Research article

A one-step multiplex PCR-based assay for simultaneous detection and classification of virulence factors to identify five diarrheagenic E. coli pathotypes

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HIGHLIGHTS

- We developed a one-step single reaction mPCR to detect DEC strains.
- 10 prominently expressed genes characteristic to the five pathotypes were assayed.
- All the strains were detected at concentrations ranging from $10^4$ to $10^7$ CFU/mL.
- We show cost- and time-effective detection of DEC in clinical cultured samples.

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ABSTRACT

Human diarrhea-causing strains of Escherichia coli are referred to as diarrheagenic E. coli (DEC). DEC can be divided into five main categories based on distinct epidemiological and clinical features, specific virulence determinants, and association with certain serotypes. In the present study, a simple and rapid one-step single reaction multiplex PCR (mPCR) assay was developed for the simultaneous identification and differentiation of five currently established DEC pathotypes causing gastrointestinal diseases. The mPCR incorporated 10 primer pairs to amplify 10 virulence genes specific to the different pathotypes (i.e., stx1 and stx2 for EHEC, elt and atl for ETEC, eaeA and bfpA for EPEC, aggR and astA for EAEC, and pilA and invE for EIEC) and to generate DNA fragments of sufficiently different sizes to be unequivocally resolved. All strains were detected at concentrations ranging from $10^4$ to $10^7$ CFU/mL. To demonstrate the utility of the mPCR assay, 236 clinically isolated strains of DEC from two hospitals were successfully categorized. One-step mPCR technique reduced the cost and effort involved in the identification of various virulence factors in DEC. Thus, we demonstrated that the newly developed mPCR assay has the potential to be introduced as a diagnostic tool that can be utilized for the detection of DEC as an additional check in clinical laboratories and for confirmation in health and environment institutes, health centers, and reference laboratories.

1. Introduction

Common diseases caused by Escherichia coli include diarrhea, acute inflammation, hemorrhagic colitis, urinary tract infections, and septicemia. These pathotypes have different attributes that help them to cause diseases via different mechanisms, resulting in variable clinical symptoms. E. coli strains that play an important role in causing enteric and diarrheal diseases are referred to as diarrheagenic E. coli (DEC). Currently, DEC are grouped into five major pathotypes that differ in their virulence factor profiles, severity of clinical manifestations, and epidemiological features, viz. enterohemorrhagic E. coli (EHEC), enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), enteroaggregative E. coli (EAEC), and enteroinvasive E. coli (EIEC) [1]. Two new pathotypes have recently emerged and are known as diffusely adherent E. coli (DAEC) and adherent invasive E. coli (AIEC); the latter is thought to be associated with Crohn’s disease. Moreover, the
Stx-producing enterohaemorrhagic E. coli (EHEC) was responsible for the 2011 E. coli outbreak in Germany [2].

In developing countries, the ETEC, EPEC, and EAEC pathotypes appear to be the major causes of infantile persistent diarrhea with potentially fatal consequences when left untreated. Bacterial enteropathogens of the EIEC pathotype have been reported to be commonly isolated in children and are the most common cause of traveler’s diarrhea. The EAEC pathotype is the second most common cause of traveler’s diarrhea after ETEC, and its prevalence in endemic and epidemic diseases is well recognized. EHEC is the main DEC pathotype associated with food poisoning outbreaks in the developed world [2]. Humans seem to be the major source of EIEC infections as no animal reservoirs have been identified, and transmission mainly occurs through the fecal–oral route. Although EIEC infections occur worldwide, they are particularly common in low-income countries, where poor general hygiene favors their spread [3].

For instance, infection with EPEC pathotypes causes the characteristic attaching-and-effacing (A/E) lesions. The bacterial eae gene products, viz. the outer membrane adhesive, intimin, are essential for the intimate bacterial attachment to epithelial cells and were first identified in A/E lesions. The bfpA gene is a major subunit of the bundle-forming pilus coding BFP-A, which is responsible for the formation of microcolonies through bacterial-bacterial interactions and a binding pattern known as localized adherence. The major toxin produced by the EHEC pathotype is the phage-encoded Stx (consisting of two subgroups Stx1 and Stx2) [2]. Stx mainly defines STEC (shiga toxin-producing E. coli) and is the key virulence factor that causes hemolytic uremic syndrome diseases. The A subunit of Stx is an RNA-glycosidase that removes an adenine from the 28S rRNA, thereby inhibiting protein synthesis and causing cell death [4]. EIEC pathotype infections are mediated by the invasion-associated plasmid antigen ipaH, which promotes bacterial survival by triggering cell death and modulating host immune responses [5] and by InvE (VirB) of the Ipa proteins that are located on the 140-MDa plasmid harbored by all EIEC strains that are involved in T3SS [6]. EPEC pathotypes generally harbor a high MW plasmid (pAA) associated with aggregative adherence (AA); the five genes located on pAA that are associated with adherence virulence are as follows: aggregative adherent fimbria I (AAF/I), AAF/II, AAF/III, aggR, and AstA. The aggR gene is responsible for transcriptional activation of AAF/I protein expression, and the AstA gene encodes the enterohaemagglutinin E. coli heat-stable enterotoxin I protein (EST1), which is a peptide of 38 amino acids with a molecular weight of 4.1 kDa [1]. ETEC pathotypes produce one or two enterotoxins, viz. the heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) [7]. LT and ST are secreted from E. coli and bind to the cell surface, inducing intestinal secretion and causing diarrhea [4]. These enterotoxins LT/ST are often encoded by virulence plasmids, viz. F18, K88, and K99 plasmids [8].

Different techniques, such as DNA hybridization and ELISA, have been developed through decades of research for the identification of different DEC pathotypes. Moreover, before PCR came to be prevalently used, these techniques for DEC detection were based on the genes and proteins related to the pathogenicity of each category [9, 10, 11]. At present, routine detection and differentiation methods for DEC, especially EHEC strains in pathology laboratories and examination centers of hospitals in Japan, are usually either selective agar-based, relying on a combination of biochemical tests and serotyping of only O157, O26, and O111, or phenotypic assays based on virulence characteristics. Molecular techniques such as multiplex PCR (mPCR) and pulse-field gel electrophoresis (PFGE), can be performed only in facilities, such as health and environment institutes, that have the equipment required to perform these techniques. Furthermore, several mPCR assays have been developed using multiple primer sets published previously in prefectural continuous journals and derived from ready-made products in certain health and environment institutes. Screening bacterial isolates for DEC strains requires a large number of individual PCR assays when only a few primer sets are used under such circumstances.

Therefore, to simplify and accelerate differential diagnosis and to reduce the number of tests required for mPCR assay-based diagnosis of DEC, we designed and evaluated a novel mPCR assay for the simultaneous detection and differentiation of the five major categories of intestinal pathogenic E. coli strains in a one-tube reaction. So far, numerous mPCR constructs have been reported [12, 13, 14, 15]. The one-step mPCR assay reported by Toma et al. detects six target genes for the categorization of DEC [16], while that of Aranda et al. distinguishes between seven target genes [17]. Kimata et al. reported the use of a one-step mPCR assay to detect 12 target genes, including the four essential genes [18], and Oh et al. detected 9 virulence genes [19]. The sequence lengths of the previously reported primers used in the routine detection of DEC in health and environment institutes are relatively short, and as a result, the annealing temperature of the primers is low. Thus, the sequences of all the primer sets had to be redesigned for this study because a high annealing temperature is necessary to avoid non-specific products. In this study, we report a simplified one-step single reaction mPCR assay to identify 10 target genes, including all virulent genes needed to identify each DEC pathotype in Japan.

2. Materials and methods

2.1. Bacterial strains

The five DEC reference strains used as positive controls in the mPCR assays included the EPEC strain PE8 (eaeA, bfpA positive), EIEC strain 63 (aggR, astA positive), EHEC strain 509952 (stx1, stx2 positive), EIEC strain 509763 (invE, ipaH positive), and ETEC strain 5091888 (elt, sth positive), and the clinical strains whose virulence markers have already been characterized also included EPEC strain O86 GB1371, EAECC strain O42, ETEC strain 12566, EHEC strain 14507, and EIEC strain 3. Control strains, EHEC strain 509952, EIEC strain 509763, and ETEC strain 5091888, were purchased from Pathogenic Microbes Repository Unit (Research Institute for Microbial Diseases, Osaka University, Japan), characterized, and confirmed to have the relevant genes using their respective PCR systems. E. coli ATCC 25922 and K-12 were used as the negative control strains.

The 236 clinical E. coli strains investigated in this study were isolated from the stool samples of patients with diarrhea. Stool specimens were collected from patients visiting different hospitals in Japan, namely Komaki Prefectural Hospital, Aichi, and Tajimi Prefectural Hospital, Gifu, between February 2015 and April 2018. These stool specimens were previously found to be negative for Salmonella enterica, Shigella spp., Yersinia enterocolitica, Vibrio spp., Salmonella spp., and Campylobacter spp. The total number of E. coli-like isolates was obtained by culturing the stool samples on a 5% sheep blood agar plate and on selective indicator media, such as deoxycholate hydrogen sulfide lactose (DHL) medium. All strains were analyzed using an automatic accurate microbial identification system and were sometimes analyzed using an automated commercial MS system for identification; then, the strains were grown on 5% sheep blood agar, and they were preserved at 4°C as described below, for future use in mPCR assays.

2.2. Preparation of DNA templates for PCR

The reference strains or clinical isolates were cultured in 3 mL of nutrient broth and incubated overnight at 37°C with shaking at approximately 115 rpm. A 100 μL aliquot of bacterial culture was suspended in 900 μL of Tris-EDTA (TE) buffer, boiled for 10 min, centrifuged at 10000 × g for 10 min, and the supernatant was subsequently maintained at -20°C until further use.

2.3. Construction of PCR primers

The target list of detectable genes of each DEC category was created, and primers were designed to recognize these genes. For the detection of
ETEC strains, primer pairs that specifically target the heat-labile toxin, elt, and the heat-stable toxin, sth, were designed. EIEC can be identified by the presence of the intermediary regulator invE of Ipa proteins and ipoH. We selected astA and aggR genes as the EAE marker genes since they are predominantly present in EAEC and have also been detected in samples at the Prefectural Health and Environment Institute. To identify EPEC strains, we designed specific primers for eaeA and bfpA. For the identification of HEC, we relied on the genes stx1 and stx2. These virulence genes are highly conserved in the representative strains of the five DEC pathotypes. The DNA sequences of the primers, expected sizes of amplification products, and concentration of each primer pair used in the final reactions are shown in Table 1. All oligonucleotides were synthesized by Life Technologies Japan Ltd. or FASMAC, Japan. Thus, 10 primer pairs were designed based on the virulence gene sequences retrieved from the National Center for Biotechnology Information (NCBI) database using the Primer 3 program of the genetic information processing software, GENETYX (Genetyx, Japan). The resulting amplicons were used in the developed mPCR assay. No PCR products were obtained with the developed mPCR assay. No PCR products were obtained using double primer set in the mPCR reaction (Table 1). mPCR assays with double primer sets for each DEC pathotype were first conducted to examine the specificity and sensitivity of each primer pair to amplify the appropriate region corresponding to the virulence target gene harbored by the corresponding strains and generate mPCR products of the expected size. As shown in Figure 1A-E, the representative strains produced mPCR products of the expected sizes for stx1 and stx2 (693 bp and 794 bp, Figure 1A) for EHEC, eaeA and bfpA (1001 bp and 326 bp, Figure 1B) for EPEC, elt and sth (494 bp and 172 bp, Figure 1C) for EIEC, invE (596 bp and 235 bp, Figure 1D) for EAEC, and astA and aggR (100 bp and 407 bp, Figure 1E) for EAF. mPCR reactions with double primer sets for the same DEC pathotype resulted in clear and specific PCR product bands, with no visible non-specific bands, indicating the specificity of the primers in the developed mPCR assay. No PCR products were obtained from negative controls. The sensitivity of detection was $10^3$ CFU/mL per assay for ipoH, $10^3$ CFU/mL per assay for aggR, astA, bfpA, and invE, and $10^4$ CFU/mL per assay for elt, sth, eaeA, stx1, and stx2 with each reference strain. All strains were detected at concentrations ranging from $10^2$ to $10^4$ CFU/mL. Therefore, the presence of $10^3$ CFU/mL per assay must be ensured to confirm detection of all pathotypes. Once the amplification using double

2.4. Detection of selected virulence determinants by mPCR

The PCR mixtures were subjected to the following cycling conditions: 98 °C (2 min, 1 cycle); 30 cycles of 98 °C (10 s), 60 °C (30 s), and 68 °C (1 min), and a final extension step at 68 °C (7 min, 1 cycle) in a thermal cycler (GeneAtlas, ASTEC, Japan). The amplification procedure was carried out in a reaction mixture (final volume of 20 μL) containing 1X PCR buffer, 0.4 mM of each deoxynucleoside triphosphate, 0.4 U of FX Neo DNA polymerase (Toyobo), 2 μL of the DNA template, and the PCR primers.

To assess the sensitivity of the mPCR, the CFU of each prototype strain culture after approximately 10 h of incubation of prototype strains was measured. Then, the supernatant described as the DNA template was diluted to $1 \times 10^7$ CFU, serially 10-fold diluted, and the limit of detection (LOD) was measured to evaluate the sensitivity of the mPCR primers. A mPCR with the reaction mixture containing all 10 primer pairs was performed using a mixture of five types of DEC strain supernatants.

Clinical fecal E. coli isolates were analyzed by mPCR to detect virulence genes (stx1, stx2, eaeA, bfpA, aggR, astA, invE, ipoH, sth, elt). The mPCR products were separated by electrophoresis in 2.5% agarose gels in TAE buffer with a 100-bp DNA ladder as a molecular mass marker (New England BioLabs Japan Inc.) and visualized under UV light after staining with ethidium bromide. The amplicons were identified based on the size of the amplified product. A negative control containing TE buffer instead of template DNA was included in each experiment to exclude the possibility of reagent contamination.

The external control consisted of the supernatant of 5 DEC strains for DNA templates that were amplified together to monitor reaction failure and PCR-induced artifacts. The external control was run in parallel with the test specimens. The internal control (IC) consisted of the DNA amplification primers for the E. coli 16S rRNA gene and was used to prevent false negative results (5'-GGTGTAGCATGCTCATGAATGAAGG-3' and 5'-CTTACTGAGCAGTGCTGAC-3', 1205-bp amplicon). The internal control was included and amplified in all 263 clinical strains analyzed.

2.5. Sequence analysis of mPCR products

The identities of representative mPCR products obtained from the reference strains were verified by nucleotide sequence analysis using the Big Dye Terminator Cycle Sequencing kit (Thermo Fisher Scientific).

3. Results

3.1. Specificity and sensitivity measurement of the mPCR primers

First, we designed the mPCR assay using EPEC strain P8, EHEC strain 509952, ETEC strain 5091888, EIEC strain 509763, and EAEC strain 63. To optimize the reaction conditions for the mPCR assay, we considered the size of the mPCR product and the sequences of each primer set in the mPCR reaction (Table 1). mPCR assays with double primer sets for each DEC pathotype were first conducted to examine the specificity and sensitivity of each pair of primers to amplify the appropriate region corresponding to the virulence target genes harbored by the corresponding strains and generate mPCR products of the expected size. As shown in Figure 1A-E, the representative strains produced mPCR products of the expected sizes for stx1 and stx2 (693 bp and 794 bp, Figure 1A) for EHEC, eaeA and bfpA (1001 bp and 326 bp, Figure 1B) for EPEC, elt and sth (494 bp and 172 bp, Figure 1C) for EIEC, invE (596 bp and 235 bp, Figure 1D) for EAEC, and astA and aggR (100 bp and 407 bp, Figure 1E) for EAF. mPCR reactions with double primer sets for the same DEC pathotype resulted in clear and specific PCR product bands, with no visible non-specific bands, indicating the specificity of the primers in the developed mPCR assay. No PCR products were obtained from negative controls.

The sensitivity of detection was $10^3$ CFU/mL per assay for ipoH, $10^3$ CFU/mL per assay for aggR, astA, bfpA, and invE, and $10^4$ CFU/mL per assay for elt, sth, eaeA, stx1, and stx2 with each reference strain. All strains were detected at concentrations ranging from $10^2$ to $10^4$ CFU/mL. Therefore, the presence of $10^3$ CFU/mL per assay must be ensured to confirm detection of all pathotypes. Once the amplification using double

| Target gene | Forward primer sequence | Conc. (nM) | Reverse primer sequence | Conc. (nM) | Product size (bp) |
|-------------|-------------------------|-----------|-------------------------|-----------|-------------------|
| astA        | tatatacgaagaagcctactacag | 275       | cagcgcgcgtgtagaccttg    | 275       | 100               |
| bfpA        | ctaccgtgctgtcttagctc    | 275       | ctacgcttccctgcttagacc   | 275       | 326               |
| elt         | tatatacgaagaagcctactacag | 550       | cgcggagaaacctgtaacct    | 550       | 494               |
| aggR        | gcctaaagagcctctgcgag    | 275       | tgtgcttgcttgcttagctt    | 275       | 407               |
| ipoH        | tatatacgaagaagcctactacag | 275       | caccgtctgagacttctctc    | 275       | 596               |
| stx1        | aggagagattactcagagaggaggg | 275       | aatgttccccagtgattag     | 275       | 693               |
| stx2        | gcctaaagagcctactacag    | 275       | cgcgcttcgccatattctctc   | 275       | 794               |
| eaeA        | ctaccgtgctgtcttagctc    | 275       | cgcggagaaacctgtaacct    | 275       | 1001              |
| sth         | ctctttctgtttctctctct    | 1100      | gcagttaccaacaacttacagac | 1100      | 172               |
| invE        | gaaattctggagcctgagcctg  | 1100      | cttcgcgcgagagcactct    | 1100      | 235               |


primer sets for DEC succeeded, the specificity of this novel mPCR assay was subsequently validated for 10 pairs of primers using a subset of reference strains.

Non-specific amplification can often occur because of non-specific reactions of mixed PCR primers (cross-priming amplification); therefore, optimizing the sensitivity and specificity of this assay is crucial. The LOD was measured to evaluate the sensitivity of the 10 pairs of mPCR primers. To optimize the reaction conditions of the mPCR assay, we integrated the best performing concentrations into a single reaction after testing each primer pair at various relative primer concentrations. Various combinations of primer sequences and primer concentrations were progressively incorporated to generate equally visible amplified bands. The LODs were as follows: $1 \times 10^3$ CFU/mL and $1 \times 10^3$ CFU/mL for stx1 and stx2 of EHEC, $1 \times 10^4$ CFT/mL and $1 \times 10^4$ CFT/mL for bfpA and eaeA of EPEC, $1 \times 10^3$ CFT/mL and $1 \times 10^3$ CFT/mL for aggR and astA of EAEC, $1 \times 10^4$ CFU/mL and $1 \times 10^4$ CFU/mL for elt and sth of ETEC, and $1 \times 10^5$ CFT/mL and $1 \times 10^5$ CFT/mL for invE and ipaH of EIEC, respectively (Figure 2). Therefore, the presence of $10^4$ CFU/mL per assay must be ensured for the detection of all categories. In the aforementioned mPCR, we identified EPEC, EAEC, EIEC, EHEC, and ETEC strains in a single reaction tube.

As demonstrated in Figure 3, the specific DNA fragments corresponding to genes defining the appropriate pathotypes, ETEC (elt positive and sth positive), EIEC (invE positive and ipaH positive), EHEC (stx1 positive, stx2 positive, eaeA positive, and astA positive), EPEC (bfpA positive and eaeA positive), and EAEC (astA positive and aggR

![Figure 1. Sensitivity of mPCR of each type of DEC as determined by the limits of detection. EHEC (stx1 and stx2) (A), EPEC (eaeA and bfpA) (B), ETEC (elt and sth) (C), EIEC (ipaH and invE) (D), EAEC (astA and aggR) (E). Lane 1: positive control mix; lane 2: $1 \times 10^7$ CFU/mL; lane 3: $1 \times 10^6$ CFU/mL; lane 4: $1 \times 10^5$ CFU/mL; lane 5: $1 \times 10^4$ CFU/mL; lane 6: $1 \times 10^3$ CFU/mL; lane 7: $1 \times 10^2$ CFU/mL; lane 8: $1 \times 10^1$ CFU/mL; lane 9: Negative control; lane 10: 100 bp DNA ladder (A–E: NEB). The primer final concentration in the reaction mixture was 0.3 μmol of each primer.](image-url)
positive), were easily detected by mPCR in a single reaction mixture. No mPCR product was obtained for the culture supernatants from *E. coli* ATCC 25922 and *E. coli* K-12, *Staphylococcus aureus*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Bacillus cereus*, *Listeria monocytogenes*, or the negative control (Figure 3).

Thus, we established a 10-gene mPCR assay that could successfully amplify mixed DNA of 10 target genes simultaneously by controlling the primer concentrations.

### 3.2. Validation of mPCR with clinical isolates from patients with diarrhea

To demonstrate the diagnostic utility of this assay, 236 clinical *E. coli* strains isolated from patients with diarrhea were subjected to the mPCR protocol. The results of the mPCR analysis of the 236 bacteria are summarized in Table 2. No virulence genes were observed in any of the 212 clinical strains examined. Twenty-four DEC strains were detected (EPEC, 1; EAEC, 14; ETEC, 3; and EHEC, 6). The PCR assay detected 1 (0.42%) atypical EPEC isolate (eaeA PCR positive), 14 (5.93%) EAEC isolates (astA PCR positive), 3 (1.27%) ETEC isolates (elt or sth PCR positive), and 6 (2.54%) EHEC isolates (stx1 and/or stx2 PCR positive) from patients with diarrhea. The results of mPCR for stx1 and/or stx2 were consistent with the results of immunochromatography for stx1 and/or stx2 performed in the hospital (Table 2).

### 4. Discussion

Simple and rapid tests are required for the detection of various infections. Recently, PCR is being employed worldwide as an applicable technique for the detection of infectious pathogens, such as the novel coronavirus infection. PCR is also the most commonly used technique for the detection of various pathotypes of DEC [20, 21]. mPCR assays have also been developed as practical and rapid diagnostic tools for the routine identification of all human DEC categories. Numerous mPCR assays have been developed for the identification of *E. coli* pathotypes [12, 13, 14, 15, 16, 18, 19, 22, 23]. However, in previous reports, the sensitivity of mPCR was generally not evaluated; in some cases where evaluation was done, the LOD was 10⁶ CFU/mL for each DNA template [18]. The LOD in our assay was 10⁴ CFU/mL for each of the pathotypes detected. Oh et al. had demonstrated the LOD of double primer sets for each DEC type; however, they did not examine the LOD using the 9 mPCR primer pairs. Specifically, their mPCR detected EAEC and EIEC using 1 gene, EHEC and EPEC using a pair of primers for 2 genes, and ETEC for 3 genes [19]. Our mPCR could detect 2 genes each for EAEC and EIEC to avoid missing the detection of EAEC and EIEC.

In health and environment institutes, the mPCR reaction conditions currently in use require multiple rounds of PCR for the detection of each strain because of differences in annealing temperatures of PCR primers and the amplified DNA fragment sizes of some PCR products, making the process laborious and leading to wastage of time and PCR tubes. In the pathology laboratories of hospitals, of the various DEC pathotypes and normal flora, only the major serotypes of EHEC strains are identified by the selective agar method and confirmed by techniques, such as ELISA or immunochromatography, which detects stx1 and/or stx2. DEC pathotypes other than EHEC are not detected. Therefore, we were interested in...
constructing a PCR method using ten primer sets for a one-tube single-reaction mPCR assay, which could save time and effort in analyzing various DEC virulence factors of E. coli isolates. For the simultaneous and rapid identification and differentiation of DEC strains belonging to the five major pathotypes, we set up a single-step mPCR. The design and development of the mPCR assay were monitored using five reference strains. All reference strains exhibited the expected gene pattern, as confirmed by DNA sequencing. Furthermore, all PCR amplicons showed comparable band intensities and were of sufficiently different sizes to be unequivocally resolved by standard agarose gel electrophoresis. This mPCR assay could help circumvent drawbacks of existing methods, such as over-cultivation and differentiation of the five well-established DEC pathotypes: EHEC, EPEC, ETEC, EIEC, and EAEC. To date, seven categories of DEC have been recognized; therefore, future studies might be necessary to develop a new mPCR that can detect all seven categories in one reaction. Further, future studies validating this mPCR for application to stool specimens are required.

In conclusion, the mPCR presented in this paper offers a practical method for the rapid identification and differentiation of DEC in a single reaction tube. It is a highly specific, robust, cost-efficient, and time-efficient diagnostic tool; therefore, it may have the potential to be introduced not only as an additional check (not in routing practice) in clinical microbiology laboratories, but also for confirmation in health and environment institutes, public health centers, and reference laboratories.

Declarations

Author contribution statement

Mari OHMURA-HOSHINO: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Yuki MIYAKI & Shigeko YASHIMA: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

The authors do not have permission to share data.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.
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