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Bacteriology

Detection of *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction

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Abstract

The magnitude of bacterial diarrhea in developing countries is largely unknown because affordable detection methods are not available. We have developed a polymerase chain reaction (PCR)-based assay for use in areas with limited resources to screen for diarrheogenic strains from clinical isolates. To simplify the assay and minimize reagents, our method implemented the use of plasmids rather than bacteria as template controls and the use of bacterial suspensions or crude DNA preparations rather than purified genomic DNA as template DNA. The assay consisted of 3 PCR reactions using 3 groups of 5 to 6 primer pairs to identify the 11 most common bacterial diarrheogenic pathogens. The 3-reaction multiplex PCR amplifies DNA targets specific for each 1 of the 6 *Escherichia coli* diarrheogenic strains and the 5 non-*E. coli* diarrheogenic strains, including *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, and *Vibrio cholerae*. The assay may provide an important epidemiologic tool to investigate the role of diarrheogenic bacterial pathogens in areas of the world with limited resources.
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1. Introduction

Infectious diarrheal disease is a leading cause of morbidity and mortality in children in developing countries (Guerrant et al., 2002; Huilan et al., 1991; Kosek et al., 2003). Travelers, including children, from industrialized nations to developing countries, are also affected by traveler's diarrhea (Adachi et al., 2002). Although the etiologic agents and their mechanisms of pathogenesis have been elucidated, information on the prevalence of these agents in developing countries is largely unknown. Assays for identification of these pathogens are limited to research laboratories, and affordable identification assays are not commercially available. Most epidemiologic data on diarrheal pathogens from developing countries is scattered and generally does not include *Escherichia coli* as a causative agent, because no approved testing system is available for the identification of the 6 known diarrheogenic *E. coli* strains (Reither et al., 2007). The only method available in most developing nations for detection of bacterial diarrheal pathogens is conventional bacterial culture from stool. This method requires a minimum of 48 h for identification of *E. coli* and non-*E. coli* spp. It is unable to discriminate between nonpathogenic and pathogenic *E. coli* strains. Furthermore, no commercially available methods exist to differentiate among the 6 different *E. coli* pathotypes associated with diarrhea, including enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffuse adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC) (Levine, 1987).

A sensitive, specific, and affordable test for rapid identification of bacterial diarrhea is necessary to determine the impact of diarrheal bacterial pathogens on childhood morbidity and mortality in developing countries. This method may be instrumental for epidemiologic surveillance

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of diarrheal diseases, for evaluation of food and water for human consumption, and possibly for rapid diagnosis of severe diarrhea.

The most common bacterial strains associated with diarrhea include *E. coli*, *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. (Huilan et al., 1991). Among *E. coli*, 6 strains are capable of inducing diarrheal disease by well-defined virulence genes and different mechanisms of pathogenesis. To our knowledge, no polymerase chain reaction (PCR)-based method has been published on the use of a comprehensive assay for detection of *E. coli* and non-*E. coli* enteropathogens from environmental sources, clinically isolated sources, or stool samples. Several multiplex PCR assays for detection of diarrheogenic *E. coli* have been reported since 1995 (Stacy-Phipps et al., 1995); all are based on amplification of gene targets specific for each *E. coli* pathotype, as shown in Table 1. Most of the assays are able to detect *E. coli* from clinical isolates (Aranda et al., 2007; Brandal et al., 2007; Nguyen et al., 2005; Rappelli et al., 2005; Rich et al., 2001; Toma et al., 2003; Vidal et al., 2005) and only 1 from both clinical isolates and directly from stools (Stacy-Phipps et al., 1995). Although most assays detect amplified DNA on agarose gels stained with ethidium bromide or an equivalent intercalating agent, some assays use fluorescent primer probes (Brandal et al., 2007; Watterworth et al., 2005). Independently, multiplex PCR assays have been developed for detection of non-*E. coli* enteropathogens from stools, food, water, and animals. Detection of *Y. enterocolitica* was described by the use of the *Yersinia* heat-stable enterotoxin gene (yst) PCR (Ibrahim et al., 1997). Detection of *Salmonella* spp. relies on amplification of the internal transcribed spacer region of the 16S-23S rRNA gene (Chiu et al., 2005; Park et al., 2006). *Campylobacter* spp. detection is based on the amplification of the hippuricase gene (hipO) (Persson and Olsen, 2005). *V. cholerae* detection uses the toxin-coregulated pilus and the cholera toxin genes (RTX-A and RTX-B) (Gubala, 2006).

Conventional PCR assays imply the need for expensive equipment (freezers) and supplies (molecular-grade solutions, genomic DNA isolation), as well as trained personnel in molecular biology techniques. These methods use prototype bacterial isolates for PCR controls, which assume the need for −70 °C freezers for bacterial strain storage. Furthermore, these techniques rely on genomic DNA as DNA templates for amplification, which requires the use of multiple molecular-grade reagents and technical expertise.

Table 1
Target genes for PCR amplification. Gene size, location, and strain of origin

Primer mix	Gene target	PCR size	Location	Strain	Primer sequence	Orientation	Source
M1	VT	518	Chromosome	EHEC	5′-GAGCGAAATAATTTATATGTG-3′	Forward	Aranda et al. (2007)
					5′-TGATGATGGCAATTCAGTAT-3′	Reverse	
	eae	917	Chromosome	EHEC, EPEC	5′-CTGAACGGCGATTACGCGAA-3′	Forward	Aranda et al. (2007)
					5′-CGAGACGATACGATCCAG-3′	Reverse	
	bfpA	326	Plasmid	EPEC	5′-AATGGTGCTTGCGCTTGCTGC-3′	Forward	Aranda et al. (2007)
					5′-GCCGCTTTATCCAACCTGGTA-3′	Reverse	
	aggR	254	Plasmid	EAEC	5′-GTATACACAAAAGAAGGAAGC-3′	Forward	Aranda et al. (2007)
					5′-ACAGAATCGTCAGCATCAGC-3′	Reverse	
M2	LT	218	Plasmid	ETEC	5′-GCACACGGAGCTCCTCAGTC-3′	Forward	Vidal et al. (2005)
					5′-TCCTTCATCCTTTCAATGGCTTT-3′	Reverse	
	ST	147	Plasmid	ETEC	5′-GCTAAACCAGTAGAG(C) TCTTCAAAA-3′	Forward	Nguyen et al. (2005)
					5′-CCCGGTACAG(A) GCAGGATTACAACA-3′	Reverse	
	daaE	542	Plasmid	DAEC	5′-GAACGTTGGTTAATGTGGGGTAA-3′	Forward	Vidal et al. (2005)
					5′-TATTCACCGGTCGGTTATCAGT-3′	Reverse	
	virF	618	Chromosome	EIEC	5′-AGCTCAGGCAATGAAACTTTGAC-3′	Forward	Vidal et al. (2005)
					5′-TGGGCTTGATATTCCGATAAGTC-3′	Reverse	
	ipaH	933	Plasmid	EIEC	5′-CTCGGCACGTTTTAATAGTCTGG-3′	Forward	Vidal et al. (2005)
					5′-GTGGAGAGCTGAAGTTTCTCTGC-3′	Reverse	
M3	ITS	312	Chromosome	*Salmonella* spp.	5′-TATGCCCCATCGTGTAGTCAGAAC-3′	Forward	Park et al. (2006)
					5′-TGCGGCTGGATCACCTCCTT-3′	Reverse	
	YST	145	Chromosome	*Y. enterocolitica*	5′-GTTAATGCTGTCTTCATTTGGAGC-3′	Forward	This study
					5′-GACATCCCAATCACTACTGACTTC-3′	Reverse	
	RTX-A	120	Chromosome	*V cholerae*	5′-AGCAAGAGCATTGTTGTTCCTACC-3′	Forward	Gubala (2006)
					5′-ACTTCCCTGTACCGCACTTAGAC-3′	Reverse	
	hipO	344	Chromosome	*C. jejuni*	5′-GACTTCGTGCAGATATGGATGCTT-3′	Forward	Persson and Olsen (2005)
					5′-GCTATAACTATCCGAAGAAGCCATCA-3′	Reverse	
	virF	618	Chromosome	*Shigella* spp.	5′-AGCTCAGGCAATGAAACTTTGAC-3′	Forward	Vidal et al. (2005)
					5′-TGGGCTTGATATTCCGATAAGTC-3′′	Reverse	
	ipaH	933	Plasmid	*Shigella* spp.	5′-CTCGGCACGTTTTAATAGTCTGG-3′	Forward	Vidal et al. (2005)
					5′-GTGGAGAGCTGAAGTTTCTCTGC-3′	Reverse	

All of these elements—equipment, techniques, reagents, and expertise—are simply not available in many geographic settings in the developing world.

In this study, we described a comprehensive assay capable of detecting not only *E. coli* diarrheogenic strains but also *Salmonella* spp., *Shigella* spp., *Y. enterocolitica*, *V. cholerae*, and *Campylobacter jejuni* enteropathogens. DNA templates used in this assay include DNA plasmid controls rather than bacterial strain controls, and bacterial suspensions or crude DNA preparations rather than purified genomic DNA. Three sets of 5 to 6 plasmids containing cloned genes unique for each diarrheogenic bacterial pathogen will be used to provide the positive control template for reference PCR amplicons. By using plasmid controls, we obviate the need of bacterial strains that require expensive freezing equipment for storage. By using crude DNA extracts as template DNA, we avoid the need for equipment and reagents essential to process bacteria for isolation of highly purified genomic DNA. The emphasis of this assay is simplification, speed of detection, sensitivity, specificity, and capability of implementation in limited-resource areas of the world where this clinical epidemiologic technology is needed the most.

2. Materials and methods

2.1. Strains and plasmids

Control bacterial strains used in this study are listed in Table 2. *E. coli* K-12 strain DH5α was used for cloning experiments. Plasmids used in this study are listed in Table 3. *E. coli*, *Shigella*, *Salmonella*, and *Y. enterocolitica* strains were grown on McConkey agar or eosin methylene blue agar, Luria agar, or Luria broth at 37 °C overnight, unless otherwise specified. *Campylobacter* spp. were grown on blood agar and microaerophilic environment using Pack-MicroAero (Mitsubishi Gas Chemical America, New York, NY). All strains were grown at 37 °C overnight from a frozen stock aliquot.

2.2. DNA templates processing for PCR assays

Bacterial suspensions and crude DNA preparations were used as DNA templates for PCR reactions. Bacterial suspensions were made from each strain by harvesting bacteria from agar plates into a 1.5-mL Eppendorf tubes containing TE buffer (10 mmol/L Tris–HCl, pH 8.0, 5 mmol/L EDTA). Bacterial suspensions were diluted to an optical density of 0.1 at 600 nm (OD_{600}) and used immediately for PCR. Preparation of crude DNA preparations required 1 mL of bacterial suspension in TE buffer adjusted to approximately 2.0, OD_{600}. The bacterial suspension was then vortexed for 1 min at maximum speed and centrifuged at maximum speed in an Eppendorf microfuge for 2 min. The supernatant containing crude genomic DNA was transferred to a new Eppendorf tube for use as DNA template. The crude DNA preparations were stored at 4 °C until used.

Table 2
E. coli and non-*E. coli* reference strains used in this study

Name	Species	Strain/ biotype	Serotype	Gene targets[a]	Source
DH5α	*E. coli*	K12		None	Laboratory collection
2060-004	*E. coli*	EHEC	O157:H7	VT, *eaeA*	UIHC[b]
E2348/69	*E. coli*	EPEC	O127:H6	*eae*, *bfpA*	Gomez-Duarte & Kaper (1995)
JM221	*E. coli*	EAEC	O78:H33	*aggR*	Nataro et al. (1995)
E9034A	*E. coli*	ETEC	O8:H9	LT, ST	Levine et al. (1984)
C1845	*E. coli*	DAEC	O75:NM	*daaE*	Bilge et al. (1989)
EC-12	*E. coli*	EIEC	–	*ipaH*, *virF*	University of Washington[c]
SH24	*Shigella flexneri*	–	–	*ipaH*, *virF*	UIHC
1999-002	*S. enterica*	–	Type B	ITS	UIHC
1992-045	*Y. enterocolitica*	–	–	YST	UIHC
33291	*C. jejuni*	–	–	*hipO*	UIHC
N16961	*V. cholerae*	El Tor, Inaba	O1	RTX	Levine et al. (1988)

[a] Gene targets: VT, EHEC VT genes 1 or 2. *eae*, EPEC–EHEC *E. coli* effacement-attaching gene. *bfpA*, EPEC bundle-forming pilus structural subunit gene. aggR, EAEC aggregative adherence fimbriae regulator. LT, ETEC heat-labile toxin gene. ST, ETEC heat-stable toxin gene. *daaE*, DAEC diffuse adherence pilus structural subunit gene. *virF*, *Shigella* virulence regulatory gene. *ipaH*, *Shigella* invasion plasmid antigen gene. ITS, *Salmonella* internal transcribed spacer region of 16S-23S rRNA. YST, *Yersinia* heat-stable enterotoxin gene. RTX-A, *V. cholerae* cholera toxin subunit A gene. *hipO*, *C. jejuni* hippuricase O gene.

[b] Microbiology Laboratory, University of Iowa Hospital and Clinics, kindly provided by Dr S. Richter.

[c] Department of Microbiology, University of Washington, Seattle, WA, kindly provided by Dr S. Moseley.

2.3. DNA amplification of target genes

Thirteen gene targets from *E. coli*, *Salmonella* spp., *Shigella* spp., *Y. enterocolitica*, *V. cholerae*, and *C. jejuni* diarrheogenic strains were amplified using oligonucleotide primer pairs listed in Table 1. One microliter of crude DNA preparation was mixed with 24 μL of a premade mix containing primers at a 0.2-μmol/L final concentration and Platinum Blue PCR SuperMix polymerase (Invitrogen, Carlsbad, CA). Combination of 5 to 6 pairs of primers per primer group, and designated M1, M2, and M3, are described in Table 1. Primer mixes were evaluated at different concentrations, and the 0.2 μmol/L final concentration for individual primers was chosen for all reactions. The PCR program used for amplification consisted of 2 min at 94 °C of denaturing temperature, followed by 40 cycles of 30 s at 92 °C of denaturing temperature, 30 s at 59 °C of annealing temperature, and 30 s at 72 °C of extension temperature. At the end of the 40 cycles, a 5-min extension at 72 °C was used before samples were ready for analysis or PCR DNA fragment purification.

Table 3
Plasmids used or generated in this study

Control group	Plasmid	Description	Reference
	pCR2.1	Cloning vector (Ap^r, Km^r)	Invitrogen
	pSC-A	Cloning vector (Ap^r, Tet^r)	Stratagene
P1	pOG401	pSC-A with a 518-bp VT PCR insert from the 2060-004 EHEC strain	This study
	pOG390	pSC-A with 482-bp *eae* gene fragment from the E2348/69 EPEC strain	This study
	pOG394	pSC-A with a 300-bp *bfpA* gene fragment from the E2348/69 EPEC strain	This study
	pOG395	pSC-A with a 630-bp *aggR* gene fragment from the EAEC JM221 virulence plasmid	This study
P2	pWD299	pBR313 with 1.8-kb insert containing LT genes A and B	(Dallas et al., 1979)
	pSLM004	pBR322 with 0.8-kb *Taq*I insert containing the ST gene	(Moseley et al., 1982)
	pOG391	pSC-A with a 542-bp *daaE* gene fragment from the C1845 DAEC strain	This study
	pOG392	pSC-A with a 933-bp *ipaH* gene fragment from the EC-12 EIEC strain	This study
	pOG393	pSC-A with a 618-bp *virF* gene fragment from the EC-12 EIEC strain	This study
P3	pOG396	pCR2.1 with a 120-bp RTX-A gene fragment from the N16961 *V. cholerae* strain	This study
	pOG397	pCR2.1 with a 312-bp *ITS* gene fragment from the 1999-02 *S. enterica* strain	This study
	pOG398	pSC-A with a 145-bp *YST* gene fragment from the 1992-045 *Y. enterocolitica* strain	This study
	pOG400	pSC-A with a 344-bp *hipO* gene fragment from the 33291 *C. jejuni* strain	This study

2.4. Cloning of target genes into plasmids

DNA fragments amplified by PCR from prototype strains were column-purified using Invitrogen PureLink PCR purification kits and following supplier recommendations. Gene target-purified DNA fragments were ligated into plasmid vectors pSC-A (Stratagene, La Jolla, CA) or pCR2.1 (Invitrogen) following conventional molecular biology techniques (Sambrook and Russell, 2001). Ligation mixes were introduced into *E. coli* competent cells by transformation as recommended by suppliers. Bacterial clones carrying each 1 of the cloned gene targets (Table 3) were stored in a −80 °C freezer for further use. DNA plasmids containing each 1 of the 12 gene targets were isolated from *E. coli* K12 strains using a HiPure midiprep kit (Invitrogen) as recommended by suppliers (Table 3). Cloned DNA targets from all plasmids were sent for DNA sequencing to confirm that the cloned DNA corresponded to the gene of interest. DNA sequences obtained from the DNA facility (University of Iowa, Iowa City, IA) were analyzed by the BLAST soft ware from the National Center for Biotechnology Information Web site. All DNA sequences analyzed corresponded to the gene targets of interest (data not shown).

2.5. DNA gel electrophoresis and image recording

DNA amplified by PCR was separated onto a 2.0% agarose gel electrophoresis containing ethidium bromide and using Tris–acetate–EDTA as running buffer (Sambrook and Russell, 2001). Images of DNA separation from gel exposed to ultraviolet (UV) light were captured with a digital camera. Images were transferred to a PowerPoint software program for analysis, storage, and printing. Material contaminated with ethidium bromide was disposed according to local guidelines. An alternative method for staining of DNA gels includes SYBR green stain (Invitrogen), a reagent not classified as a toxic agent and disposable with conventional waste.

3. Results

3.1. Three-sample multiplex PCR assay

A 3-sample multiplex PCR was designed for identification of the 11 most common bacterial strains associated with diarrhea in a single PCR program run. The 6 *E. coli* strains included EHEC, EPEC, EAEC, ETEC, DAEC, and EIEC,

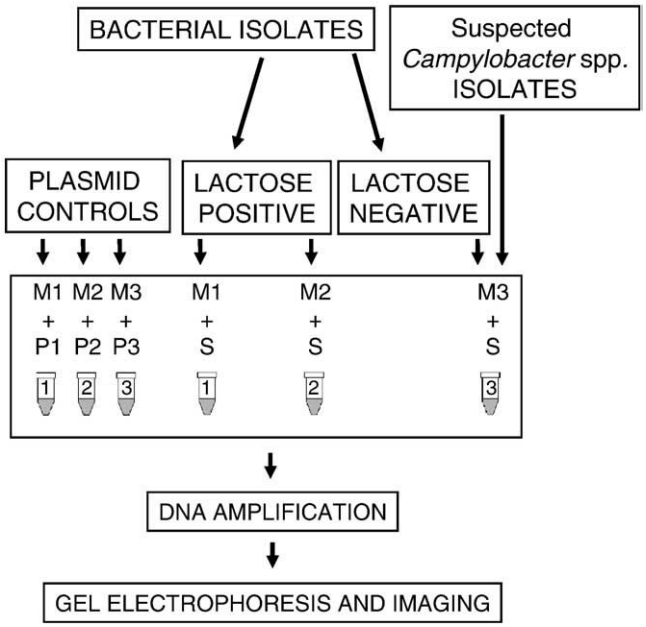

Fig. 1. Three-sample multiplex PCR diagram. Diagram shows the sequential steps on the PCR assay beginning with the lactose fermentation screening of bacterial isolates, followed by the addition of the DNA templates (S or P) to the PCR reaction mixes (M), PCR amplification, and the DNA fragment separation onto a 2% agarose gel electrophoresis, and digital imaging capture for further analysis. Mixes M1, M2, and M3 contain all reagents except DNA template. P1, P2, and P3 correspond to plasmid controls templates. S corresponds to sample DNA templates, which may be crude DNA preparations or diluted bacterial suspensions. Tube 1 containing M1 and P1 reagents will amplify gene targets specific for EHEC, EPEC, and EAEC. Tube 2 will identify specific targets for ETEC, DAEC, and EIEC. Tube 3 will amplify targets specific for *Campylobacter* spp., *Salmonella* spp., *Y. enterocolitica*, *V. cholerae*, and *Shigella* spp.

and the 5 non-*E. coli* strains included *Shigella* spp., *Salmonella*, *Y. enterocolitica*, *Campylobacter* spp., and *V. cholerae*. The multiplex PCR consisted of 3 individual PCR reactions containing a premade primer mix (M) of Taq polymerase enzyme, enzyme buffer, nucleotides, and a set number of specific primer pairs and the DNA template control (P) or DNA template sample (S) (Fig. 1). All clinical isolates were screened for lactose fermentation before being tested with the 3-sample multiplex PCR. The assay required the addition of 1 μL of DNA template to the 24-μL premade solution mix. Polymerase chain reaction samples 1 to 3 (M1, M2, and M3) had all reagents except DNA templates. M1 mix contained primers for amplification of 5 gene targets including verotoxins (VTs) 1 and 2 from EHEC, intimin (*eae*) from EHEC and EPEC, bundle-forming pilus structural subunit (*bfpA*) from EPEC, and regulatory gene *aggR* from EAEC. M2 mix contained primers for amplification of the heat-labile toxin (LT) and the heat-stable toxin (ST) genes from ETEC, for the diffuse adherence structural subunit gene (*daaE*) from DAEC, for the invasion plasmid antigen H (*ipaH*), and for the virulence invasion factor (*virF*) genes from EIEC (or *Shigella* spp.). M3 contained primers for amplification of non-*E. coli* genes including cholera toxin (RTX1) from *V. cholerae*, YST from *Y. enterocolitica*, hippuricase gene (*hipO*) from *Campylobacter* spp., 16S ribosomal RNA region specific for *Salmonella* spp. (ITS), invasion plasmid antigen H (*ipaH*), and virulence invasion factor (*virF*) from *Shigella* spp. (or EIEC). Primer pair mixes are described and shown in Table 1. Primers for YST of *Yersinia* were modified from previously described primers (Ibrahim et al., 1997). Three nucleotides were added at the 5-prime end of each forward and reverse primer to make the

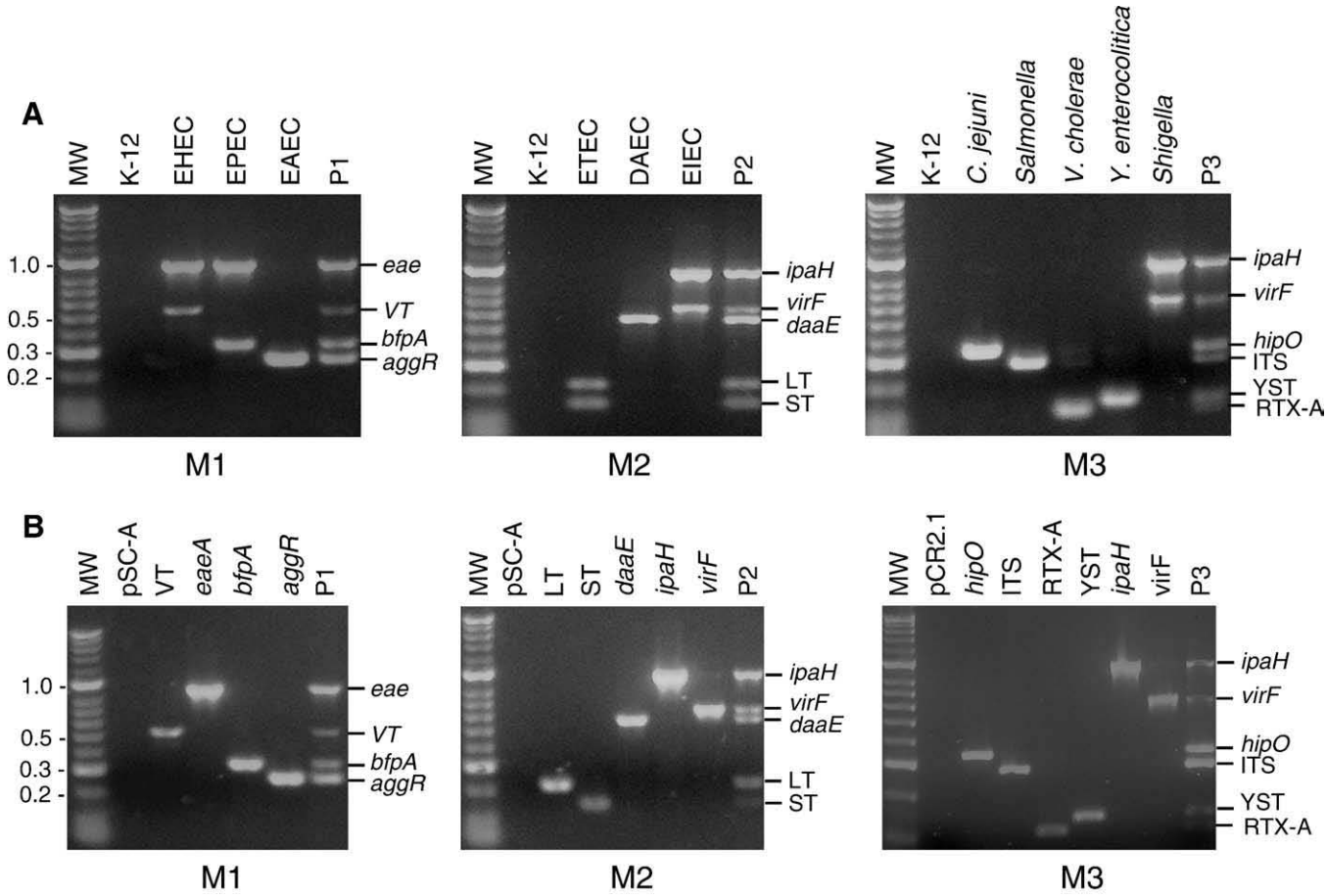

Fig. 2. Three-sample multiplex PCR assay using plasmid DNA carrying gene target controls. Ethidium bromide-stained 2% agarose gel electrophoresis showing amplified products from crude DNA preparations from control strains and plasmid controls. M1, M2, and M3 correspond to primer mixes. M1 contains primers specific for EPEC, EHEC, and EAEC gene targets. M2 contains primers for ETEC, DAEC, and EIEC gene targets. M3 contains primers for *Campylobacter* spp., *Salmonella* spp., *V. cholerae*, *Y. enterocolitica*, and *Shigella* spp. gene targets (Table 1). (A) Three-reaction multiplex PCR of control strains using crude DNA preparations. Strains used are described in Table 2. P1, P2, and P3 correspond to plasmid control mixes. The K12 HB101 *E. coli* strain is included as a negative control. (B) Three-reaction multiplex PCR of individual plasmid DNA templates described in Table 2 representing individual gene targets. pSC-A plasmid vector; VT: pOG401; *eae*: pOG390; *bfpA*: pOG394; *aggR*: pOG395; P1: pOG401, pOG390, pOG394, and pOG395 plasmids; LT: pWD299; ST: pSL0004; *daaE*: pOG391; *ipaH*: pOG392; *virF*: pOG393; P2: pWD299, pSL0004, pOG391, pOG392, and pOG393 plasmids; pCR2.1: plasmid vector; *hipO*: pOG400; ITS: pOG397; RTX-1: pOG396; YST: pOG398; and P3: pOG400, pOG392, pOG393, pOG397, pOG396, and pOG398 plasmids. MW = molecular weight marker (Hyperladder II, BIOLINE Taunton, MA).

melting time temperature similar to the remaining primers, as indicated in Table 1. Each reaction had a total volume of 25 μL. Polymerase chain reaction mixes were amplified in a thermocycler using a single program and processed as described in Materials and methods.

3.2. Gene target controls for the 3-sample multiplex PCR

Because long-term storage of bacterial control strains are not possible in many geographic regions, we have implemented the use of plasmid DNA as gene target controls. Gene targets amplified from control strains were cloned in conventional plasmid vectors, and purified plasmids carrying individual gene targets were used as gene target controls. Gene targets from control strains and from control plasmids were amplified by using the 3-sample multiplex PCR. As shown in Fig. 2, identical DNA bands were amplified from both plasmid controls and bacterial control strains. The molecular masses for each gene target were as expected (Table 3). Plasmid controls were stored dried or in solution at 4 °C. They did not require freezing and were ready to use anytime. The control reaction P1 contained plasmids pOG390 (*eae*), pOG394 (*bfpA*), pOG395 (*aggR*), and pOG401 (VT); the control template P2 contained plasmids pSLM004 (ST), pWD299 (LT), pOG391 (*daaE*), pOG392 (ipaH), and pOG393 (virF); and the control template P3 contained plasmids pOG392 (*ipaH*) and pOG393 (*virF*), pOG396 (RTX-A), pOG397 (ITS), pOG398 (YST), and pOG400 (*hipO*). Plasmid vectors pSC-A and pCR2.1 used for cloning of gene targets did not amplify DNA bands when used as DNA template on the multiplex PCR assay (Fig. 2).

3.3. Bacterial suspensions versus crude DNA preparations as DNA templates

To simplify the multiplex PCR and facilitate its use in clinical laboratories with limited resources, we evaluated the efficiency of the multiplex PCR assay by comparing 2 different DNA templates including bacterial suspensions and crude DNA preparations isolated by the vortex method. Both DNA templates were able to amplify the expected DNA fragments. As shown in Fig. 3, both EPEC DNA templates amplified the *eae* and *bfpA* gene targets, and the ETEC DNA templates amplified the LT and ST gene targets. The DNA template did not amplify unspecific bands. All strains were tested at decreasing DNA template concentrations, and they were still able to amplify gene targets when the DNA was diluted to 1:1000 or more from the original concentration (data not shown).

3.4. The 3-sample multiplex PCR is sensitive

Three-sample multiplex PCR was performed using control template plasmid. All 3 samples had the Blue mix taq polymerase, primer mix (either M1, M2, or M3), and plasmid mix (either P1, P2, or P3). Plasmid mixes P1, P2, and P3 were sequentially diluted from a concentration

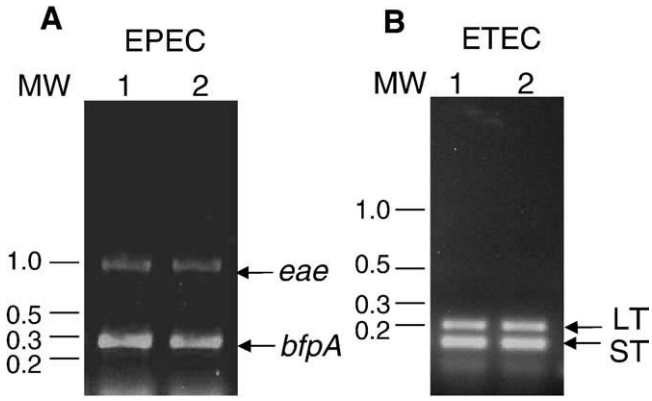

Fig. 3. Crude DNA preparations versus bacterial suspension templates for use with the PCR assay. Crude DNA preparations or bacterial suspensions from E2348/69 EPEC and E9034A ETEC bacterial strains were tested on the multiplex PCR assay. Panel A shows a PCR reaction using M1 PCR reaction mix and EPEC DNA template. Lane 1, crude DNA preparation; lane 2, bacterial suspension. Panel B shows multiplex PCR assay using M2 PCR reaction mix and ETEC DNA template. Lane 1, crude DNA preparation; lane 2, diluted bacterial suspension. Arrows in panel A indicate *eae* and *bfpA* amplified DNA fragments. Arrows in panel B indicate LT and ST. MW represents molecular weight markers.

starting with 10 mg/mL to as low as 0.0001 ng/mL. The multiplex PCR was able to detect specific target DNA at concentration as low as 0.001 ng/mL, which corresponds to a detection level of 1 to 10 molecules of target DNA per reaction sample (data not shown). Although PCR assays reported use a single sample for detection of all 5 of 6 *E. coli* strains (Nguyen et al., 2005; Vidal et al., 2005), we observed decreased sensitivity and specificity when more than 6 pairs of primer pairs were present in a single PCR reaction. This phenomenon has been previously described (Watterworth et al., 2005). To minimize the presence of nonspecific amplified DNA, we used a 3-sample PCR for detection of all 6 *E. coli* pathotypes and all 5 non-*E. coli* enteropathogens.

3.5. Validation of the multiplex PCR method

A collection of previously characterized bacterial strains from different countries including the United States was tested with the 3-sample PCR assay to validate the method. *E. coli*, *Shigella* spp., *Salmonella* spp., *Y. enterocolitica*, *Campylobacter* spp., and *V. cholerae* strains were initially classified into lactose fermenters and non-lactose fermenters. All strains were tested with the 3-sample multiplex PCR, and each strain was recognized based on the DNA banding pattern on the agarose gel as *E. coli* enteropathogens, *Shigella* spp., *Salmonella* spp., *Y. enterocolitica*, *Campylobacter* spp., or *V. cholerae*. Enteroinvasive *E. coli* and *Shigella* spp. strains in this study were differentiated by the lactose-fermenting phenotype present in all *E. coli* and absent in all *Shigella* spp. strains. Negative control strains, including normal flora *E. coli* strains, *Klebsiella* spp., and *Proteus* spp., were also tested with the 3-sample multiplex PCR. No

Table 4
Bacterial strains tested with the 3-sample multiplex PCR

No. of strains positive by PCR/no. of strains tested														
Strain	VT	*eae*	*bfpA*	*aggR*	LT	ST	*daaE*	*virF*	*ipaH*	*hipO*	ITS	RTX	YST	Lactose fermenter
EHEC	5/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5
EPEC	0/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5
EAEC	0/4	0/4	0/4	4/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4
ETEC	0/21	0/21	0/21	0/21	16/20	17/20	0/21	0/21	0/21	0/21	0/21	0/21	0/21	21/21
DAEC	0/5	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5
EIEC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5	5/5	0/5	0/5	0/5	0/5	5/5
Negative *E. coli*	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	10/10
C. jejuni	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	4/4	0/4	0/4	0/4	ND
Salmonella spp.	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	7/7	0/7	0/7	0/7
Shigella spp.	0/7	0/7	0/7	0/7	0/7	0/7	0/7	7/7	0/7	0/7	0/7	0/7	0/7	0/5
V. cholerae	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11	0/11	11/11	0/11	0/11
Y. enterocolitica	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	0/2
Klebsiella spp.	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5
Proteus spp.	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

ND = not determined.

amplified DNA bands were observed from these negative controls (Table 4). Furthermore, there was no cross-reactivity observed among the *E. coli* or non-*E. coli* templates, indicating that the assay was specific. Although the primers tested in this assay have been validated independently for *E. coli* pathotypes, *Shigella* spp., *Salmonella*, *Y. enterocolitica*, *Campylobacter* spp., and *V. cholerae*, we were able to validate the assay as a 3-sample multiplex PCR assay.

4. Discussion

Diarrhea is a major cause of morbidity and mortality in children in the developing world and an important cause of morbidity to travelers from industrialized nations to developing countries. Although rapid, specific, sensitive, affordable, and commercially available methods exist for the identification of enteroviral agents such as rotavirus, no equivalent method exists for identification of bacterial diarrheal pathogens. Current methods of identification are slow, expensive, and time consuming, and require a high level of expertise. Furthermore, identification of any of the 6 *E. coli* pathotypes associated with diarrhea cannot be done by conventional clinical microbiology. These pathogens are identified in research laboratories because no approved method of identification is commercially available.

Evaluation of the epidemiology of infectious diarrhea in developing countries requires affordable methods for identification of bacterial agents. Information on incidence, endemicity, and epidemics of diarrhea in developing countries is, at present, vastly unknown. Rapid identification methods may provide important information regarding the prevalence of bacterial diarrheal pathogens associated to diarrhea and the type of bacterial contaminants of drinkable water and food products. This information may facilitate epidemiologic surveillance and public health measures leading to improved prevention measures, and ultimately decrease infant mortality due to infectious diarrhea. The methods may potentially be used for diagnosis and better medical management of children with severe infectious diarrhea. Technology transfer will be necessary to provide formal training on the use, processing, and interpretation of the rapid PCR test to local health authorities in developing countries.

In the present manuscript, we show that a rapid and affordable PCR-based method may be used for the identification of the most common bacteria associated with diarrhea in developing countries, including the 6 pathotypes of *E. coli* and the 5 most common non-*E. coli* enteropathogens, including *Shigella* spp., *Salmonella* spp., *Y. enterocolitica*, *Campylobacter* spp., and *V. cholerae*. The PCR assay allowed the specific detection of each bacterial strain, except *Shigella* spp. and EIEC, which were differentiated based on the lactose fermentation phenotype, which was only expressed in the *E. coli* strains tested. The lactose fermentation prescreening of bacterial samples may decrease the number of assays required per strain because lactose fermenter strains may only be tested with reaction 1 and 2 for detection of *E. coli* pathotypes, whereas lactose nonfermenter isolates may be tested only with PCR reaction 3 for detection of *Shigella* spp., *Salmonella* spp., *Y. enterocolitica*, *Campylobacter* spp., and *V. cholerae* (Fig. 1). All *E. coli* strains tested in our assay were lactose fermenters, and all non-*E. coli* isolates, except *Klebsiella* spp. and *Campylobacter* spp., were lactose nonfermenters. Although lactose-nonfermenting *E. coli* strains have been reported in some geographic areas (Colonna et al., 1992), a decision for testing lactose nonfermenter strains with all 3-sample PCR assay should be made if local epidemiologic information suggest the presence of these uncommon *E. coli* strains.

The assay is also capable to detect atypical EPEC strains, which carry the locus for *E. coli* effacement and lack bundle-forming pilus. These strains may be recognized by the presence of the *eae* gene and the absence of the *bfpA* gene. This

pattern of detection is important because atypical EPEC strains are considered emerging pathogens in several developing countries as well as in Europe (Alikhani et al., 2006; Jenkins et al., 2006; Moreno et al., 2008; Wani et al., 2006).

To our knowledge, this is the 1st description of a PCR-based method used for detection of 11 *E. coli* and non-*E. coli* enteropathogens in a single assay. Prior reports on multiplex PCR assays have concentrated on either identification of *E. coli* pathotypes (Brandal et al., 2007; Matar et al., 2002; Nguyen et al., 2005) or non-*E. coli* strains (Gubala, 2006; Ibrahim et al., 1997; Park et al., 2006). Our assays have modified the PCR methodology from a single PCR reaction to a 3-sample reaction. This method was used to increase the specificity of the reaction, because this may decrease when more than 5 pairs of primers are used in a single reaction (Watterworth et al., 2005). The assay is sensitive, as demonstrated by the level of detection to almost single molecule. It is specific, because multiple PCR primers were specific for each target, and no cross-reactivity was detected with heterologous DNA. Specificity was also conserved within a significant template concentration range. Validation of the PCR method confirmed that approximately 100 strains previously characterized were correctly identified by the 3-sample PCR method. The assay is rapid, as in 4 h, it is possible to isolate crude DNA preparations from a bacterial suspension, amplify the DNA, separate the DNA fragments in an agarose gel, and have a visual report of the banding pattern.

The PCR assay has been simplified to facilitate its use in limited settings. When testing pure bacterial isolates, the assay can use bacterial suspensions or crude DNA preparations rather than purified genomic DNA. It includes premade reactions already containing all reagents necessary for the PCR except the template. It also includes plasmid controls to obviate the need to have −70 °C freezing conditions for bacterial storage, not feasible in many areas with limited resources. After amplification, samples are separated in agarose gel electrophoresis, DNA bands visualized under UV light, and images recorded by a conventional digital camera. Although ethidium bromide is a relatively inexpensive reagent for DNA gel staining, it has human and environmental safety concerns. Ethidium bromide is a toxic and carcinogenic compound that requires specific disposal condition and decontamination protocols. Therefore, strict safety regulations must be followed or alternative DNA gel staining reagents should be advised to avoid contamination in the work place or the environment.

The minimal requirement for the assay's implementation include access to electricity, water, and refrigeration (−10 to 4 °C). These conditions are met in most hospitals and reference laboratories in developing nations. The assay is also flexible with respect to the number of primer pairs and type of pathogens to identify. Detection of *V. cholerae*, for instance, may be more relevant for Africa and Asia than for Latin America. Furthermore, the assay may potentially identify enteropathogens from environmental sources, clinical isolates, or stool samples. In collaboration with Latin-American research institutions, we have been successful in implementing the system in Cartagena and Bogotá, Colombia, and have found that ETEC is the most prevalent pathogen associated with diarrhea in children younger than 5 years. Enterotoxigenic *E. coli* was also identified as the most common contaminant of food products obtained from public markets in several Colombian cities (our unpublished results).

We believe that the multiplex PCR assay may provide valuable information on the prevalence of bacterial pathogens associated with diarrhea in developing nations. Similarly, it may facilitate epidemiologic surveillance of water and edible products for human consumption to determine the risk of acute diarrheal disease at any single time in any densely populated geographic region. Implementation of this assay may not only improve epidemiologic information on the dimension of childhood diarrhea in the developing world, but more importantly, it may also facilitate the implementation of preventive measures to decrease morbidity and mortality due to bacterial diarrhea in children living in the developing world.

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