Method

Rapid and Accurate Diagnosis Based on Real-Time PCR Cycle Threshold Value for the Identification of Campylobacter jejuni, astA Gene-Positive Escherichia coli, and eae Gene-Positive E. coli

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SUMMARY: We previously developed a multiplex real-time PCR assay (Rapid Foodborne Bacterial Screening 24 ver.5, [RFBS24 ver.5]) for simultaneous detection of 24 foodborne bacterial targets. Here, to overcome the discrepancy of the results from RFBS24 ver.5 and bacterial culture methods (BC), we analyzed 246 human clinical samples from 49 gastroenteritis outbreaks using RFBS24 ver.5 and evaluated the correlation between the cycle threshold (CT) value of RFBS24 ver.5 and the BC results. The results showed that the RFBS24 ver.5 system was more sensitive than BC for Campylobacter jejuni and Escherichia coli harboring astA or eae, with positive predictive values (PPV) of 45.5–87.0% and a kappa coefficient (KC) of 0.60–0.92, respectively. The CTs were significantly different between BC-positive and negative samples (p < 0.01). All RFBS24 ver.5-positive samples were BC-positive under the lower confidence interval (CI) limit of 95% or 99% for the CT of the BC-negative samples. We set the 95% or 99% CI lower limit to the determination CT (d-CT) to discriminate for assured BC-positive results (d-CTs: 27.42–30.86), and subsequently the PPVs (94.7%–100.0%) and KCs (0.89–0.95) of the 3 targets were increased. Together, we concluded that the implication of a d-CT-based approach would be a valuable tool for rapid and accurate diagnoses using the RFBS24 ver.5 system.

INTRODUCTION

There are approximately 1,000 foodborne illness outbreaks in Japan each year (1). To prevent the expansion and recurrence of these outbreaks, public health officials must rapidly and accurately identify the causative pathogen (2–3). Although real-time polymerase chain reaction (PCR) assays detect viral pathogens within a few hours, bacterial culture methods (BCs), used to identify the causative pathogen and facilities causing food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This method for the detection and confirmation of the causative agents of foodborne outbreaks. Therefore, public health officials currently use the BC approach to determine the causative pathogen and facilities causing food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads us to propose the necessity for improving the diagnostic accuracy of RFBS24 ver.5 to rapidly determine the causative agent in the food poisoning. This leads we.
to reduce discrepancies in identifying the above pathogens when compared with the BC method.

MATERIALS AND METHODS

Clinical samples: We investigated 49 outbreaks (fecal [n = 243] or vomit samples [n = 3]) of bacterial foodborne illnesses or infections that occurred from April 2010 to February 2016 in Shimane Prefecture, Japan. These were composed of mixed infections of: enteroaggregative E. coli (EAEC), enterotoxigenic E. coli (ETEC), astA gene-positive E. coli (8 fecal samples from one outbreak), astA gene-positive E. coli (12 fecal samples from one outbreak), enterohemorrhagic E. coli (EHEC) O157 (11 fecal samples from one outbreak), Clostridium perfringens (24 fecal samples from 4 outbreaks), Yersinia enterocolitica (one fecal sample from one outbreak), C. jejuni (65 fecal samples from 14 outbreaks); Salmonella enterica Typhi (one fecal sample from one outbreak); unknown origin (30 fecal samples and 3 vomit samples from 8 outbreaks); and norovirus or sapovirus (90 fecal samples from 17 outbreaks).

Bacterial culture (BC): BC was performed by direct-enrichment-culture techniques as described previously (16–18). For isolation of diarrheagenic E. coli, we screened 5–40 colonies grown on Chromocult coliform agar (Merck, Darmstadt, Germany) using PCR as described (19). If C. jejuni and astA gene-positive E. coli were detected with RFBS24 ver.5 but not with BC, isolation of C. jejuni was performed using modified charcoal-cеfoperazone-deoxycholate (CCDA) agar (Oxoid, Basingstoke, UK) followed by enrichment culture at 42 °C for 24 h in Preston Campylobacter selective enrichment broth (Oxoid). For the enrichment of the astA gene-positive E. coli, mEC broth (Nissui, Tokyo, Japan) was used.

DNA extraction and RFBS24 ver.5 analysis: DNA samples were extracted from fecal and vomit samples as described previously (1).

RFBS24 ver.5, which includes 8 sets (A–H) of SG-mPCR primers for the detection of the 24 foodborne bacterial targets, was performed with a Thermal Cycler Dice Real-Time System TP860 (Takara TP860; Takara Bio, Shiga, Japan) as described previously (1). The concentration of IAC was adjusted to be approximately 5.6 × 10^{2} copies/reaction to achieve a CT (cycle number at the fluorescence signal crosses the threshold line) between 31 and 34, calculated using Thermal Cycler Dice Real Time System Software ver.5.11. Among these, we compared the CTS in the RFBS24 ver.5 results of cj0414, astA, and eae genes, with BC results for C. jejuni and diarrheagenic E. coli, respectively.

The specificity of the reaction by RFBS24 ver.5 was confirmed by the analysis of the melting temperature of the amplification products after the last reaction cycle. Furthermore, for RFBS24 ver.5-positive but BC-negative samples, we confirmed the specificity of the reaction by 2 semi-nested PCRs for detection of eae and astA gene, and real-time TaqMan PCR for C. jejuni, as reported previously (20). To perform the 2 semi-nested PCRs, we designed 2 primers using the Primer3 program and with sequences submitted to GenBank (21) (eae-950R 5'-TCGGCATTATTCGCCACCAAA-3' and astA-150R 5'-TTCCATGACAGAAGCGCAG-3'). Our previously reported (1) (Primers of eae, eae-F2 and eae-950R; Primers of astA, EAST-1-S and astA-150R) were used to amplify the 2 targets (eae, 52 bp; astA, 88 bp) in a 20 μL PCR mixture containing 10 × Ex Taq buffer (Takara Bio), 10 μM primers, and 2 μL of the RFBS24 ver.5-PCR products (diluted 100-fold), according to Takara’s protocol. Amplification was performed as follows: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and elongation at 72°C for 1 min; and final extension at 72°C for 7 min. The PCR products were separated by electrophoresis in 2% NuSieve 3:1 agarose gels (Takara Bio) and stained with ethidium bromide.

Correlation analysis: The DNA samples were then analyzed using RFBS24 ver.5, and the results of the 3 bacterial targets (cj0414, astA, and eae) were compared with the BC results. We calculated the positive predictive value (PPV) and negative predictive value (NPV) using the following equation: PPV = A/(A + B), NPV = C/(C + D), where A was the number of BC- and RFBS24 ver.5-positive samples, B was the number of BC-negative but RFBS24 ver.5-positive samples, C was the number of BC- and RFBS24 ver.5-negative samples, and D was the number of BC-positive but RFBS24 ver.5-negative samples.

Statistical analysis: Statistical analysis was performed using IBM SPSS Statistics ver. 23.0 software (IBM Corporation, Armonk, NY, USA). We calculated kappa coefficients (KCs) for cj0414, eae, and astA to assess the agreement between the RFBS24 ver.5 and BC results. KCs were categorized as excellent agreement (0.93–1.00), very good agreement (0.81–0.92), good agreement (0.61–0.80), fair agreement (0.41–0.60), slight agreement (0.21–0.40), and poor agreement (0.01–0.20) (22). We used the Mann-Whitney test to compare the CT of BC-negative with BC-positive samples with reference to cj0414, eae, and astA RFBS24 ver.5-positive samples. A p value of < 0.05 was considered statistically significant. We calculated the 95% and 99% confidence intervals (CIs) of 3 bacterial targets’ CTS (cj0414, eae, and astA) for RFBS24 ver.5-positive but BC-negative samples or RFBS24 ver.5-positive and BC-positive samples. We set the lower limit of 95% or 99% CI (i.e., CT for RFBS24 ver.5-positive and BC-negative samples) for the determination CT (d-CT) to determine whether the cj0414, eae, and astA results were positive and consistent with those of the analyses using RFBS24 ver.5 and BC.

Ethical considerations: Patient fecal and vomit samples were tested in accordance with the Food Sanitation Law and the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections, and informed consent was waived. The ethics committees of the Shimane Prefectural Institute of Public Health and Environmental Science approved this study, including the waiver of informed consent. This study was performed according to the Ethical Guidelines for Epidemiology Research of the Ministry of Education, Culture, Sports, Science and Technology of Japan.
RESULTS

Evaluation of RFBS24 ver.5 for clinical samples from 49 gastroenteritis outbreaks: The PPVs and NPVs of 3 bacterial targets (cj0414, astA, and eae) by the RFBS24 ver.5 were 45.5%–87.0% and 100.0% respectively (Table 1). The KC for eae was identified as fair agreement (0.60), while the KC either for astA or cj0414 were as good agreement (0.74), or very good agreement (0.92), respectively.

For the 3 bacterial targets, RFBS24 ver.5-positive but BC-negative results were obtained in 6 to 13 patient’s samples (Table 1). We investigated the specificity of the RFBS24 ver.5 methods reaction by real-time TaqMan PCR for C. jejuni and 2 semi-nested PCRs for eae and astA genes. The real-time TaqMan PCR successfully detected C. jejuni from all RFBS24 ver.5-C. jejuni-positive but BC-negative samples. Moreover, the all amplification products of the 2 semi-nested PCRs were confirmed to have the correct molecular weight by agarose gel electrophoresis. Thus, these results implied that there were no false-positive results for the 3 bacterial targets.

The CTs of RFBS24 ver.5 associate with the BC results: The CTs of either cj0414, eae, or astA gene-positive samples, were compared with the BC results to assess their association (Fig. 1). The median CTs of BC-positive and BC-negative samples for cj0414 were 26.34 (95% CI, 19.96–31.92; 99% CI, 17.49–34.39) and 31.46 (95% CI, 30.86–32.34; 99% CI, 30.56–32.64), respectively. The median CTs of BC-positive and BC-negative samples for eae were 22.77 (95% CI, 16.82–29.42; 99% CI, 14.21–32.03) and 30.93 (95% CI, 29.43–33.39; 99% CI, 28.61–34.21), respectively. The median CTs of BC-positive and BC-negative samples for astA were 23.38 (95% CI, 16.34–30.21; 99% CI, 13.46–33.08) and 30.73 (95% CI, 28.32–32.65; 99% CI, 27.42–33.55), respectively. Thus, these data indicated that the CTs were significantly lower for the BC-positive group compared with those of the BC-negative group in the 3 bacterial targets (p < 0.01).

RFBS24 ver.5 CTs statistically correlate with BC results: We calculated the d-CT to determine whether the cj0414, eae, and astA results were truly positive for the RFBS24 ver.5 and BC. Namely, the lower limits of the 95% CI or 99% CI (i.e., CT for RFBS24 ver.5-positive but BC-negative samples) were investigated to determine the correlations to the d-CT. For cj0414 and eae, all RFBS24 ver.5-positive samples were BC-positive at the < 95% CI or 99% CI lower limit (Tables 2 and 3). In contrast, the RFBS24 ver.5-astA-positive samples were BC-positive results at the < 99% CI lower limit, although only one positive sample was BC-negative at the < 95% CI lower limit (Table 4). The proportion

Table 1. Number of bacterial pathogens detected using RFBS24 ver.5 and BC (n = 246)

| Bacterial pathogen | Results of RFBS24 ver.5(1) | No. of positive samples with BC(2) | d-CT (3) | No. of RFBS24 ver.5 positive samples | No. of RFBS24 ver.5 negative samples | PPV/NPV (%)(4) | No. of RFBS24 ver.5 positive samples | No. of RFBS24 ver.5 negative samples |
|-------------------|-----------------------------|-----------------------------------|---------|-------------------------------------|--------------------------------------|--------------|-------------------------------------|--------------------------------------|
| C. jejuni (cj0414) | NA(5)                       | 40                                | 6       | 0                                   | 200                                  | 87.0/100.0   | 0.92                                | 40                                   |
|                   | Lower limit of 95% CI       | 37                                | 0       | 3                                   | 206                                  | 100/98.6     | 0.95                                | 10                                   |
|                   | (30.86)                     | Lower limit of 99% CI             | 35      | 0                                   | 5                                    | 100/97.6     | 0.92                                | 10                                   |
| E. coli (eae)     | NA(5)                       | 10                                | 0       | 0                                   | 224                                  | 45.5/100.0   | 0.60                                | 21                                   |
|                   | Lower limit of 95% CI       | 9                                 | 0       | 1                                   | 236                                  | 100/99.6     | 0.95                                | 21                                   |
|                   | (29.43)                     | Lower limit of 99% CI             | 9       | 0                                   | 1                                    | 100/99.6     | 0.95                                | 21                                   |
| E. coli (astA)    | NA(5)                       | 21                                | 13      | 0                                   | 212                                  | 61.8/100.0   | 0.74                                | 21                                   |
|                   | Lower limit of 95% CI       | 18                                | 1       | 3                                   | 224                                  | 94.7/98.7    | 0.89                                | 21                                   |
|                   | (28.32)                     | Lower limit of 99% CI             | 18      | 0                                   | 3                                    | 100/98.7     | 0.92                                | 21                                   |

(1): RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5.
(2): d-CT, the determination cycle threshold (CT) value for discrimination of assured-positive result.
(3): PPV/NPV, positive predictive value/negative predictive value.
(4): KC, kappa coefficient.
(5): BC, bacterial culture method.
(6): NA, not applicable.
(7): The lower limit of the 99% and 95% confidence intervals (CIs) of CT value for RFBS24 ver.5-positive but BC-negative samples.
of eae or astA gene-positive samples at the < 99% CI lower limit was 41.7%–87.5% in the 3 diarrheagenic E. coli outbreaks (e.g., outbreak No. 1 in Table 3; outbreak Nos. 1 and 2 in Table 4) and 0%–28.6% in the remaining outbreaks.

For cj0414 and astA, RFBS24 ver.5-positive samples were BC-positive or BC-negative at the ≥ 99% CI lower limit (Tables 2 and 4). Twelve samples of RFBS24 ver.5-eae-positive samples gave a BC-negative result and one sample gave a BC-positive result at the ≥ 95% CI lower limit (Table 3). For BC-negative samples at ≥ 95% CI lower limit, we isolated C. jejuni and astA gene-positive E. coli from 3 of 5 and 2 of 4 fecal samples, respectively, by BC (Tables 2 and 4). We evaluated the PPVs and NPVs of the 3 bacterial targets after applying the d-CT (Table 1). The PPVs and NPVs were 94.7%–100.0% and 97.6%–99.6%, respectively, at the < 95% or 99% CI lower limit. Similarly, KCs of the 3 bacterial targets were 0.49–0.95 (very good-excellent agreement) at the < 95% or < 99% CI lower limit. Together, these data clearly showed that the CT-based detection method correlates with the BC results.

**Table 2. Number of C. jejuni positive sample using RFBS24 ver.5 to analyze 14 gastroenteritis outbreaks caused by Campylobacter**

| Outbreak No. | No. of patient samples | Causative pathogen | No. of C. jejuni positive samples with BC1) | CT of RFBS24 ver.52) for C. jejuni (cj0414) | Total |
|-------------|----------------------|--------------------|-------------------------------------------|---------------------------------------------|-------|
| 1           | 11                   | EHEC O157          | 10                                        | < 99% CI (30.56)2)                           | 35    |
| 2           | 8                    | EPEC, ETEC, and astA gene-positive E. coli | 0                        | 94.7% CI (28.61)3)                           | 2     |
| 3           | 12                   | astA gene-positive E. coli | 0                        | 99% CI (30.56) ≤ CT < 97% CI (30.86)         | 3     |
| 4           | 8                    | C. perfringens      | 0                                        | 95% CI (30.86) ≤ CT  ≤ 99% CI (30.93)       | 6 (3) |
| 5           | 4                    | C. perfringens      | 0                                        | CI lower limit (30.56)2)                    | 6 (3) |
| 6           | 8                    | Norovirus           | 0                                        | CI lower limit (30.56)2)                    | 6 (3) |
| Total       | 61                   |                    |                                           |                                             | 40    |

1)RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5.
2)CI, The lower limit of 95% confidence intervals (CIs) of cycle threshold (CT) value for RFBS24 ver.5-positive but BC-negative samples.
3)BC, bacterial culture method.
4)The numbers in parentheses indicate BC-negative but BC-positive result combined with enrichment culture.

**Table 3. Number of eae gene-positive samples using RFBS24 ver.5 to analyze 6 gastroenteritis outbreaks**

| Outbreak No. | No. of patient samples | Causative pathogen | No. of eae positive samples with BC1) | CT of RFBS24 ver.52) for eae | Proportion of eae positive sample at the < 99% CI (%) |
|-------------|----------------------|--------------------|--------------------------------------|------------------|-----------------------------------------------|
| 1           | 11                   | EHEC O157          | 10                                   | 94.7% CI (28.61)3) | 81.82                                         |
| 2           | 8                    | EPEC, ETEC, and astA gene-positive E. coli | 0                        | 99% CI (28.61) ≤ CT < 95% CI (29.43)          | 0/0.00                                       |
| 3           | 12                   | astA gene-positive E. coli | 0                        | 95% CI (29.43) ≤ CT  ≤ 99% CI (30.86)         | 0/0.00                                       |
| 4           | 8                    | C. perfringens      | 0                                        | CI lower limit (28.61)2)                    | 0/0.00                                       |
| 5           | 4                    | C. perfringens      | 0                                        | CI lower limit (29.43)2)                    | 0/0.00                                       |
| 6           | 8                    | Norovirus           | 0                                        | CI lower limit (28.86)2)                    | 0/0.00                                       |
| Total       | 51                   |                    |                                           |                                             | 1/12                                          |

1)BC, bacterial culture method.
2)RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5.
3)CI, The lower limit of 99% confidence intervals of cycle threshold (CT) value for RFBS24 ver.5-positive but BC-negative samples.
4)Number of BC-positive and -negative samples are listed.

**Table 4. Number of astA gene-positive samples using RFBS24 ver.5 to analyze 13 gastroenteritis outbreaks**

| Outbreak No. | No. of patient samples | Causative pathogen | No. of astA positive samples with BC1) | CT of RFBS24 ver.52) for astA | Proportion of astA positive sample at the < 99% CI (%) |
|-------------|----------------------|--------------------|--------------------------------------|--------------------------|-----------------------------------------------|
| 1           | 8                    | EAE, ETEC, and astA gene-positive E. coli | 7                        | 94.7% CI (27.42)3)       | 87.50                                         |
| 2           | 12                   | astA gene-positive E. coli | 7                        | 99% CI (27.42) ≤ CT < 95% CI (28.32)          | 41.67                                         |
| 3           | 6                    | C. perfringens      | 0                                        | 95% CI (28.32) ≤ CT  ≤ 99% CI (29.43)         | 0/0.00                                       |
| 4           | 3                    | C. jejuni           | 0                                        | CI lower limit (27.42)2)                    | 0/0.00                                       |
| 5           | 2                    | C. jejuni           | 0                                        | CI lower limit (28.32)2)                    | 0/0.00                                       |
| 6           | 3                    | Cause unknown       | 0                                        | CI lower limit (28.43)2)                    | 0/0.00                                       |
| 7           | 6                    | Cause unknown       | 0                                        | CI lower limit (29.43)2)                    | 0/0.00                                       |
| 8           | 7                    | Cause unknown       | 2                                        | CI lower limit (28.43)2)                    | 0/0.00                                       |
| 9           | 10                   | Saprospirillum     | 2                                        | CI lower limit (28.43)2)                    | 0/0.00                                       |
| 10          | 4                    | Norovirus           | 1                                        | CI lower limit (28.43)2)                    | 0/0.00                                       |
| 11          | 9                    | Norovirus           | 1                                        | CI lower limit (28.43)2)                    | 0/0.00                                       |
| 12          | 4                    | Norovirus           | 0                                        | CI lower limit (28.43)2)                    | 0/0.00                                       |
| 13          | 7                    | Norovirus           | 0                                        | CI lower limit (28.43)2)                    | 0/0.00                                       |
| Total       | 81                   |                    |                                           |                                             | 3/12 (2)                                      |

1)BC, bacterial culture method.
2)RFBS24 ver.5, rapid foodborne bacterial screening 24 ver.5.
3)CI, The lower limit of the 99% and 95% confidence intervals (CIs) of cycle threshold (CT) value for RFBS24 ver.5-positive but BC-negative samples.
4)Number of BC-positive and -negative samples are listed.
5)The numbers in parentheses indicate BC-negative but BC-positive result combined with enrichment culture.
DISCUSSION

This study evaluated the correlation of the RFBS24 ver.5 method with the results of traditional culturing techniques, normally used as golden standard for the identification of foodborne outbreaks, based on newly provided data (e.g., the CT of patient’s samples, PPV, NPV, KC, and d-CT). The comparative study showed that the results of KCs for eae and astA were identified to be in fair-good agreement. Although the KC for cj0414 was identified as in very good agreement, the PPV was under 100.0%. Moreover, we found that eae and astA were detected in the samples from 16 non-E. coli foodborne outbreaks. After multiple statistical analyses, we could conclude that RFBS24 ver.5 CT-based analysis could be an appropriate diagnostic method, and results correlated to the traditional culturing technique.

A CT less than the lower limit of 95% or 99% CI (i.e., the CT for RFBS24 ver.5-positive but BC-negative samples), indicated that the BC result was not negative at significance level of 0.025 or 0.005. There were 100% BC-positive results at the < 95% CI or 99% CI lower limit in actual gastroenteritis outbreaks (Tables 2–4), and the 95% CI or 99% CI lower limit was applied as the d-CT. Consequently, NPVs of the 3 bacterial targets were slightly decreased by the application of the 95% CI or 99% CI lower limit as the d-CT, and KCs and PPVs were increased compared with those before the application of d-CT. Taken together, we set the d-CT to obtain the most appropriate results of KCs and PPVs. The 99% CI lower limit was optimal as the d-CT for astA, whereas the 95% or 99% CI lower limit was optimal for eae, and the 95% CI lower limit was optimal for cj0414. A previous study (14) reported the CT cutoffs of the 3 pathogens (Cryptosporidium, ETEC, and Shigella) for the identification of the pathogen causing acute childhood diarrhea in Zanzibar. For C. jejuni and diarrheagenic E. coli harboring the eae or astA gene, no study has previously investigated setting the CT for the discrimination of an assured BC-positive result using patient feces, whereas our method combined with d-CT is useful for this.

Public Health Ontario Laboratories utilize a real-time PCR assay to identify B. pertussis, and CTs < 36 are reported as positive and CTs ≥ 36 and < 40 are considered indeterminate (12). Similarly, in an actual outbreak of gastroenteritis, public health officials would be informed if a RFBS24 ver.5-positive result of < d-CT is an assured BC-positive result and that the RFBS24 ver.5-positive result at the ≥ d-CT is a BC-indeterminate result, as the NPVs of the 3 bacterial targets were < 100.0%. The determination of the causative pathogen may be based on the BC result when a RFBS24 ver.5-positive sample at the ≥ d-CT is epidemiologically important for determining the causative pathogen or the facilities that caused the food poisoning. For fecal samples where ≥ d-CT for cj0414 and astA, C. jejuni and astA gene-positive E. coli were isolated from some fecal samples using BC. Thus, it is better to combine BC with enrichment culture for RFBS24 ver.5-positive samples at ≥ d-CT. Furthermore, the proportion of eae or astA gene-positive samples at < d-CT was higher in the diarrheagenic E. coli outbreaks than in the non-E. coli outbreaks. A previous study (14) reported that the CTs of ETEC and Shigella spp. were lower in patients than in healthy carriers. Thus, diarrheagenic E. coli harboring eae or astA gene are not likely to be implicated as a causative pathogen when the proportion of these positive samples at < d-CT are low (0%–28.6%) in an actual outbreak of gastroenteritis.

The limitations to the present study are as follows: Iijima et al. (23) reported that traditional laboratory methods might not detect most diarrheagenic E. coli infections, particularly in the early stage of gastroenteritis, because the proportion of diarrheagenic E. coli is < 10.0% of the E. coli population. Moreover, bacterial viability may be compromised by the conditions of fecal sample transport and administration of antibiotic treatments. Therefore, further studies are required to confirm the correlation between CTs and BC results for a sufficiently large number of outbreaks of gastroenteritis. Furthermore, development of an appropriate culture method including enrichment culture, testing greater numbers of bacterial isolates (e.g., E. coli), and discriminating viable from dead cells using RFBS24 ver.5 combined with use of the viable/dead stain ethidium monoazide (24–25), for example, may be required to overcome these limitations.

In conclusion, the d-CTs for cj0414, eae, and astA can be used as an effective indicator for the discrimination of an assured BC-positive result and for improving diagnostic accuracy. For bacterial pathogens with a large discrepancy between the RFBS24 ver.5 and BC results, arriving at a rapid diagnosis according to the d-CT would facilitate providing public health officials with accurate information about the causative pathogen.

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Conflict of interest None to declare.

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