



Research article

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

Mari Ohmura-Hoshino^{a,*}, Yuki Miyaki^b, Shigeko Yashima^c^a Department of Medical Technology, School of Nursing and Medical Care, Yokkaichi Nursing and Medical Care University, Yokkaichi, Japan^b Laboratory, Komaki Hospital, Japan^c Laboratory, Tajimi Prefectural Hospital, Japan

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 10⁴ to 10⁷ CFU/mL.
- We show cost- and time-effective detection of DEC in clinical cultured samples.

ARTICLE INFO

Keywords:

Diarrheagenic *Escherichia coli*
 Multiplex PCR
 Diagnostic tool

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 *sth* for ETEC, *eaeA* and *bfpA* for EPEC, *aggR* and *astA* for EAEC, and *ipaH* 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 10⁴ to 10⁷ 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

* Corresponding author.

E-mail address: hoshino@y-nm.ac.jp (M. Ohmura-Hoshino).<https://doi.org/10.1016/j.heliyon.2022.e10231>

Received 20 November 2021; Received in revised form 22 March 2022; Accepted 4 August 2022

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Stx-producing enteroaggregative *E. coli* (STEAEC) 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 ETEC 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 *eaeA* gene products, viz. the outer membrane adhesin, 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]. EAEC pathotypes generally harbor a high MW plasmid (pAA) associated with aggregative adhesion (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 enteroaggregative *E. coli* heat-stable enterotoxin I protein (EAST1), 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), EAEC 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, EAEC strain O42, ETEC strain 12566, EHEC 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

EPEC strains, primer pairs that specifically recognize 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 *ipaH*. We selected *astA* and *aggR* genes as the EAEC 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 EHEC, 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 ranged from 100 to 1000 bp in length. The predicted product sizes of the virulence genes in the corresponding reference strains were as follows: *eaeA* (1001 bp) and *bfpA* (326 bp) for EPEC, *elt* (494 bp) and *sth* (172 bp) for ETEC, *astA* (100 bp) and *aggR* (407 bp) for EAEC, *invE* (235 bp) and *ipaH* (596 bp) for EIEC, and *stx1* (693 bp) and *stx2* (794 bp) for EHEC.

The optimal concentration of each primer pair in the reaction mixture was determined empirically until the PCR products exhibited equal intensities on 2.5% agarose gels. The concentration of each primer pair was independently varied.

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 1×10^7 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. The culture supernatants of all pathotypes with the same CFU were mixed and then 10-fold serially diluted.

Clinical fecal *E. coli* isolates were analyzed by mPCR to detect virulence genes (*stx1*, *stx2*, *eaeA*, *bfpA*, *aggR*, *astA*, *invE*, *ipaH*, *sth*, and *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'-GTTTGATCATGGCTCAGATTGAACGC-3' and 5'-CGTAAGGGCCATGATGACTTGAC-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 PE8, 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 ETEC, *ipaH* and *invE* (596 bp and 235 bp, Figure 1D) for EIEC, and *astA* and *aggR* (100 bp and 407 bp, Figure 1E) for EAEC. 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 10^2 CFU/mL per assay for *ipaH*, 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 10^4 CFU/mL per assay must be ensured to confirm detection of all pathotypes. Once the amplification using double

Table 1. Primer pairs used for detection of marker virulence genes indicative of the pathogenic *E. coli* types.

Target gene	Forward primer sequence	Conc. (nM)	Reverse primer sequence	Conc. (nM)	Product size (bp)
<i>astA</i>	tatatccgaagcccgatccag	275	caggtcgcgagtgacgctttg	275	100
<i>bfpA</i>	ctaccagctcgcgtctgattcc	275	cgtagccttcgctgaagtacc	275	326
<i>elt</i>	tataccgtgctgacttagacccc	550	cggtgggaacctgctaatac	550	494
<i>aggR</i>	gcctaaaggatgcctgatg	275	tgctgctttgctcattcttg	275	407
<i>ipaH</i>	ctttcgcataaccgtctctgc	275	caccctctgagagtactcattctcc	275	596
<i>stx1</i>	agagggatagatccagaggaaggg	275	aattgccccagagtgatg	275	693
<i>stx2</i>	gtatacgcagacagatatcgacccc	275	cgctgcagctgtattacttccc	275	794
<i>eaeA</i>	cttcagtcgcatctctgaacg	275	ggtagctttgctgctttggct	275	1001
<i>sth</i>	ctttctgtattcttttcaaccttc	1100	gcaggattacaacaattcacagc	1100	172
<i>invE</i>	gaaattctggtgacactctagaag	1100	ctttcgcgcagacagattctct	1100	235

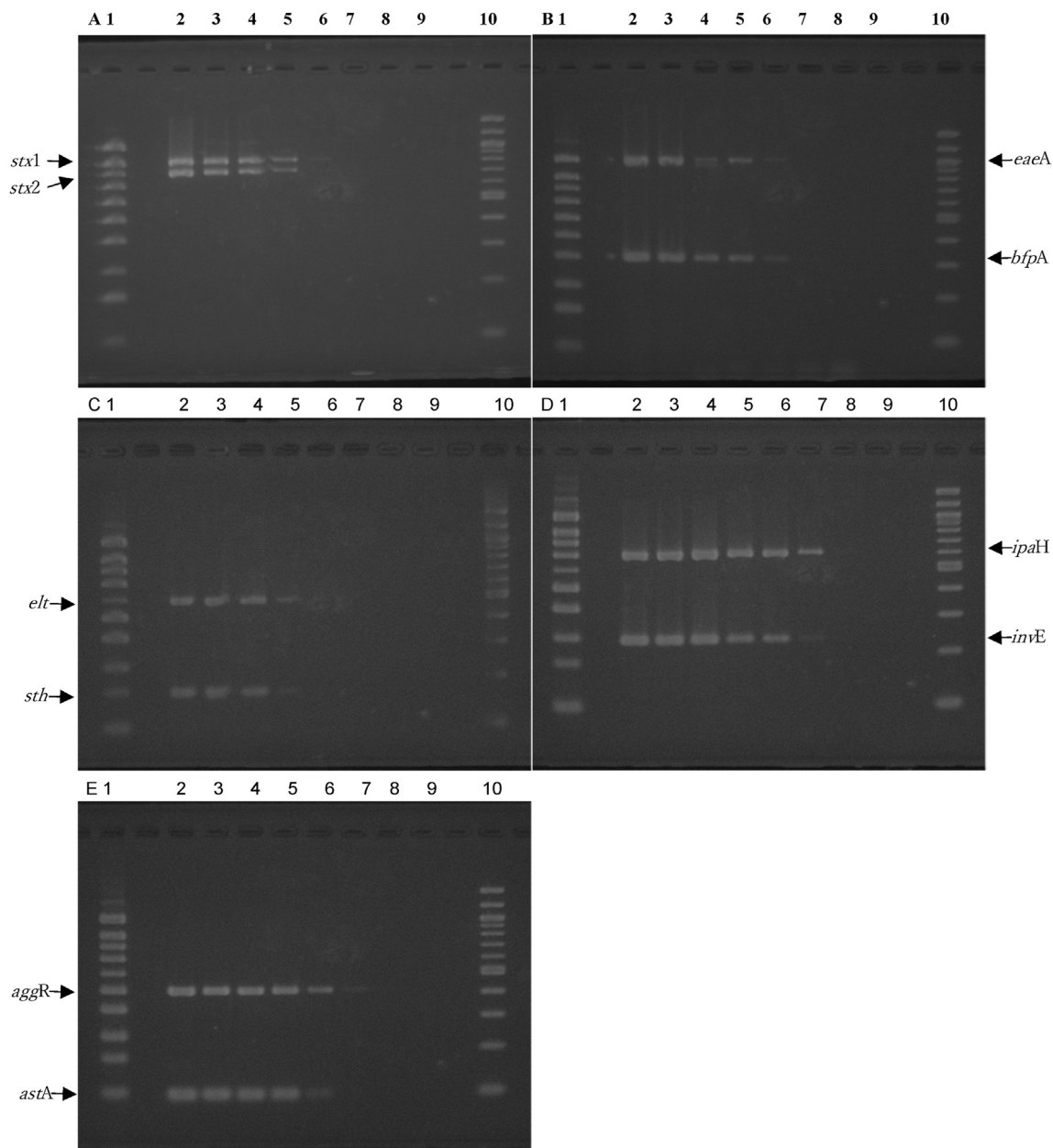


Figure 1. Sensitivity of mPCR of each type of DEC as determined by the limits of detection. EHEC (*stx1* and *stx2*) (A), EPEC (*eaeA* and *bfpA*) (B), ETEC (*elt* and *sth*) (C), EIEC (*ipaH* and *invE*) (D), EAEC (*astA* and *aggR*) (E). Lane 1: positive control mix; lane 2: 1×10^7 CFU/mL; lane 3: 1×10^6 CFU/mL; lane 4: 1×10^5 CFU/mL; lane 5: 1×10^4 CFU/mL; lane 6: 1×10^3 CFU/mL; lane 7: 1×10^2 CFU/mL; lane 8: 1×10^1 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 μ mol of each primer.

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: 1×10^3 CFU/mL and 1×10^3 CFU/mL

for *stx1* and *stx2* of EHEC, 1×10^4 CFT/mL and 1×10^3 CFU/mL for *bfpA* and *eaeA* of EPEC, 1×10^3 CFT/mL and 1×10^4 CFU/mL for *aggR* and *astA* of EAEC, 1×10^4 CFU/mL and 1×10^4 CFU/mL for *elt* and *sth* of ETEC, and 1×10^4 CFT/mL and 1×10^1 CFU/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, and *astA* 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*

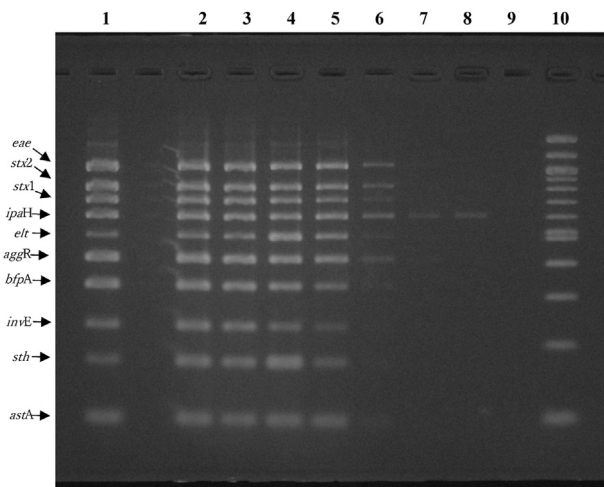


Figure 2. Sensitivity of mPCR of positive control (reference strain) mix as determined by the limits of detection. Lane 1: positive control mix; lane 2: 1×10^7 CFU/mL of each strains; lane 3: 1×10^6 CFU/mL of each strains; lane 4: 1×10^5 CFU/mL of each strains; lane 5: 1×10^4 CFU/mL of each strains; lane 6: 1×10^3 CFU/mL of each strains; lane 7: 1×10^2 CFU/mL of each strains; lane 8: 1×10^1 CFU/mL of each strains, respectively; lane 9: negative control; lane 10: 100 bp DNA ladder (NEB).

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 10^6 CFU/mL for each DNA template [18]. The LOD in our assay was 10^4 CFU/mL for each of the pathotypes detected. Oh et al. had demonstrated the LOD of double primer sets for each DEC types; 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

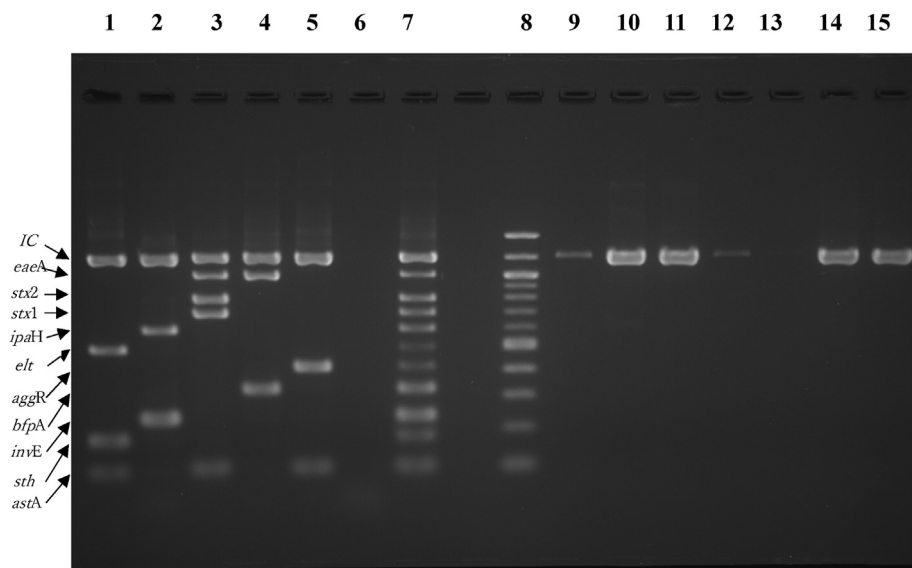


Figure 3. Specificity of mPCR against diarrheagenic *E. coli* strains and non-*E. coli* strains. lane 1: ETEC 12566; lane 2: EIEC 3; lane 3: EHEC 14507; lane 4: EPEC O86 GB1371; lane 5: EAEC O42; lane 6: negative control; lane 7: positive control; Lane 8: 100 bp DNA ladder (NEB); lane 9: *Staphylococcus aureus*; lane 10: *Salmonella typhimurium*; lane 11: *Yersinia enterocolitica*; lane 12: *Bacillus cereus*; lane 13: *Listeria monocytogenes*; lane 14: *E. coli* ATCC 25922; lane 15: *E. coli* K-12.

Table 2. Detection of virulence genes of 236 *E. coli* isolates.

Strain No.	Marker identified in the hospital	Results of mPCR	
		Toxin gene	Pathotype
1	stx1 (-)、stx2 (+)	astA, stx2, eaeA	EHEC
12		astA	EAEC
35	stx1 (-)、stx2 (+)	astA, stx2, eaeA	EHEC
36	stx1 (-)、stx2 (+)	astA, stx2, eaeA	EHEC
44		astA	EAEC
50	stx1 (+)、stx2 (+)	astA, stx1, stx2, eaeA	EHEC
53		elt	EPEC
84	stx1 (+)、stx2 (-)	astA, stx1, eaeA	EHEC
92		astA	EAEC
94	stx1 (-)、stx2 (+)	astA, stx2, eaeA	EHEC
97		astA	EAEC
124		astA	EAEC
130		astA	EAEC
133		eaeA	EPEC
144		astA	EAEC
155		astA	EAEC
158		astA	EAEC
167		astA	EAEC
169		astA	EAEC
180		sth	EPEC
186		astA	EAEC
208		elt	EPEC
210		astA	EAEC
221		astA	EAEC

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 with no visible non-specific bands. We estimated the LOD of DEC by our mPCR assay to be approximately 10^4 CFU/mL. Sufficient specificity of the mPCR assay was demonstrated using several DEC strains and non-*E. coli* strains. Our 10-gene mPCR assay is a one-shot technique wherein we require only one tube for PCR of one strain, reducing the number of reaction-mixture-loaded wells for gel electrophoresis. This mPCR assay could help circumvent drawbacks of existing methods, such as overlapping PCR products sizes, high costs, high consumption of time, and resources (PCR tubes and reagents) due to multiple iterative assays performed to detect and classify one clinical strain from clinical samples obtained from diarrhea patients. Our mPCR assay takes less than 1.5 h, while the complete sample analysis takes 2–2.5 h, including the time required for mPCR product generation from clinical samples and separation of mPCR products by agarose gel electrophoresis.

The robustness of the developed mPCR assay was verified on *E. coli* isolated from stool samples of diarrhea patients. In addition, we performed mPCR on 14 clinical EHEC strains. The mPCR product sizes obtained were as expected; the EHEC strains possessed *stx1* and/or *stx2* in agreement with the results of *stx1*- and *stx2*-detecting ELISA carried out in the hospital (unpublished data). Furthermore, we examined whether a pair of primer used to detect *stx2* in this study could amplify *stx2* variant (a to g) genes. While the *stx2f* could not be detected, all other variants (*stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, and *stx2g*) were detectable (data not shown).

Savarino et al. established the concept that *astA* encodes EAST-1 [24], and further studies reported that EAST1 is associated with *E. coli* pathotypes. This gene was detected in 100% of O157:H7 EHEC, 41% of ETEC, and 22% of EPEC, except EAEC, and in other bacterial genera, such as *Yersinia enterocolitica* and *Vibrio cholerae* non-O1 strains [25]. In this study, 100% of EHEC contained *astA* and 6.19% of non-classified clinical *E. coli* strains contained *astA*, indicating that the index virulence factor *astA* is not, in general, restricted to the EAEC pathogroup but instead can also occur in other pathotypes, such as EHEC and ETEC, which might contribute to their virulence potential.

Although the exact reason for the low detection (10%) rate of DEC strains in clinical samples was not determined, one must note that it is not easy to identify the actual pathogen because the differentiation of pathogenic organisms from nonpathogenic members of the normal flora is difficult. Moreover, there is the possibility that the infection could be viral and not bacterial. Lack of information on the use of antimicrobial agents by a large number of patients prior to stool sampling may have confounded the results, considering that the use of antimicrobial agents would lead to negative results for certain strains in the stool culture tests. A significant number of patients with negative stool examination results may have used antimicrobial agents.

Taken together, we have developed a one-step mPCR incorporating five primer pairs that have been designed and optimized to yield DNA fragments of sufficiently different sizes to be unequivocally resolved by standard agarose gel electrophoresis that can be used for the simultaneous identification 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 routine 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.

Funding statement

This work was supported by JSPS KAKENHI (grant number, 17K19849).

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.

Acknowledgements

The EPEC strain PE8, EAEC strain 63, and the clinical strains, including EPEC strain O86 GB1371, EAEC strain O42, ETEC strain 12566, EHEC 14507, and EIEC strain 3, and *stx2* variants were kindly provided by Dr. Yamasaki (Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Japan). The virulence markers of the strains have already been characterized previously and confirmed that each of the provided strains had the relevant genes using single PCR.

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