (listed previously) of food-borne bacteria, which included 3 groups of *E. coli*, were detected from 110 (57.6%) of 191 feces in 21 cases by duplex SG-PCR. Then these causative agents were isolated and identified after 2 to 4 days. With the exception of two cases (cases 10 and 11), the first run of duplex SG-PCR confirmed the presence of a pathogen in 54 (58.1%) of 93 feces in 19 (90.5%) cases within 2 h. The exceptions were case 10 where a confirmation test was necessary to detect the *eaeA* gene of EHEC O26 and case 11 where *astA*-positive *E. coli* was detected on the third run. In the first run, DNA samples extracted from 5 feces (1, 3, 4 or 7 feces in 6 cases) of symptomatic patients were used and the causative pathogens were detected from 1 to 5 samples; 1 (in 8 cases: 1, 2, 4, 7, 8, 15, 19 and 21), 2 (in 3 cases: 9, 13, and 20), 3 (in 3 cases: 16, 18, and 21) , 4 (in 3 cases: 5,

6, and 17) and 5 samples (in 3 cases: 3, 12, and 14). Then the causative pathogens were later isolated in a routine laboratory. In cases 11 and 12, *C. perfringens* or *C. jejuni* was detected by duplex SG-qPCR with more than $10^5$ CFU/g feces from only 1 sample and *C. perfringens* was then also isolated from only 1 of 46 samples and *C. jejuni* from only 1 of 16 samples by culture method. Therefore, the infections with both these pathogens were determined to be sporadic cases and they were immediately eliminated as causative pathogens in cases 11 and 12. It was confirmed that duplex SG-PCR analysis of 5 feces collected from symptomatic patients was ultimately the most effective screening method for food-borne pathogens in food-borne outbreaks [9, 10].

Duplex SG-PCR rapidly and accurately demonstrated that 12 (57.1%) of 21 cases were caused with a single food-borne pathogen such as *C. jejuni* (6 cases), *C. perfringens* (3 cases), *B. cereus* (2 cases) and TDH-producing *V. parahaemolyticus* (one case). There were also 7 (33.3%) cases with plural food-borne bacterial pathogens (such as *astA*-positive *E. coli,* EPEC, *C. jejuni*, *C. perfringens, A. hydrophila* and *P. shigelloides*) and 2 (9.5%) cases with food-borne bacterial pathogens (*astA*-positive *E. coli* or EHEC O:26) and norovirus. In cases 2 and 10, although detection of norovirus is out of the scope of our work, norovirus and food-borne bacterial pathogens were concomitantly detected by conventional PCR analysis in our virological laboratory. In case 2 in which norovirus was detected in 6 of 7 feces, the *astA* gene of EAEC was detected from 7 of 10 feces and then *astA*-positive *E. coli* strains were isolated from 6 samples. In case 10 in which norovirus was detected from 20 of 22 feces, the *eae* gene of EPEC or EHEC was detected from 8 of 22 feces and EHEC O26 strains were isolated from 8 of 22 feces. In 7 cases (cases 1, 11,12, 13, 16, 20 and 21), the pathogenic *E. coli* strains belonging to *astA*-positive *E. coli* and/or EPEC were concomitantly detected with other food-borne bacterial pathogens. In case 1, the *eae* gene of EPEC or EHEC was detected from 4 of 22 feces and the *astA* gene of EAEC was detected in 3 other feces. However, duplex SG-PCR could not detect other virulent genes, including the *stx*1 and *stx*2 genes of EHEC. Then EPEC strains were later isolated from 5 feces and *astA*-positive *E. coli* from 4 other feces. In case 12, the *astA* gene of EAEC was detected in all 5 feces and the *eae* gene of EPEC or EHEC in 2 feces, but duplex SG-PCR could not detect other *E. coli* virulent genes. The subsequent bacteriological examination could not isolate pathogenic *E. coli* among nonpathogenic *E. coli* flora. In case 16, the *C. jejuni* specific gene was detected in 6 of 9 feces and the *astA* gene of EAEC was detected in 5 feces (both genes from 3 feces). *C. jejuni* strains were then isolated from 9 of 14 feces, but we were not able to isolate the pathogenic *E. coli* strain among nonpathogenic *E. coli* flora. In cases 19 to 21 analyzed improved real time PCR using 8 primers for the detection of 7 main food-borne bacteria and *astA*-positive *E. coli*, *C. jejuni*, EPEC or *astA*-positive *E. coli* ware detected from 1 to 3 fecal samples on the first run and the absence of the other main food-borne bacteria in the analyzed samples was readily

confirmed. In case 20, the *eae* gene of EPEC or EHEC was detected from 2 of 5 fecal samples on the first run and the *gyrB* gene of *P. shigelloides* were detected separately from other 2 fecal samples on the second run. Then *P. shigelloides* strains were isolated from 2 feces, but isolation of the EPEC strain was very difficult due to the presence of large nonpathogenic *E. coli* flora in the feces.

In almost all cases, the duplex SG-PCR assay first run detected these causative agents from the more than one of the five feces. Then, in almost all cases, the presence of a causative agent (presumed from duplex SG-PCR assay) was confirmed by the results of the final SG-PCR assay run and the bacteriological cultivation of additional feces. These findings confirmed that for food-borne outbreaks duplex SG-PCR is a useful tool for the rapid detection of both single and multiple pathogens.

**Quantification of the causative agent in 14 food-borne outbreak cases.**

Fig 2 shows the relationship between CFU and DNA copy of food-borne pathogens using SG-quantitative PCR (qPCR) assay in 71 feces from 14 cases examined by viable cell counting. There was no correlation ($r^2 = 0.1183$) between CFU and DNA copy of food-borne pathogens in feces, although almost all pathogens were detected by SG-PCR from feces registering more than $10^3$ CFU/g by viable cell counting. By using SG- qPCR assay combined with DNA extraction using the QIAamp DNA Stool Mini kit, Bibbal *et al.* [3] reported a significant correlation between CFU and DNA copy of ampicillin-resistant *Enterobacteriaceae* in swine feces. Fu *et al.* [8] reported a significant correlation between CFU and DNA copy of *Lactobacillus* and total anaerobic bacteria in dog feces, but found no correlation between CFU and DNA copy of *C. perfringens*. Although accurate quantifications of food-borne pathogens, including *C. jejuni* and *C. perfringens,* in feces were not completely performed by SG-qPCR in this study, the presence of any food-borne pathogens at more than $10^3$ CFU/g feces was certainly confirmed by melting curve analysis. There are two major problems for these differences. One case is different sample preparation that was used for CFU from the feces stored in the transport medium and for qPCR using the mass sample collected for virological inspection. Another cause is the approach used to construct the standard curves that were prepared from pure bacterial cultures. These curves do not relate with the "real" situation of a bacterial quantification in a faecal sample and can in part explain the absence of correlation between CFU and DNA copy of food-borne pathogens in faeces.

In our routine bacteriological diagnostic laboratory, we used duplex SYBR Green I PCR assay combined with DNA extraction via QIAamp DNA Stool Mini kit for the detection of food-borne bacteria from 21 food-borne outbreak cases. The causative bacteria were detected in almost all cases in 2 h or less. The first run was for the detection of 8 main food-borne bacteria and the second run was for the detection of other unusual suspect bacteria. The results proved that for comprehensive and rapid molecular diagnosis in food-borne outbreaks, duplex SG-PCR assay is not only very useful, but is also economically viable for

one-step differentiation of causative bacteria in fecal specimens obtained from symptomatic patients. This then allows for effective diagnosis and management of food-borne outbreak.

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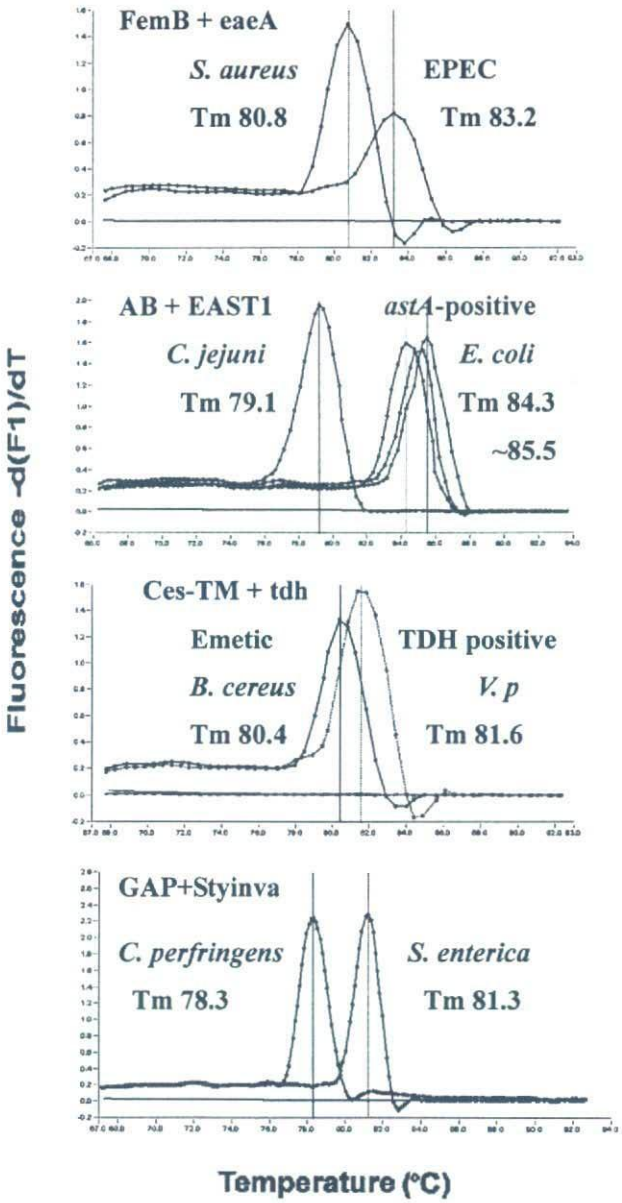


Figure 1 Melting curve analysis of duplex SYBR Green I PCR products in the first run using four primer sets; FemB plus eaeA, AB plus EAST1, ces plus tdh and GAP plus Styinva..

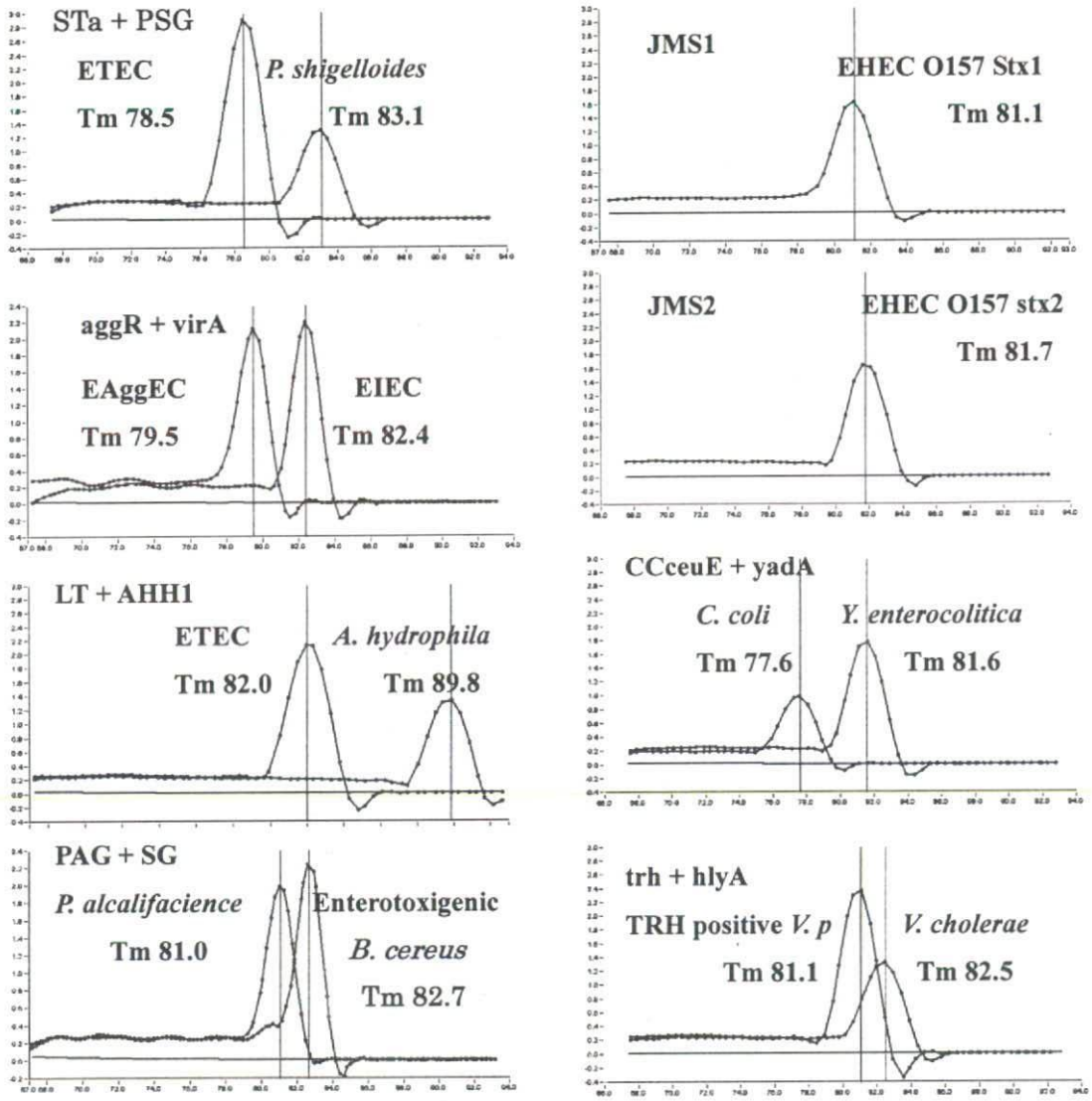


Figure 2 Melting curve analysis of duplex SYBR Green I PCR products in the second run using four primer sets; ST plus PSG, aggR plus virA, LT plus AHH1 and PAG plus SG: the third run using two primer sets; CCcesE plus yadA and trh plus hlyA: and simple PCR with primers JMS 1 and JMS2.

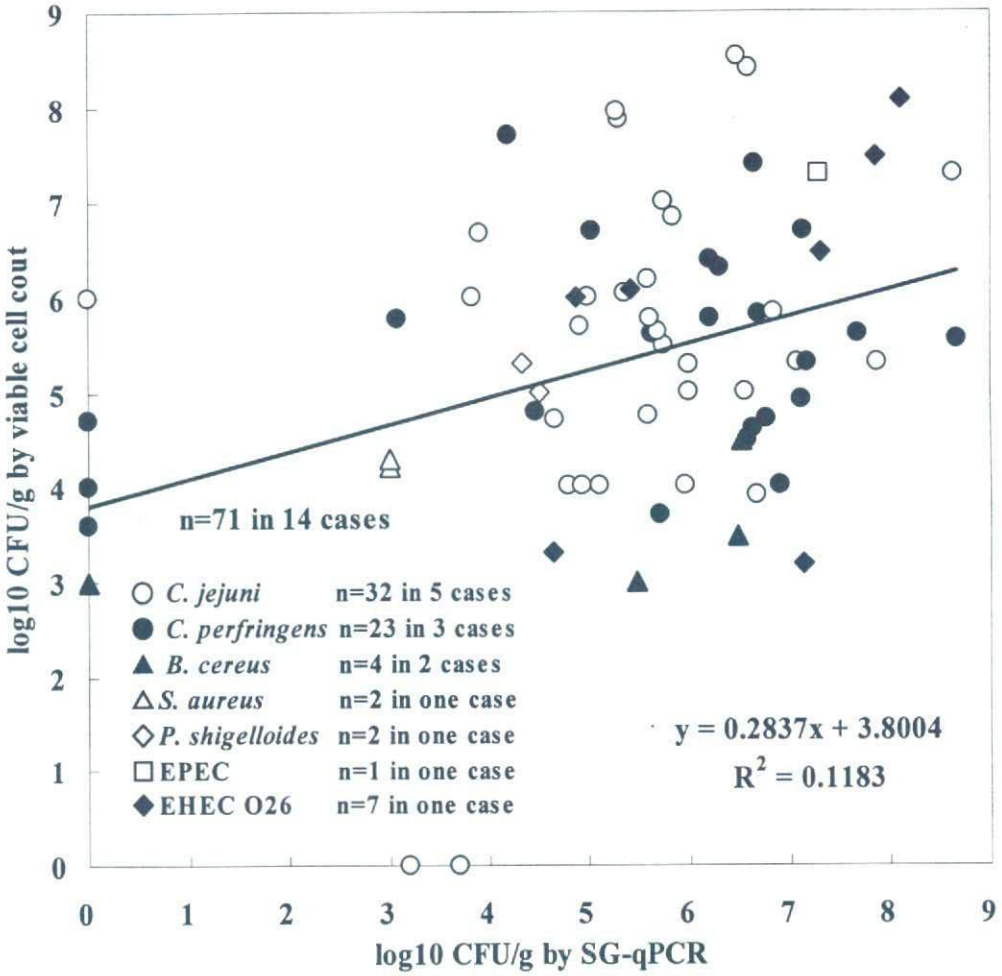


Figure 3 The relationship between CFU and DNA copy of food-borne pathogens in 71 food-borne pathogens-positive feces in 14 food-borne outbreak cases examined by viable cell counting.

**Table 1.** Bacterial strains assayed by SYBR Green I PCR

| Bacterial strains | Sources[e] | PCR results with each primer set (see Table 2) | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | eae | JMS1 | JMS2 | LT | STa | EAST-1 | aggR-Z | VirA | Styinva | yadA-X | PAG | PSG | AB | CC ceuE | hlyA | Tdh | Trh | AHH1 | FemB | ces-TM | SG | GAP |
| *Escherichia coli*-EPEC O55 (*eaeA*) | EC-2736[b] | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E.. coli*-EPEC O153 (*eaeA* and *astA*) | EC-2649[b] | + | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli*-EHEC O26:H11(*Stx1*) | SE-02005 | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli*-EHEC O157:H7 (*Stx2*) | SE020025 | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli*-EHEC O157:H7 (*Stx1* and *Stx2*) | SE-02027 | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli*-ETEC O148 (LT, ST and *astA*) | EC-3515[b] | - | - | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli*-ETEC O169 (ST and *astA*) | EC-4725[b] | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli*-EAEC O111 (*aggR* and *astA*) | EC-4131[b] | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *E. coli*-EIEC O124:HNM (*virA*) | EA32[a] | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Shigella sonnei* | I00031 | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Salmonella* Enteritidis | Sal-2339 | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Yersinia enterocolitica* O3/B4 | Pa241 | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| *Y. pseudotuberculosis* O4b | SP988 | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| *Providencia alcalifaciens* | NIID124[C] | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| *Plesiomonas shigelloides* | NIID123[C] | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| *Campylobacter jejuni* | SC 009 | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| *Campylobacter coli* | SC 011 | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| *Vibrio cholerae* O1 | ATCC14035 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| *V. cholerae* O139 | NIID63-93[C] | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| *V. cholerae non*-O1 | SVP84 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| *V. parahaemoliticus* O3:K6 (tdh) | SVP02 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - |
| *V. parahaemoliticus* O3:K6 (trh) | NIIDK4[C] | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| *Aeromonas hydrophila* O1 | ATCC7966 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| *Staphylococus aureus* | SS 05[e] | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| Emetic *Bacillus cereus* | No. 127[e] | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| Enterotoxigenic *B. cereus* | No. 1[e] | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - |
| *Clostridium perfringens* | H2[d] | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |

Strain kindly donated by K. Sugiyama[a], Shizuoka Prefectural Institute of Public Health, Shizuoka; J. Yatsuyanagi[b], Akita Prefectural Institute of Public Health, Akita; M. Tamura and E. Arakawa[c], [f]Other strains except for ATCC numbers are our own collections.

**Table 2** 22 pairs of specific primers for SYBR Green I PCR

| Primer set for duplex PCR | | Species and subgroups | Target gene | | PCR primers | | | GenBank accession no. | location | Product size (bp) | $T_m$ values[a] | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Name | Forward or revers | primers' sequences (5' - 3') | | | | | |
| First run | 1 | *Escherichia coli* | *eaeA* | *[b] | eae | F2 | CATTGATCAGGATTTTTCTGGTGATA | Z11541 | 899-924 | 106 | 83.2±0.2 | 21 |
| | | EPEC and EHEC | | | | R | CTCATGCGGAAATAGCCGTTA | | 1000-979 | | | |
| | | *Staphylococcus aureus* | *femB* | * | FemB | fw | AATTAACGAAATGGGCAGAAACA | AF106850 | 277-299 | 93 | 80.8±0.3 | 18 |
| | | | | | | rv | TGCGCAACACCCTGAACTT | | 370-351 | | | |
| | 2 | *Campylobacter jejuni* | *C. jejuni*- specific DNA | * | AB | F | CTGAATTTGATACCTTAAGTGCAGC | AL111168 | 381135 | 86 | 79.1±0.4 | 24 |
| | | | | | | R | AGGCACGCCTAAACCTATAGCT | | 381185 | | | |
| | | EAEC | *astA* | | EAST-1 | S | GCCATCAACACAGTATATCC | L11241 | 63-82 | 106 | 84.9±0.6 | 30 |
| | | | | | | AS | GAGTGACGGCTTTGTAGTCC | | 168-148 | | | |
| | 3 | *Vibrio parahaemoliticus* | *tdh* | | Tdh199 | F | GGTACTAAATGGCTGACATC | X54341 | 601-582 | 251 | 81.6±0.3 | 22 |
| | | | | | | R | CCACTACCACTCTCATATGC | | 351-370 | | | |
| | | Emetic *Bacillus cereus* | *ces* | * | ces-TM | F | GATGTTTGCGACGATGCAA | DQ360825 | 8689-8707 | 65 | 80.4±0.1 | This study |
| | | | | | | R | CTTTCGGCGTGATACCCATT | | 8793-8734 | | | |
| | 4 | *Salmonella* spp. | *invA* | * | Styinva | JHO-2-right | TCGTCATTCCATTACCTACC | M90846 | 167-186 | 119 | 81.3±0.4 | 13 |
| | | | | | | JHO-3-left | AAACGTTGAAAAACTGAGGA | | 285-234 | | | |
| | | *Clostridium perfringens* | *cpe* | | GAP | 11 | GGTTCATTAATTGAAACTGGTG | X81849 | 583-604 | 154 | 78.3±0.4 | 17 |
| | | | | | | 12 | AACGCCAATCATATAAATTACAGC | | 712-736 | | | |
| | 5 | ETEC (ST) | ST | | STa-F | F | GCTAATGTTGGCAATTTTTATTTCTGTA | M25607 | 294-321 | 190 | 78.5±0.2 | 6 |
| Second and third runs | | | | | | R | AGGATTACAACAAAGTTCACAGCAGTAA | | 483-456 | | | |
| | | *Plesiomonas shigelloides* | *gyrB* | | PSG | 237-F | TTCCAGTACGAGATCCTGGCTAA | AJ300545 | 237-259 | 68 | 83.1±0.2 | 9 |
| | | | | | | 304-R | TGAATCGACACGCCAGAGTTC | | 304-284 | | | |
| | 6 | EAggEC | *aggR* | * | aggR-Z | F | CAGAATCGTCAGCATCAGCTACA | Z18751 | 432-454 | 97 | 79.5±0.3 | This study |
| | | | | | | R | GATGCCCTGATGATAATATACGGAA | | 358-382 | | | |
| | | EIEC & Shigella spp. | *virA* | | virA-F | F | CTGCATTCTGGCAATCTCTTCACA | D26468 | 1589-1622 | 215 | 82.4±0.3 | 27 |
| | | | | | | R | TGATGAGCTAACTTCGTAAGCCCTCC | | 1813-1788 | | | |
| | 7 | *Aeromonas hydrophila* | *ahh1* | * | AHH1 | F | GCCGAGCGCCCAGAAGGTGAGTT | CP000462 | 1653360-82 | 133 | 89.8±0.4 | 28 |
| | | | | | | R | GAGCGGCTGGATGCGGTTGT | | 1653492-73 | | | |
| | | ETEC (LT) | LT | | LT | 1 | AGCAGGTTTCCCACCGGATCACCA | S60731 | 613-636 | 132 | 82.0±0.3 | 15 |
| | | | | | | 2 | GTGCTCAGATTCTGGGTCTC | | 744-725 | | | |
| | 8 | *Providencia alcalifaciens* | *gyrB* | | PAG | 38F | TCTGCACGGTGTGGGTGTT | AJ300547 | 38-56 | 73 | 81.0±0.2 | 9 |
| | | | | | | 110R | ACCGTCACGGCGGATTACT | | 110-92 | | | |
| | | Enterotoxigenic *B. cereus* | *nheB* | * | SG | F3 | GCACTTATGGCAGTATTTGCAGC | DQ153257 | 2101-2123 | 152 | 82.7±0.4 | 29 |
| | | | | | | R3 | GCATCTTTTAAGCCTTCTGGTC | | 2252-2231 | | | |
| | 9 | *Yersinia enterocolitica* | *yadA* | * | yadA-X | F | CCAGAACCAATTGCAATGCCT | X13882 | 1564-1543 | 100 | 81.6±0.2 | This study |
| | | *Y. pseudotuberculosis* | | | | R | CTTTAAACAGCTTGTTCCAGCCA | | 1465-1487 | | 81.1±0.3 | |
| | | *Campylobacter coli* | *ceuE* | * | CCceuE | 825F | ACGCGCACAAGGCATACTT | X88849 | 3513-3531 | 91 | 77.6±0.3 | This study |
| | | | | | | 915R | CCAGTATTCAGGATCAAGATAAATGATTT | | 3603-3575 | | | |
| | 10 | *Vibrio cholerae* | *hlyA* | | hlyA | 2272-F | AGCAGCGTGTGGGACAAGA | X51746 | 2272-2291 | 71 | 82.4±0.1 | 9 |
| | | | | | | 2272-F | GCGGACCCTAATGCATCAAT | | 2342-2323 | | | |
| | | *Vibrio parahaemoliticus* | *trh* | | Trh | 250-F | GGCTCAAAATGGTTAAGCG | DQ359748 | 256-274 | 250 | 81.1±0.1 | 22 |
| | | | | | | 251-R | CATTTCCGCTCTCATATGC | | 505-487 | | | |
| Singl PCR | | EHEC (Stx 1) | *Stx1* | | JMS1 | F | GTCACAGTAACAAACCGTAACA | EF441598 | 509-488 | 95 | 81.1±1.0 | 16 |
| | | | | | | R | TCGTTGACTACTTCTTATCTGGA | | 415-437 | | | |
| Singl PCR | | EHEC (Stx 2) | *Stx2* | | JMS2F | F | CGACCCCTCTTGAACATA | EF441616 | 140-157 | 108 | 81.7±0.3 | 16 |
| | | | | | | R | GATAGACATCAAGCCCTCGT | | 247-228 | | | |

a: Average ±standard deviation of *Tm* values of 10 tests; b: *:new selected or designed primer