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REVIEW

Status and Prospects of PCR Detection Methods for Diagnosing Pathogenic *Escherichia coli*: A Review

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Abstract

Escherichia coli are the predominant facultative bacteria found in the gastrointestinal tract of animals and humans. Some strains of *E. coli* that acquire virulence factors and cause foodborne and waterborne diseases in humans are called pathogenic *E. coli* and can be divided into five pathotypes according to the virulence mechanism: EAEC, EHEC, EIEC, EPEC, and ETEC. Although selective media have been developed to detect *E. coli*, distinguishing pathogenic strains from non-pathogenic ones is difficult because of their similar biochemical properties. Therefore, it is very important to find a new and effective diagnostic method to identify pathogenic *E. coli*. With recent advances in molecular biology and whole genome sequencing, the use of polymerase chain reaction (PCR) is increasing rapidly. In this review paper, we provide an overview of pathogenic *E. coli* and present a review on PCR detection methods that can be used to diagnose pathogenic *E. coli*. In addition, the possibility of real-time PCR incorporating IAC is introduced. Consequently, this review paper will contribute to solving the current challenges related to the detection of pathogenic *E. coli*.

Keywords

pathogenic *Escherichia coli*, polymerase chain reaction (PCR), real-time PCR, internal amplification control (IAC), virulence genes

Introduction

Pathogenic *Escherichia coli* are one of the major causative agents of food poisoning accidents occurring in Korea and abroad. Pathogenic *Escherichia coli* infect human through contaminated food and drinking water [1-3] Pathogenic *Escherichia coli* can be divided into five types according to the pathological mechanism, and some *Escherichia coli* have high pathogenicity [2]. In 2011, numerous food poisoning accidents caused by *Escherichia coli* O104 were reported in Europe, most of which were fatal [3,4]. Therefore, detecting and discriminating pathogenic *Escherichia coli* in food are necessary. However, the biochemical properties of pathogenic *Escherichia coli* are similar to those of normal *Escherichia coli* conventional medium except for *Escherichia coli* O157:H7, rendering it difficult to discriminate them [5].

The currently available method for discriminating pathogenic *Escherichia coli* according to the pathological mechanism requires skilled technicians. Nonetheless, pathogenic *Escherichia coli* can be detected using polymerase chain reaction (PCR) [6]. Although PCR has the advantage of rapid detection, it requires considerable time and resources to discriminate the five kinds of *Escherichia coli*. In addition, when PCR is

used, the test results can be considered valid only when false-positive or false-negative results can be discriminated. Therefore, in this review paper, we tried to present the possibility of developing multiplex PCR that can simultaneously distinguish 5 types of pathogenic *Escherichia coli* using an internal amplification control (IAC).

Therefore, this review paper was organized to provide general information about (1) tan summary of PCR detection methods that could be used to confirm pathogenic *Escherichia coli* and also (2) the possibility of real-time PCR incorporating IAC would be introduced.

Pathogenic *Escherichia coli*

Escherichia coli are the part of the normal flora found in the intestine of human beings and animals [7]. However, several strains of *Escherichia coli* are identified as pathogenic and cause severe diseases in their host [8]. Pathogenic *Escherichia coli* have different virulence strategies, and the symptoms vary according to pathogenicity [9]. Pathogenic *Escherichia coli* can be classified according to their pathogenicity into five types: enteroaggregative *Escherichia coli* (EAEC), enterohemorrhagic *Escherichia coli* (EHEC), enteroinvasive *Escherichia coli* (EIEC) enteropathogenic *Escherichia coli* (EPEC), and enterotoxigenic *Escherichia coli* (ETEC) [10].

The most common virulence factor of pathogenic *Escherichia coli* is the production of various toxins within the host [11]. The following toxins are produced by pathogenic *Escherichia coli,* Shiga toxins (Stx1 and/or Stx2), heat-labile enterotoxins (LT), and heat-stable enterotoxins (ST) [12]. Moreover, specific invasion plasmids, colonization factors, fimbriae, and adhesions are known to affect the pathogenic properties of *Escherichia coli* isolates [13].

Virulence factors are determined by the genetic properties acquired through plasmids, phages, or other gene transfer events [14]. The common symptoms due to pathogenic *Escherichia coli* are diarrhea, acute inflammation, hemorrhagic colitis, urinary tract infections, and septicemia [15].

1. Enteroaggregative *Escherichia coli*

EAEC have a plasmid of 60-65 MDa, which encodes the aggregative adherence fimbriae AAFI or AAFII [16]. In addition, EAEC produce several toxins, of which Pic and Shigella enterotoxin 1 (ShET1) share the same chromosomal locus on opposite strands [17]. EAEC have a unique LT plasmid that encodes the entero-aggregative toxin EAST1 [18]. The virulence factors of EAEC are regulated by a single transcriptional activator called AggR, a member of the AraC family of transcriptional activators [19].

2. Enterohemorrhagic *Escherichia coli*

EHEC is characterized by the production of Shiga toxins (Stx) [20]. The Stx causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) [21]. EHEC also has the locus of enterocyte effacement (LEE), which is characterized by the ability to attach to the enterocyte [22]. Although more than 200 serotypes produce Stxs, most serotypes do not

have the LEE [23]. Stx-producing *Escherichia coli* (STEC) or verotoxin-producing *Escherichia coli* (VTEC) produce Stx, but do not have the LEE, whereas EHEC produce Stx and have the LEE [23,24].

3. Enteroinvasive *Escherichia coli*

The pathogenic mechanism and clinical symptoms (dysentery-like diarrhea with fever of EIEC) are similar to those of *Shigella* spp. EIEC invade and proliferate within the epithelial cells of the colon, causing extensive cell destruction [25]. EIEC pathogenesis occurs via a plasmid-borne type III secretion system that secretes several proteins such as IpaA, IpaB, IpaC, and IpgD [26]. Among them, IpaH, which encodes the invasive plasmid antigen H, is present on both the chromosome and invasion plasmid [27].

4. Enteropathogenic *Escherichia coli*

EPEC are characterized by attaching and effacing (A/E) lesions on the intestinal epithelium [28]. The genetic element responsible for the A/E lesions is located on a 35 kb pathogenicity island called the LEE, which encodes an intimin, a type III secretion system, many secreted (Esp) proteins, and the translocated intimin receptor named Tir [29]. A typical EPEC has 70-100 kb of EPEC adherence factor (EAF) plasmid, and this plasmid encodes a type IV pilus called the bundle-forming pilus (BFP) [30]. In a typical EPEC, BFP mediates interbacterial adherence and epithelial cell adhesion [31]. Atypical EPEC has only the LEE plasmid, but not the EAF plasmid [32].

5. Enterotoxigenic *Escherichia coli*

ETEC produce enterotoxins and cause fever-free diarrhea [33]. ETEC can produce LT and/or ST enterotoxins; they can produce one or two toxins simultaneously, each with one or more colonization factors [34]. LT toxins are structurally and functionally similar to cholera enterotoxin and are classified as LT I (associated with humans and animals) and LT II (associated primarily with animals) [35]. ST toxin variants include ST1a and STb [34].

Serotyping of *Escherichia coli*

Serotyping by using somatic (O) and flagellar (H) antigens is the most basic method of classifying *Escherichia coli* [36]. However, serology is not always sufficient to identify the pathotypes because it does not involve checking for the presence of virulence factors [36]. Better strain identification requires specialized knowledge and the use of various detection methods, but these methods are difficult to perform and to apply to routine investigation [36].

Occurrence of Pathogenic *Escherichia coli*

1. Pathogenic *Escherichia coli* in world

Over the past 10 years, food poisoning has been mainly caused by EPEC, STEC/EHEC,

EIEC, ETEC, and EAEC. Vegetables, fruits, meat products, and cooked foods were mainly contaminated by bacteria from food handlers. Pathogenic *Escherichia coli* originate from contaminated environments (water and soil), animals, and humans. Food poisoning due to pathogenic *Escherichia coli* is attributed to the consumption of less cooked and contaminated food and by contamination from food workers [36]. STEC is more commonly responsible for food poisoning, and contamination by STEC strains O104:H4, O157 PT8, and O111:NM leads to death [35].

The most serious food poisoning accident in Germany in 2011 was caused by STEC O104:H4 [37]. A large-scale food poisoning outbreak resulted in 3816 STEC infections and 54 deaths, of which 32 died from HUS, which is known to mainly affect children, but 89% of all patients with HUS were adults. The source of infection was found to be raw sprouts. In addition to Germany, STEC O104:H4 infection incidents have been reported in Europe and North America (Table 1). Six cases of STEC O104:H4 infection were confirmed in the United States, and five of them had traveled to Germany during the outbreaks. Of the 6 patients, 4 developed HUS, and 1 died. In France, 24 cases of STEC O104:H4 infection were reported, of which 22 (92%) were reported in adults: 7 cases (29%) developed HUS; 5 cases (21%), bloody diarrhea; and 12 cases (50%), diarrhea [38].

2. Pathogenic *Escherichia coli* in South Korea

The Korea Centers for Disease Control and Prevention (KCDC) analyzed the epidemic pattern and pathotype of pathogenic *Escherichia coli* between 2010 and 2019 and isolated 6,485 pathogenic *Escherichia coli*, of which 5,785 (89.2%) and 700 (10.8%) were isolated from domestic and foreign samples, respectively [39]. By pathotype, EPEC were the highest (3,921 [60.5%]), followed by ETEC (2,025 [31.2%]), EIEC (101 [1.5%]), and EHEC (438 [6.8%]). Of the ETEC isolated, 556 (27.5%) were of foreign origin, which required continuous monitoring and quarantine (Table 2).

Pathogenic *Escherichia coli* were mostly isolated in summer from June to September, accounting for 61.7% of the total, and were more frequent in children under 9 years of age (37.9%). In children under the age of 9 years, EHEC was more common (51.7%) than other pathogenic *Escherichia coli*. The major virulence genes for each pathogenic *Escherichia coli* were detected in the following order (Table 3): EIEC *ipaH* (100%), EPEC *eaeA* (97.4%), ETEC *st* (53.4%), EHEC *stx1* (45.7%), and EHEC with both Stx gene and *eaeA* (57.5%).

Polymerase Chain Reaction and Internal Amplification Control for diagnosing Pathogenic *Escherichia coli*

1. Conventional PCR and real-time PCR for pathogenic *Escherichia coli*

PCR is an easy alternative tool for the identification of *Escherichia coli* that can be used for diagnosis by amplifying specific genes of interest present in the target pathotype [40]. Multiplex PCR simultaneously amplifies more than one target sequence

Table 1. Foodborne outbreaks caused by pathogenic *Escherichia coli* during 2010–2019

Country	Year	Pathotype	Serotypes	Source	Incidence
USA	2010	EHEC	O157:H7	Cheese and beef	59
		EHEC	O145	Romaine lettuce	26
	2011	EHEC	O104	Sprouts	6
		EHEC	O157:H7	Romaine lettuce	58
	2014	EHEC	O121	Raw clover sprouts	19
		EHEC	O157:H7	Ground beef	12
	2015	EHEC	O26	Restaurant	55
		EHEC	O157:H7	Chicken salad	19
	2016	EHEC	O157:H7	Beef product	11
		EHEC	O121, O26	Flour	63
		EHEC	O157	Alfalfa sprouts	11
	2017	EHEC	O157:H7	Butter, leafy greens	57
	2018	EHEC	O157:H7	Romain lettuce	210
		EHEC	O157:H7	Ground beef	18
	2019	EHEC	O157:H7	Salad kit and romaine lettuce	177
		EHEC	O103	Ground beef	209
		EHEC	O26	Flour	21
		EHEC	O103 and O121	Bison	33
Korea	2012	EPEC	O169	Kimchi	230
	2013	ETEC	O157:H45	Egg soup and tuna bibimbap	33
Japan	2011	EHEC	O111 and O157	Raw beef dishes	181
	2012	ETEC	O169:H41	Japanese restaurant	102
China	2010	EPEC	O127a:K63	Dining room	112
Italy	2012	EIEC	O96H19	Cooked vegetables	109
German	2011	EHEC	O104:H4	Sprouts	3,816
France	2011	EHEC	O104:H4	Fenugreek seeds	24
		EHEC	Sorbitol-fermenting O157:H7	Frozen ground beef products	18
England	2010	EHEC	O157 PT8	Raw leeks and potatoes	252
	2013	EAEC	O131:H27, O104:H4, O20:H19	Food festival	592
Denmark	2010	ETEC	O6:K15:H16	Lettuce	264
Norway	2012	ETEC	O78	Imported chives and scrambled eggs	>300

Adapted from Yim with permission of author [7].
EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; EIEC, enteroinvasive *Escherichia coli*; EAEC, enteroaggregative *Escherichia coli*.

Table 2. The number of pathogenic *Escherichia coli* collected by the Enteric Pathogens Active Surveillance Network (Enter-Net), 2010–2019

Pathotype	No. of isolates (%)		
	Domestic strains	Imported strains	Total
Enteropathogenic *Escherichia coli* (EPEC)	3,855 (66.6)	66 (9.4)	3,921 (60.5)
Enterotoxigenic *Escherichia coli* (ETEC)	1,469 (25.4)	556 (79.4)	2,025 (31.2)
Enteroinvasive *Escherichia coli* (EIEC)	47 (0.8)	54 (7.7)	101 (1.5)
Enterohemorrhagic *Escherichia coli* (EHEC)	414 (7.2)	24 (3.4)	438 (6.8)
Total	5,785 (100)	700 (100)	6,485 (100)

Rearranged by referring to the Table in Yun et al. [39] with public domain.

in the same reaction mixture [41]. Multiplex PCR can be applied to various virulence-associated genes to differentiate between different pathotypes.

Until now, the various methods that are explored to diagnose *Escherichia coli* and diarrheagenic *Escherichia coli* in water samples using multiplex PCR [42], multiplex

Table 3. Virulence gene profiles of pathogenic *Escherichia coli* collected by the Enteric Pathogens Active Surveillance Network (Enter-Net), 2010–2019

Pathotype	No. of isolates (%) according to virulence genes									
	stx1	*stx2*	*stx1+stx2*	*eaeA*	*bfpA*	*eaeA+bfpA*	*lt*	*st*	*lt+st*	*ipaH*
Enterohemorrhagic *Escherichia coli* (EHEC)	200 (45.7)	123 (28.1)	115 (26.3)							
Enteropathogenic *Escherichia coli* (EPEC)				3,818 (97.4)	8 (0.2)	95 (2.4)				
Enterotoxigenic *Escherichia coli* (ETEC)							407 (20.1)	1,081 (53.4)	537 (26.5)	
Enteroinvasive *Escherichia coli* (EIEC)										101 (100.0)
Total	438 (100)			3,921 (100)			2,025 (100)			101 (100)

Rearranged by referring to the Table in Yun et al. [39] with public domain.
Stx, Shiga toxins.

real-time PCR [43], nucleic acid based sequence amplification real-time PCR [44], propidium monoazide real-time PCR [45], real-time PCR and quantitative real-time PCR [46], reverse transcriptase PCR [47], and so on.

The main advantages and disadvantages (limitations) of each method are as follows.

The advantages of standard PCR are (a) Higher sensitivity and specificity than culture-based methods, (b) Possibility of multiplex PCR for multiple pathogen detection, (c) Detects viable but nonculturable cells, (d) Simultaneous detection of different targets within the same species is possible (multiplex PCR), and the disadvantages are (a) Post-PCR confirmation step needed (for example, electrophoresis), (b) Non-quantitative, (c) No distinction between viable and dead cells (detects both), (d) Inhibition of the amplification when environmental samples are analyzed due to the presence of contaminants (for example, organic, inorganic and biomass content), (e) Low nucleic acid concentration causes frequent variability on the results, which leads to tube-to-tube variability [42,48].

The advantages of real-time PCR are (a) Faster than conventional PCR, (b) High level of sensitivity and specificity, (c) Real-time detection, (d) Quantification of the target in the sample is possible (quantitative real-time PCR), and the disadvantages are (a) Inhibition of the amplification when environmental samples are analyzed due to the presence of contaminants, (b) No distinction between viable and dead cells (detects both) [43,48].

The advantages of nucleic acid based sequence amplification real-time PCR are (a) Distinguishes viable from dead cells, (b) No interference from background DNA, and the disadvantage is (a) The same as in RT-PCR [44, 48].

The advantages of propidium monoazide real-time PCR are (a) Distinguishes live from dead cells and from free DNA, (b) Simple to perform, and the disadvantages are (a) Possible inhibition from high solid content samples, (b) Use of an extremely toxic compound [45,48].

The advantage of reverse transcriptase PCR is (a) Distinguishes viable from dead cells, and the disadvantages are (a) Complexity of the procedures, (b) Short half-life of RNA,

(c) Technical expertize is necessary, (d) Environmental samples can inhibit the detection [47,48].

Mendes Silva and Domingues [48] reported in detail the target gene and the method used to detect pathogenic *Escherichia coli*. It is summarized in detail in Table 4.

Waturangi et al [49] reported that prevalence of pathogenic *Escherichia coli* from salad vegetable and fruits sold in Jakarta. Fruits and Vegetables were analyzed by multiplex conventional PCR which consisted of six sets of primer encoding virulence genes were used such as *aggr* (EAEC), *stx* (EHEC), *ipah* (EIEC), *eae* (EPEC), and *elt & est* (ETEC) [49].

And Rani et al [50] demonstrated that trends in point-of-care diagnosis for *Escherichia coli* O157:H7 in food and water. Various strategies could be applied to manage the outbreak of infection from *Escherichia coli* O157:H7. However, since early diagnosis of *Escherichia coli* O157:H7 was not easy, prevention strategies to minimize infection were difficult. Unfortunately, the gold standard method currently used to detect *Escherichia coli* O157:H7 was the culture methods. For the purpose of overcoming the limitations of *Escherichia coli* O157 diagnosis, mobile PCR and CRISPR-Cas diagnosis platforms have been recently developed [50].

Furthermore, various methods are currently being used for the diagnosis of *Escherichia coli* O157, for example, isothermal amplification method, biosensor, surface-enhanced Raman spectroscopy, paper-based diagnosis, and smart phone-based digital method [50].

Table 4. PCR methods used to detect pathogenic *Escherichia coli* in samples

Type of pathogenic *Escherichia coli*		Target gene	Detection method used
Escherichia coli		clpB-mRNA	Nucleic acid based sequence amplification real-time PCR (Molecular beacon probe)
Escherichia coli		Internal transcribed spacer (ITS) region between 16S-23S rRNA subunit genes	Quantitative real-time PCR (SYBR Green)
Escherichia coli, *Helicobacter pylori*		*lacZ*	Quantitative real-time PCR (TaqMan probe)
Enterococcus spp., *Enterococcus faecalis/faecium*, *Escherichia coli*, and *Shigella* spp.		23S rRNA, *mtlf*, *ddl*, and *atpD*	Reverse transcriptase PCR (TaqMan probe)
Escherichia coli O157:H7	Normal	*rfbE*	PCR
		rfbE and *fliC*	Real-Time PCR and electronic microarray
		stx1 and/or *stx2*	Multiplex-Reverse transcriptase PCR (SYBR Green)
		stx1, *stx2*, and *rfbE*	Reverse transcriptase PCR (TaqMan probe)
	Stressed	*eae*, *stx1*, and *stx2*	Multiplex-Quantitative real-time PCR (Minor groove binding probes)
Enterohemorrhagic *Escherichia coli* (EHEC)		*stx1*, *stx2*, and *eae*	Multiplex-Reverse transcriptase PCR (SYBR Green)
Enterotoxigenic *Escherichia coli* (ETEC)		LT1	Quantitative real-time PCR (Molecular beacon probe)
		LT1 and ST1	Quantitative real-time PCR (SYBR Green)
Shiga toxin-producing *Escherichia coli* (STEC)		*stx2*	Quantitative real-time PCR (Molecular beacon probe)

Rearranged by referring to the Table in Mendes Silva and Domingues [48] with permission of Elsevier.
PCR, polymerase chain reaction; Stx, Shiga toxins; LT, heat-labile enterotoxins; ST, heat-stable enterotoxins.

2. Internal amplification control for effectively eliminating false-negative results in PCR

Although PCR is a routinely used method, it may be difficult to reproduce the results owing to the differences in the performance of PCR thermal cyclers and the efficiency of DNA polymerase and presence of various PCR inhibitors in the environment [51].

IAC is a nontarget DNA sequence that can be added to the sample and is amplified simultaneously with the target sequence [52]. IAC can prevent false-negative results that may be caused by PCR inhibitors [53]. The European standardization committee, in cooperation with the International Standard Organization, proposed the guidelines for testing pathogens by using PCR, including IAC [54].

The approach used for developing an IAC largely depends on whether it will act competitively or non-competitively with the target sequence. In a competitive strategy, the target sequence and IAC are amplified using a common primer set under the same conditions [55]. In this strategy, the amount of IAC used is very important because it affects the limit of detection of the target sequence [56]. In a noncompetitive strategy, target sequence and IAC are amplified using different primer sets [57].

Conflict of Interest

The authors declare no potential conflict of interest.

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