

Novel multiplex PCR for detection of diarrheagenic *Escherichia coli* strains isolated from stool and water samples

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ABSTRACT. Diarrhea is considered the second most common cause of infant mortality worldwide. The disease can be caused by many different pathogens, including diarrheagenic *Escherichia coli* (DEC), which includes the pathotypes enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC), and enteropathogenic *E. coli* (EPEC). To develop a multiplex PCR system for the safe and accurate identification of the five main pathotypes of DEC, seven pairs of primers were determined for the following genes: *aaiC*, *escV*, *bfpA*, *ipaH*, *elt*, *stx1*, and *stx2*. To validate the system, 413 isolates from different sources (water and both animal and human stool) were analyzed that had been characterized previously. The sensitivity data were grouped by pathotype, in which 92.7% of the atypical EPEC were correlated, as were 92.8% of the STEC, 91.35% of the EAEC, and 100% of the typical EPEC, ETEC, and EIEC. These findings indicate that it is possible to detect the major five pathotypes of DEC from different sources, which can aid in determining the epidemiology of diarrhea with a low cost,

high sensitivity and specificity, and the easy and safe viewing of the resulting PCR products.

Key words: Multiplex PCR; Diarrheagenic *Escherichia coli*; Epidemiology; Diarrhea; Virulence

INTRODUCTION

Diarrhea, which is considered the second most common cause of infant mortality worldwide, is a neglected clinical manifestation, especially because of its labor-intensive microbiological diagnosis and epidemiology.

Escherichia coli diarrheagenic (DEC) pathotypes represent the dominant bacterial pathogens that are epidemiologically associated with diarrhea: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Shiga-toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC) (Clements et al., 2012).

Because the virulence genes that characterize *E. coli* are genetically encoded by chromosome, pathogenicity islands, plasmids, and bacteriophages, it follows that polymerase chain reaction (PCR) would provide a reliable system for amplifying a specific region of DNA (Pass et al., 2000).

Several multiplex PCR systems for DEC detection have been developed; however, the analyses have been restricted to *E. coli* strains isolated from fecal samples from humans or animals (Pass et al., 2000; Rumi et al., 2012; Fujioka et al., 2013; Oh et al., 2014).

Considering the importance of DEC as the cause of diarrhea, the aim of this study was to perform a multiplex PCR assay to detect the five main pathotypes of DEC isolated from different sources, water and both human and animal feces. This technique can aid in epidemiological surveillance and control efforts in endemic areas.

MATERIAL AND METHODS

Bacterial strains

The strains used in this study were obtained from collections of the Bacteriology and Bacteriology Basic and Applied Laboratories of the State University of Londrina between 2012 and 2015. A total of 413 *E. coli* isolates were sampled from the following different sources: 189 from animal stools (cat, dog, and bovine), 54 from water, and 170 from human stools. Among these isolates, positivity for one or more DEC genes corresponded to 109 (57.7%), 9 (16.7%), and 65 isolates (38.2%), respectively, and the total included 183 isolates of DEC and 230 isolates of nonpathogenic *E. coli*. This collection was characterized using the following primers: EAEC (*aggR*) (Boisen et al., 2012), EIEC (*ipaH*) (Aranda et al., 2007), STEC (*stx1*, *stx2*, and *eae*) (Paton and Paton, 1998), EPEC (*eae* and *bfpA*) (Gunzburg et al., 1995; Paton and Paton, 1998) and ETEC (*elt*) (Aranda et al., 2007). The *E. coli* DH5a and other Enterobacteriaceae were used as negative controls: *Shigella flexneri* M90T, *Salmonella enterica* serovar Enteritidis ATCC 13076, *Klebsiella pneumoniae* ATCC 700603 K6, and *Proteus mirabilis* ATCC 7002.

Multiplex PCR design

Five primer sets were designed based on an analysis of gene sequences published

in the National Center for Biotechnology Information (NCBI) database. The MPPrimer program (Shen et al., 2010) was used to obtain, analyze, and assess the specificity of the primer sequences. The following reference strains were used: EAEC 042 (GenBank accession No. FN554766.1), EIEC 53638 O144 (GenBank accession No. CP001064.1), EPEC O127:H6 (GenBank accession No. FM180569.1), and ETEC H104:O7 (GenBank accession No. EU113248.1). Additionally, two pairs of primers that had been previously described in the literature were used (*stx1* and *stx2*) (Leotta et al., 2005).

Multiplex PCR assay

The strains analyzed by PCR were grown on Luria-Bertani agar and incubated at 37°C for 24 h. Bacterial DNA of 3 colonies was extracted by boiling. Multiplex PCRs were performed in a thermocycler (Veriti 96-Well Thermal Cycler, Applied Biosystems™, California, USA) to the following cycling conditions: 95°C/5 min, 15 cycles of 95°C/1 min, 62°C/2 min, and 72°C/2 min, 20 cycles of 95°C/1 min, 58°C/2.5 min, and 72°C/1 min and final extension at 72°C/7 min. These conditions were used in all analyses and resulted in three multiplex PCR systems with a final volume of 25 µL each (Table 1). PCR products were separated on 2.5% agarose gels (70 V) and assessed for potential differences after 1 h (Figure 1).

Table 1. Description of oligonucleotides and reagent concentrations used in the three systems.

Target gene (GenBank accession No.)	Type of PCR	Primer sequence (5'→3')	Amplicon size (bp)	Final concentration of primers (mM)	Reagents of the system	Reference		
<i>aaiC</i> (FN554766.1)	System 1	AGAGCGTCCACTGTCAGAGCGT	183	400	Buffer: 1X MgCl ₂ : 2.2 mM dNTP: 0.46 mM each Taq polymerase: 2.5 U	This study		
		GCGACCTGCTCTGGCGTGAAAT						
<i>escV</i> (FM180568.1)	System 1	TAACGCCTGCGCGCATATACCC	266	400		Buffer: 1X MgCl ₂ : 2.2 mM dNTP: 0.46 mM each Taq polymerase: 3 U	This study	
		GTTGATGCGCCTGTCGCTAGT						
<i>bfpA</i> (FM180569.1)	System 1	TCTGCAATGGTGTGCGCTTTG	478	480			Buffer: 1X MgCl ₂ : 2.2 mM dNTP: 0.46 mM each Taq polymerase: 3 U	This study
		CAGTTGCCGCTTCAGCAGGAGT						
<i>stx1</i> (JX206444.1)	System 2	AGCGATGCACGATTAATAA	130	480	Buffer: 1X MgCl ₂ : 2.2 mM dNTP: 0.46 mM each Taq polymerase: 3 U			Leotta et al., 2005
		GAAGAGTCCGTGGATTACG						
<i>ipaH</i> (CP001064.1)	System 2	CAGGTCGCTGCATGGCTGGAAA	383	480		Buffer: 1X MgCl ₂ : 2.2 mM dNTP: 0.46 mM each Taq polymerase: 3 U		This study
		GGCAGTGGCGAGGTCATTTCCT						
<i>stx2</i> (AY633471.1)	System 3	TTAACCACACCCACCGGGCAGT	346	560			Buffer: 1X MgCl ₂ : 2.2 mM dNTP: 0.46 mM each Taq polymerase: 3 U	Leotta et al., 2005
		GCTCGGATGCATCTCTGGT						
<i>Elt</i> (EU113248.1)	System 3	AGGCGTATACAGCCCTACCCA	550	480	Buffer: 1X MgCl ₂ : 2.2 mM dNTP: 0.46 mM each Taq polymerase: 3 U			This study
		ACCTGAAATGTTGCCCGCTCT						

Genes and pathotypes: *aaiC* (EAEC), *escV* (atypical EPEC), *escV* and *bfpA* (typical EPEC); *escV* and *stx1* and/or *stx2* (Enterohemorrhagic *Escherichia coli*, EHEC); *stx1* and/or *stx2* (STEC); *ipaH* (EIEC); and *elt* (ETEC).

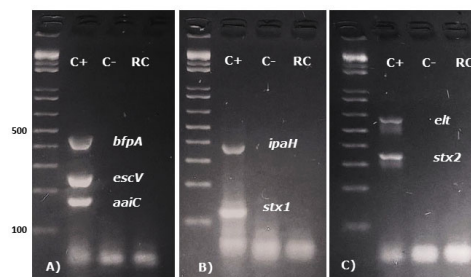


Figure 1. Profile electrophoretic of the specific loci amplified in multiplex PCR systems 1, 2, and 3 on a 2.5% agarose gel. **A.** System mPCR1 genes: *bfpA*, *escV*, and *aaiC*. **B.** System mPCR2 genes: *ipaH* and *stx1*. **C.** System mPCR3 genes: *elt* and *stx2*. C+: positive control (**A**) EPEC O127:H6 and EAEC 042; (**B**) EIEC 53638 O144 and EHEC EDL933; (**C**) ETEC H104:O7 and EHEC EDL933. C-: negative control (*E. coli* DH5 α). RC: reaction control (no DNA).

Sensitivity of the multiplex PCR

To verify the sensitivity of the multiplex PCR, serial 10-fold dilutions of bacterial DNA were performed. Then, the dilutions were subjected to multiplex PCR assay.

RESULTS

All primers were shown to be specific for the respective patterns of *E. coli*, with a minimum sensitivity of 5.2 ng/ μ L. For other Enterobacteriaceae strains verified, the *ipaH* gene was positive for *S. flexneri*.

The results were compared to previously conducted analyses based on the presence of specific genes for each pathotype. This analysis indicated that 183 strains had DEC-positive results, whereas positivity was detected in 160 isolates when using novel multiplex PCR system. Of these, 88 isolates were isolated from animal stool samples, 45 were from human stool samples, and 27 were from water samples (Table 2).

According to comparisons between the pretest identities and the results of our multiplex PCR (Table 2), for atypical EPEC, the sensitivity reached 91.3% for the isolates from the animal stool, 86.7% for the human stool, and 100% for water. For STEC strains, the sensitivity reached 81.7% for the animal stool, 86.7% for the human stool, and 100% for water samples. The sensitivity for typical EPEC, ETEC, and EIEC strains was 100%.

Table 2. Comparison between the collection and novel multiplex PCRs.

Pathotype	Collection			Multiplex PCR		
	Water	Human stool	Animal stool	Water	Human stool	Animal stool
aEPEC	6	15	23	6	13	21
tEPEC	-	10	-	-	10	-
EIEC	-	-	2	-	-	2
STEC	3	3	60	3	2	31
EAEC	-	37	11	18	20	21
ETEC	-	-	13	-	-	13
Positive	9	65	109	27	45	88
Negative	45	105	80	27	125	101
Total (isolates)	54	170	189	54	170	189

aEPEC = atypical EPEC; tEPEC = typical EPEC.

DISCUSSION

The molecular details of virulence factors have been extensively studied and characterized, thus enabling their possible applications in epidemiology studies. However, the genetic diversity of DEC strains makes the identification of species labor-intensive and involves a series of molecular tests to precisely define the pathotype involved in an infection.

The present study aimed to develop this tool to be capable of differentiating among the five main DEC categories by using 7 pairs of primers in 3 different systems with the same programming thermocycler. This would allow the possibility of analyzing strains of different origins by visualizing PCR products by agarose gel electrophoresis, maintaining good resolvability between the PCR products to ensure that the visualization of the results was unambiguous. Fujioka et al. (2013) reported the development of a multiplex PCR to sort 10 genes into five main categories of DEC in a single reaction, but the visualization of results was affected by not achieving a safe distance between the PCR products.

Moreover, this study aimed to allow different strains that were isolated from different sources (water, and both human and animal feces) to be analyzed. Different multiplex PCR systems for DEC detection have been developed; however, the analyses have been restricted to fecal samples from humans or animals or did not distinguish among the five major classes of DEC and required other PCRs to verify the results (Pass et al., 2000; Rumi et al., 2012; Fujioka et al., 2013; Oh et al., 2014).

The heterogeneity of the results of STEC present by us is likely indicated by the genetic variability for *stx2* (Paton and Paton, 1998). About *stx2* our intention was to detect the gene and not the specific subtype. We tested four different set of primers, two designed by us and two from literature (data not shown). The primer set described by Leotta et al. (2005) presented the best sensitivity and was used by us.

Our system recognized 100% of typical EPEC, ETEC, and EIEC. These findings demonstrate that the target genes probably do not have much variability in the sequence. Although the occurrence for these pathotypes was low, its importance is indicated by the occurrence of outbreaks (Ochoa et al., 2008).

For searches of EAEC strains, the collection of laboratories was chosen for the application of primers for *aggR* to detect this pathotype. This study opted to study a chromosomal gene (*aaiC*). As shown by Lima et al. (2013) the *aaiC* gene is a good candidate for molecular diagnosis because it has a chromosomal origin and is more prevalent than plasmid genes in EAEC. Our findings for *aaiC* and *aggR* revealed that the difference between the molecular diagnosis of chromosomal and plasmid genes was 59 strains positive for *aaiC* and only 48 for *aggR* (Table 2).

Therefore, the findings indicate that there is a possibility to detect the major 5 pathotypes of DEC from different sources, which may aid in assessments of the epidemiology of diarrhea, with low cost, high sensitivity and specificity, and the easy and safe viewing of PCR products.

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