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Design and standardization of four multiplex polymerase chain reactions to detect bacteria that cause gastrointestinal diseases

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Gastrointestinal diseases and diarrhea are the major cause of morbidity and mortality in children in the developing world, including Mexico. In order to diagnose acute diarrhea and dysentery, four-multiplex polymerase chain reaction (mPCR) sets were designed and standardized in this study. Main virulence genes of relevant enteropathogens, that is, *Escherichia coli* pathotypes, *Shigella* spp., *Salmonella enterica*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Aeromonas* spp., were detected. Sixteen primer pairs were designed using extensive *in silico* analysis. As predicted *in vitro*, specific amplicons were obtained and four multiplex PCR sets were standardized. Our sixteen primer pairs and four multiplex PCR results demonstrate the simultaneous amplification of different pathogens according to the type of diarrhea in the same reaction. In the future, these methods might be used as rapid, sensitive and specific epidemiological and diagnostic tools.

Key words: Gastrointestinal diseases, diarrhea, multiplex polymerase chain reaction, design, standardization, bioinformatics.

INTRODUCTION

Diarrhea occurs worldwide and is responsible for 4% of all deaths. It is most commonly caused by gastrointestinal infections that kill around 2.2 million people globally each year, mostly children in developing countries according to the World Health Organization (WHO, 2013).

In Mexico, gastrointestinal diseases are the main cause of medical consultation and death, so they are considered a public health problem. The most common bacterial strains associated with diarrhea include *Escherichia coli*, *Salmonella* spp., Shigella spp., *Yersinia enterocolitica*, *Vibrio cholerae* and *Campylobacter* spp. (Gómez et al., 2009). Moreover, in Mexican clinical laboratories, diagnosis, identification and research focus mainly on classical pathogens, such as *Salmonella*, *Shigella* and *Escherichia*, so it is important to search for other underestimated relevant pathogens, such as *Campylobacter*, *Yersinia* and

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Aeromonas. Diagnosis of diarrhea can be achieved by diverse methods, such as culture on selective and differential media, biochemical tests, serological methods, trading systems and molecular techniques like polymerase chain reaction (PCR). Multiplex polymerase chain reaction (mPCR) is a PCR variant assay to detect in a single-tube reaction, the presence of three or more genes by amplification, using more than one pair of primers in the same reaction (Markoulatos et al., 2002; Henegariu et al., 1997; Shuber et al., 1995). Also, this molecular technique approach will be useful as a rapid and effective routine diagnostic tool for detection of major pathogens causing diarrhea in less than 24 h in contrast with traditional culture technique. The cost to perform this mPCR may be high for some regions, but with the application of the diagnostic method and epidemiology tool in many samples, this cost will be reduced.

When more than a single target is to be amplified from the template, it is necessary to adapt PCR conditions to avoid interferences and the primers should be carefully designed to avoid formation of primer dimmers and to minimize such non-specific interactions. Primer design becomes a critical step in the PCR method to ensure specific and efficient amplification of a target sequence. PCR primer design includes analyzing several physico-chemical parameters, homology of primers with the target nucleic acid sequence (complementarily), primers length and guanine-cytosine content (% GC) (Kalendar et al., 2011).

In the field of infectious diseases, mPCR has been shown to be a valuable tool not only for the identification of diverse microorganisms, but also for epidemiological purposes (Markoulatos et al., 2002).

Many mPCR techniques have been developed around the world; most of them focused on the identification of the main diarrheagenic *E. coli* (Tobias and Vutukuru, 2012; Taniuchi et al., 2012; Antikainen et al., 2009; Müller et al., 2007; Aranda et al., 2004; Toma et al., 2003). Only one study was found in the literature dealing with detection of *E. coli*, *Salmonella* spp., *Shigella* spp., *Y. enterocolitica*, *V. cholerae* and *Campylobacter* spp., by three multiplex PCR (Gómez et al., 2009). Other multiplex PCR approaches using real time cycles have been used for the detection of enteric pathogens but nor include all the important pathogens in our country (Liu et al., 2012; Wiemer et al., 2011).

In Mexico, few studies have been performed for the detection of enteropathogens, among them is the study by López-Saucedo et al. (2003) which standardized a multiplex PCR for the detection of diarrheagenic *E. coli*. Additional mPCRs have been standardized, for example, for the detection of *Aeromonas* virulence genes (Aguilera-Arreola et al., 2011) as well as an mPCR for the detection of three plasmid-borne EAEC strains (Cerna et al., 2003). However, none of them allows performing the simultaneous detection of other bacterial genera, such as *Yersinia*, *Campylobacter*, *Shigella, Salmonella* and *Aeromonas.*

Although, the simultaneous amplification of many targets reduces the number of reactions that need to be performed, the design of multiplex PCR assays can be difficult because it involves extensive computational analyses of primer pairs for cross interactions (Kalendar et al., 2011). Fortunately, several programs have been developed in order to automatically design primers.

The aim of this study was to design *in silico* four-mPCR sets and standardized to detect the main enteropathogens to establish the method as a new identification and epidemiological tool in our country.

MATERIALS AND METHODS

Multiplex PCR primers design (*in silico analysis*)

A literature review was performed to collect primer sequences previously published and addressed at virulence genes of the main enteropathogens: *E. coli* pathotypes (*stx1*, *stx2*, *eae, bfpA*, *aap, aggR, lt* and *ipaH), Shigella* spp. (*ipaH, set1A* and *set1B*), *Salmonella enterica* (*spvC* and *invA*), *Y. enterocolitica* (*ail* and *yst*), *Campylobacter jejuni* (*hipO*) and *Aeromonas* spp. (*gcat*). The PCR was divided according to the type of diarrhea produced by each microorganism in four mPCR sets: dysentery (mPCR1-B), acute (mPCR2-B/NB, mPCR3-B/NB) and acute non-bloody (mPCR4-NB) diarrhea (Table 1).

All downloaded primer sequences were analyzed with the Genosensor Probe Designer (GPD) version 1.0a and WinOligo version 1.0 softwares (both developed *in house* by Méndez-Tenorio A from the Escuela Nacional de Ciencias Biológicas). The primer sequences were evaluated with GPD, software to determine physic-chemical parameters (length, guanine-cytosine content [%GC], melting temperature [Tm]) and search for repeats on the 3' end terminal. Whereas, WinOligo software was used to evaluate structural properties, such as hairpins and dimmers.

When the *in silico* analysis was finished, all primers were modified until they reached improvement (Table 2) to avoid future unspecific interactions during *in vitro* mPCR assays. All sequences that could not be improved were designed *de novo* by using the same software.

Bacterial strain (*in vitro analysis*)

For *in vitro* analysis, the reference and type strains used to standardize the four mPCR sets were enterohemorrhagic *E. coli* (EHEC) O157:H7 CECT 4076, enteroaggregative *E. coli* (EAEC) 042:II, enteroinvasive *E. coli* (EIEC) 1124, enteropathogenic *E. coli* (EPEC) E2348, enterotoxigenic *E. coli* (ETEC) H10407, *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, serovar Typhi ATCC 6539 and serovar Choleraesuis ATCC 10708, *Aeromonas hydrophila* ATCC 7966^T, *Shigella flexneri* ATCC 9199, *C. jejuni* ATCC 33250, *Y. enterocolitica* ATCC 23715, *Shigella sonnei, Shigella boydii* and *Shigella dysenteriae* from CDC (Centers for Disease Control and Prevention), and *C. jejuni* ATCC 33250. All strains were previously identified phenotypically by specific biochemical tests and genetically by sequencing *16S rRNA* gene as previously described (Aguilera-Arreola et al., 2005).

Table 1. Multiplex PCR classification in this study according to the type of diarrhea.

Designation	Type of diarrhea	Bacteria	Target detection (gene)
mPCR1-B	Bloody (Dysentery)	*Shigella* spp./EIEC	*ipaH/set1A/set1B*
		EHEC	*stx1/stx2*
mPCR2-B/NB	Bloody/Non Bloody (Acute)	*Salmonella enterica*	*invA/spvC*
		Yersinia enterocolitica	*yst/ail*
		EAEC	*aap/aggR*
mPCR3-B/NB	Bloody/Non Bloody (Acute)	*Aeromonas* spp.	*gcat*
		Campylobacter jejuni	*hipO*
mPCR4-NB	Non Bloody (Acute)	EPEC	*bfpA/eae*
		ETEC	*lt*

B = Bloody, NB = non bloody, mPCR = multiplex polymerase chain reaction. EIEC = enteroinvasive *E. coli*. EHEC = Enterohemorrhagic *E. coli*. EAEC = Enteroaggregative *E. coli*. EPEC= Enteropathogenic *E. coli*. ETEC= Enterotoxigenic *E. coli*.

Stock cultures of each strain were maintained for short periods at room temperature on blood agar base slants and for longer storage, they were either frozen at -70°C in 20% (w/v) glycerol –Todd – Hewitt broth (Oxoid, Mexico) or lyophilised in 7.5% horse glucose serum as a cryoprotector (Aguilera-Arreola et al., 2005).

Genomic DNA extraction

Genomic DNA was extracted by using Kit InstaGene™ Matrix (BioRad, Mexico) according to manufacturer´s instruction. DNA purity and quantity were determined by using the Ampli Quant AQ-07 spectrophotometer. DNA was stored at -20°C until use.

Multiplex PCR amplification (*in vitro analysis*)

To standardize mPCR, the protocol established by Henegariu et al. (1997) was followed. Initially, single PCR reactions were performed by using 50 µl of a reaction mixture consisting of 1X PCR buffer (Invitrogen, Mexico), 1.8 mM $MgCl_2$ (Invitrogen, Mexico), 200 µM deoxynucleoside triphosphates (dNTP´s) (AMRESCO, Mexico), 0.10 µM of the respective primers (Invitrogen, Mexico), 2.5 U of *Taq* DNA polymerase (Invitrogen, Mexico) and 200 ng of DNA. The single PCR program used for amplification consisted of 5 min at 94°C of denaturing temperature, followed by 35 cycles of 1 min at 94°C of denaturing temperature, 1 min at 55°C of annealing temperature, and 1 min at 72°C of extension temperature, at the end of the 35 cycles, a 10-min extension at 72°C was used. In a second phase, the four mPCR sets were standardized, performing PCR temperature gradients (45 to 65°C) and variations in concentrations of $MgCl_2$ (1.5 to 3.5 mM), primers (0.1 to 0.3 µM) and of DNA quantity (30 to 500 ng/µl of each strain). PCR product fragments were analyzed in 2% w/v agarose gel at 80 V for 2.5 h. A 100-bp ladder DNA molecular marker or 1 Kb Plus ladder DNA molecular marker were included in each electrophoresis (Invitrogen, Mexico). The gels were stained with ethidium bromide solution (0.5 µg/ml), visualized on a UV light transilluminator, photographed and digitalized (Stratagene Eagle Eye Frame Integrator, USA).

Amplicon sequencing

The products of simultaneous PCR reactions of each gene were confirmed via DNA sequencing on an ABI-PRISM 310 (Applied Biosystems, Foster City, CA) following the standard methodology outlined by the manufacturer. As in the single PCR assay, each reaction was performed in duplicate. Sequencing was performed at the Instituto de Biología*, UNAM* (Mexico). Analysis of the obtained sequence was performed with the reference genomes deposited in the NCBI (refseq_genomics) databases, employing the Megablast program of the BLAST platform.

RESULTS

Multiplex PCR primers design (*in silico analysis*)

PCR primers were designed for use in mPCR with the GPD program, evaluating physicochemical properties, such as melting temperature (Tm), which did not vary by more than 2°C between the forward and reverse primers, length between 16 to 22 bp, guanine-cytosine content (%GC) between 29 and 50%, not more than 2 repeats, and the amplicon size was between 130 and 779 bp, as shown in Table 2. Structural properties were evaluated with the WinOligo program, determining that primers were neither dimers nor hairpins. ΔG° values were shown to be lower in the 5' end than in the 3' end (values not shown). Table 3 shows modifications between original and new primers sequences to be used in the multiplex PCR standardized in this work.

Multiplex PCR amplification (*in vitro analysis*)

First, single PCR was optimized (Figure 1A). The four mPCR were divided according to the type of diarrhea produced by each enteropathogen. Initially, equimolar primer concentrations of 0.1 to 0.5 µM each were used in the mPCR, the final concentration of the primers was 0.3 µM. Different concentrations of $MgCl_2$ were tested (1.5 to 4 mM) and the optimal concentration was 2.8 mM, dNTP´s

Table 2. Results of the *in silico* study, new primer sequences for the multiplex PCR.

Bacteria	Gene	Primer sequence		Tm (°C)		%GC		Repeats		Length (bp)		Amplicon size(bp)
		Forward (5→3)	Reverse (5→3)	F	R	F	R	F	R	F	R	
ETEC	*lt*	LTF: ATGGCGACAAATTATACC	LTR: ACCGGTCTCTATATTCC	62.99	62.93	39	47	0	2	18	17	426
EPEC	*bfpA*	BFPF: CTGTCTTTGATTGAATCTG	BFPR: TAAGCGTCAGATAGTAAC	61.26	61.16	37	39	2	2	19	18	306
	eae	EAEF: GTGCCGAACCTAAAAG	EAER: GCACAAGCATAAGCTA	63.02	64.06	50	44	2	2	16	16	343
EHEC	*stx1*	STX1F: AGCAGTCATTACATAAGAAC	STX1R: TGGATTTAATGTCGCATAG	62.75	62.42	35	37	0	2	20	19	144
	stx2	STX2F: TCCTGTGTATACGATGA	STX2R: CCAGTTATCTGACATTCT	62.39	61.43	41	39	2	2	17	18	217
EAEC	*aap*	AAPF: GCATCTTGGGTATCAG	AAPR: TTCGGTTAGAGCACTA	62.76	62.90	50	44	2	0	16	16	292
	aggR	AGGRF: CGATGTATACACAAAAGAAG	AGGRR: AGTCCATCTCTTTGATAAG	61.20	61.29	35	37	2	2	20	19	422
EIEC/*Shigella* spp.	*ipaH*	IPAHF: ATGGCTGGAAAAACTC	IPAHR: AACCAGTCCGTAAATTC	63.26	62.62	44	41	2	2	16	17	413
Shigella spp.	*set1A*	SHET1F: CGCTACCATCAAAGATTA	SHET1R: CCGACGATTGGAAATATATC	62.23	63.03	39	40	0	2	18	20	304
	set1B	SHET2F: AAGAAGAAAAGGCTATTAATG	SHET2R:AGCAAATTTTTCAGCTTATTG	61.53	62.72	32	29	2	2	22	21	779
C. jejuni	*hipO*	HIPOF: TGCTAAGGCAATGATAG	HIPOR: CTAGCTTCGCATAATAACT	62.11	62.12	41	37	0	2	17	19	697
Y. enterocolitica	*yst*	YSTF: AATGCTGTCTTCATTTG	YSTR: CAATCCCAATCACTACT	61.13	61.60	35	41	2	2	17	17	130
	ail	AILF: GCCTGTTTATCAATTGC	AILR: ATCGAGTTTGGAGTATTC	62.40	61.83	41	39	0	2	17	18	439
S. enterica	*invA*	INVAF: TGGTACTGATCGATAATG	INVAR: TACGACCTGAATTACTG	61.22	61.25	39	41	2	2	17	18	207
	spvC	SPVCF: CTTGCACAACCAAATG	SPVCR: CTGTCTCTGCATTTCA	62.80	61.60	44	44	2	2	16	16	567
Aeromonas spp.	*gcat*	GCATF: TGGAATCCCAAGTATCA	GCATR: GTTGAACAGCAGTATCT	63.09	62.27	41	41	2	2	17	17	211

EIEC = Enteroinvasive *E.coli*, EHEC= Enterohemorrhagic *E. coli*, EAEC = Enteroaggregative *E. coli*, EPEC = Enteropathogen *E.coli*, ETEC= Enterotoxigenic *E. coli*, %GC = Guanine-cytosine content, bp = base pairs, F = forward, R = reverse, Tm = melting temperature.

and *Taq* DNA polymerase concentrations were not modified (200 µM and 2.5 U/µL, res-pectively), different amounts of DNA template were tested and concentrations of 10 to 500 ng/µl were employed, the final amount was 2.5 µl of each DNA for the mPCR1-B, mPCR2-B/NB and mPCR4-NB; and 2 µl of each DNA for the mPCR3-B/NB.

We performed a PCR temperature gradient, from 45 to 60°C to determine optimal alignment temperature, for mPCR1-B and mPCR2-B/NB it was 55°C; and for mPCR3-B/NB and mPCR4-NB it was 50°C, as shown in Figure 1B. The conditions obtained in the four standardized mPCR were 5 µl of 10X PCR buffer, 2.8 mM $MgCl_2$, 200 mM dNTP, 0.3 µM of each primers F and R, 2.5 U *Taq* DNA polymerase, 5 µl DNA template for mPCR1-B, mPCR2-B/NB and mPCR4-NB, and 6 µl DNA template for mPCR3-B/NB, the concentrations of DNA ranged between 10 and 200 ng/µl. The sizes of the PCR amplified products were equal to those predicted by the *in silico* study.

Amplicon sequencing

We obtained an expectation value (E) near zero and DNA sequence analysis (DNA blast search) of the PCR product of each of the tested primer pairs showed high identity levels ranging from 95 to 100% to the Gen-Bank sequence database, confirming the specificity of the primers.

DISCUSSION

The prevalence of diverse enteropathogens has not been investigated previously in cases of non-bloody diarrhea; the prevalence is high but quite often the implicated microorganism is not known. The Epidemiology Bulletin of Mexico City only reports the number of cases of digestive tract

Table 3. Comparison between original and new primer sequences.

Microorganism	Gene	Original primer sequence (5'→3')	References	New primer sequence (5'→3')
ETEC	*lt*	F: GGCGACAGATTATACCGTGC	López-Saucedo et al., 2003	ATGGCGACAAATTATACC
		R: CGGTCTCTATATTCCCTGTT		ACCGGTCTCTATATTCC
	bfpA	F: AATGGTGCTTGCGCTTGCTGC		CTGTCTTTGATTGAATCTG
		R: GCCGCTTTATCCAACCTGGTA		TAAGCGTCAGATAGTAAC
EPEC				
	eae	F: GACCCGGCACAAGCATAAGC		GTGCCGAACCTAAAAG
		R: CCACCTGCAGCAACAAGAGG		GCACAAGCATAAGCTA
EHEC	*stx*1	F: CTGGATTTAATGTCGCATAGTG		AGCAGTCATTACATAAGAAC
		R: AGAACGCCCACTGAGATCATC		TGGATTTAATGTCGCATAG
	stx2	F: GGCACTGTCTGAAACTGCTCC		TCCTGTGTATACGATGA
		R: TCGCCAGTTATCTGACATTCTG		CCAGTTATCTGACATTCT
EAEC	*aap*	F: CTTGGGTATCAGCCTGAATG	Cerna et al., 2003	GCATCTTGGGTATCAG
		R: AACCCATTCGGTTAGAGCAC		TTCGGTTAGAGCACTA
	aggR	F: CTAATTGTACAATCGATGTA		CGATGTATACACAAAAGAAG
		R: AGAGTCCATCTCTTTGATAAG		AGTCCATCTCTTTGATAAG
EIEC/*Shigella* spp.	*ipaH*	F: TGGAAAAACTCAGTGCCTCT	Lin et al., 2005	ATGGCTGGAAAAACTC
		R: CCAGTCCGTAAATTCATTCT		AACCAGTCCGTAAATTC
***Shigella* spp.**	*set1A*	F: TCA CGC TAC CAT CAAAGA		CGCTACCATCAAAGATTA
		R: TAT CCC CCT TTG GTGGTA		CCGACGATTGGAAATATATC
	set1B	F: GTG AAC CTG CTG CCGATA TC		GAAGAAGAAAAGGCTATTAATG
		R: ATT TGT GGA TAA AAATGA CG		AGCAAATTTTTCAGCTTATTG
Campylobacter jejuni	*hipO*	F: GAAGAGGGTTTGGGTGGTG	Al et al., 2007	TGCTAAGGCAATGATAG
		R: AGCTAGCTTCGCATAATAACTTG		CTAGCTTCGCATAATAACT
Yersinia enterocolitica	*yst*	F: AATGCTGTCTTCATTTGGAGC	Thisted and Danielsson-Tham, 2005	AATGCTGTCTTCATTTG
		R: ATCCCAATCACTACTGACTTC		CAATCCCAATCACTACT
	ail	F: GTTTATCAATTGCGTCTGTTAATGTGTACG		GCCTGTTTATCAATTGC
		R: CTATCGAGTTTGGAGTATTCATATGAAGCG		ATCGAGTTTGGAGTATTC

Table 3. Contd.

Salmonella enterica	*spv*C	F: ACTCCTTGCACAACCAAATGCGGA R: TGTCTTCTGCATTTCGCCACCATCA	Cheng and Ou, 1996	CTTGCACAACCAAATG CTGTCTCTGCATTTCA
	invA	F: ACAGTGCTCGTTTACGACCTGAAT R: AGACGACTGGTACTGATCGATAAT		TGGTACTGATCGATAATG TACGACCTGAATTACTG
***eromonas* spp.**	*gcat*	F: CTCCTGGAATCCCAAGTATCAG R: GGCAGGTTGAACAGCAGTATCT	Chacón et al., 2003	TGGAATCCCAAGTATCA GTTGAACAGCAGTATCT

Blue color shows the differences between original and new primers, red colour shows nucleotides that are equal, and green color shows primer sequences that were reported in opposite sequences in the literature.

diseases, classified as shigellosis, typhoid, paratyphoid, *Salmonella*-induced, cholera, bacterial food poisoning, intestinal infection by other and ill-defined organisms, and intestinal infectious diseases without specifying the organism involved Centro Nacional de Vigilancia Epidemiológica y Control de Enfermedades (CENAVECE, 2013). We decided to search for *Shigella*, *Salmonella* and five pathotypes of *E. coli* because these continue to be a major health problem worldwide, occurring predominantly in children younger than five years of age in developing countries, and are the cause of persistent diarrhea in children and adult travelers in developing countries. *Campylobacter* and *Yersinia* are not routinely searched for; therefore, the actual incidence of these pathogens is unknown. It was also decided to include *Aeromonas*, because some species have a broad host spectrum, causing intestinal and extra-intestinal infections in humans. Besides, in Mexico, it has been shown in the last fifteen years that *Aeromonas* represent an important group of pathogens involved in diarrhea (Castro-Escarpulli et al., 2003; Aguilera-Arreola et al., 2005; Aguilera-Arreola et al., 2011).

Therefore, to investigate the epidemiology and etiology of bloody diarrhea and non-bloody diarrhea and the prevalence of diverse bacteria that cause gastrointestinal diseases in Mexico, it is necessary to establish new methods of identification, such as molecular biology techniques (mainly PCR and its variants, and multiplex PCR [mPCR]), allowing for a timely diagnosis and fast, sensitive, and economical identification of the microorganisms for epidemiological purposes.

In this paper, we designed and standardized four mPCR sets to detect the main enteropathogens (five pathotypes of *E. coli*, *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter* and *Aeromonas*). In addition, the new PCR, here proposed, was designed according to the type of diarrhea caused by the bacteria under study (Table 1). The advantages of these four mPCR standardized are their ability to detect bacterial genera for which no search is performed routinely, such as *Yersinia*, *Aeromonas* and *Campylobacter*. In addition, this methodology would facilitate the diagnosis at the time of observing the clinical, food and water samples.

It is important to optimize each mPCR proposed because it can pose several difficulties, including poor sensitivity and specificity in the amplification of certain specific targets, the presence of more than one primer pair in the mPCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers. The alteration of other PCR components, such as PCR buffer constituents, dNTP´s, $MgCl_2$ and enzyme concentrations in mPCR, is greater than that reported for most single PCR and usually results in considerable improvement in the sensitivity and/or specificity of the test (Markoulatos et al., 2002).

Primer design is one of the key steps for successful PCR. Virulence genes were selected from the referenced literature and sequences of the original primers (Table 3) were used to design or adapt improvements to be used in an mPCR with the GPD program. The values obtained for length, Tm, %GC, repeats and end stability correspond to those reported in the literature for mPCR applications (Henegariu et al., 1997; Markoulatos et al., 2002; Kalendar et al., 2011). Primer dimers involving one or two sequences may occur in a PCR reaction, but with the WinOligo

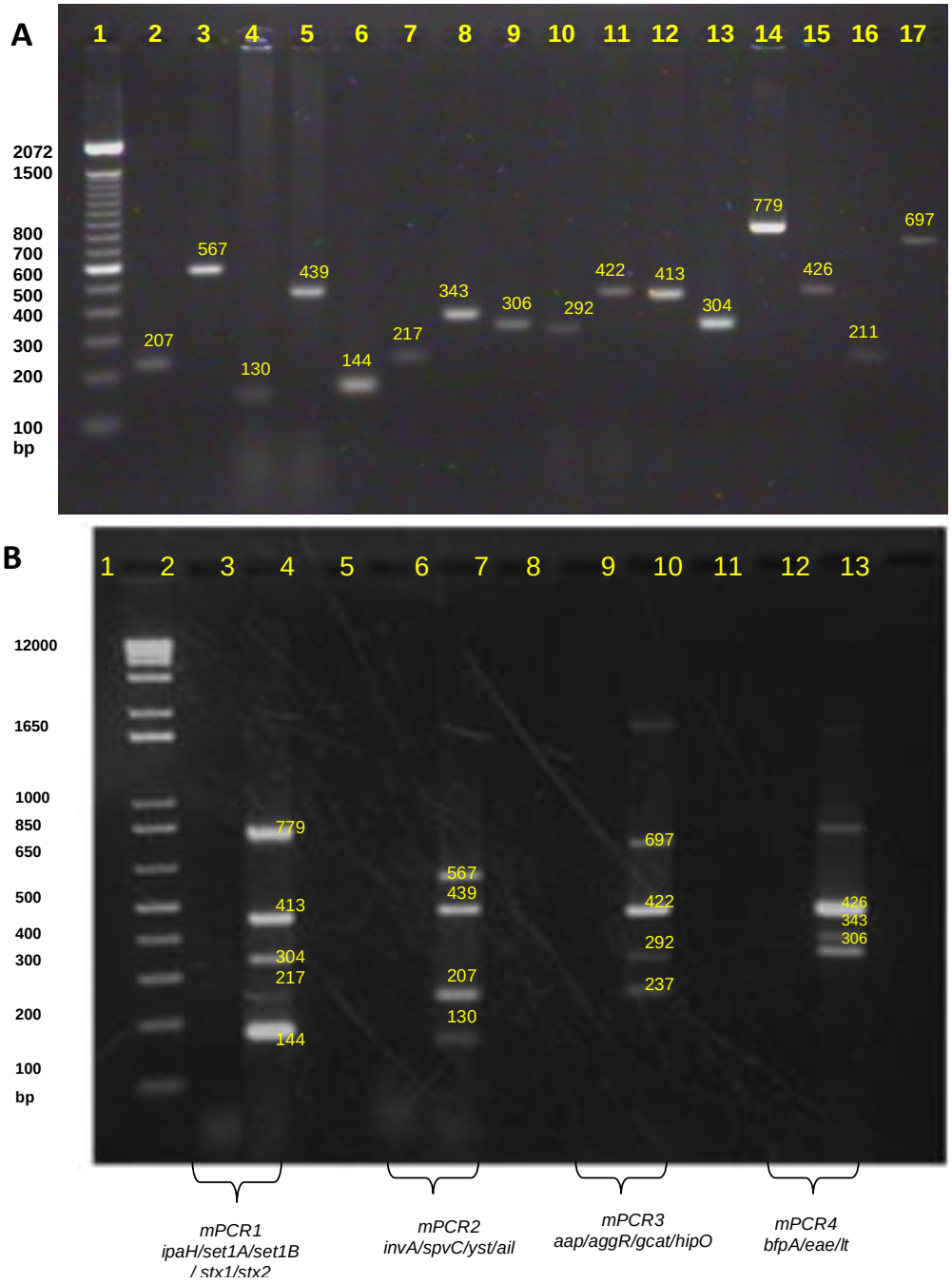

Figure 1. Electropherograms of the PCR products obtained from (A) single and (B) multiplex PCR for all enteropathogens studied.A) Single PCR Lanes: 1, 100-bp ladder DNA molecular marker (100 to 2072 bp) (Invitrogen, Mexico); 2, 3, *S. enterica Typhimurium ATCC 14028* (*spv*C and *inv*A); 4, 5, *Y. enterocolitica* ATCC 23715 (*ail* and *yst*); 6, 7, EHEC O157:H7 CECT 4076(*stx*1 and *stx*2); 8, 9, EPEC E2348 (*eae* and *bfp*A); 10, 11, EAEC 042II (*aap* and *agg*R); 12, *S. flexneri* ATCC 9199/EIEC 1124 (*ipa*H); 13, 14, *S. flexneri* ATCC 9199 (*set*1A and *set*1B); 15, ETEC H10407 (*lt*); 16, *A. hydrophila* ATCC 7966 (*gcat*); 17, *C. jejuni* ATCC 33250 (*hip*O). B) Multiplex PCR Lanes: 1, 1 Kb Plus ladder DNA molecular marker (100-12,000 bp) (Invitrogen, Mexico); 2, 4, 5, 7, 8, 10, 11 and 13, Negative controls; 3, mPCR1-B EHEC/*Shigella* spp.; 6, mPCR2-B/NB *Salmonella*/*Y. enterocolitica*; 9, mPCR3-B/NB EAEC/*A.hydrophila*/*C. jejuni*; 12, mPCR4-NB EPEC/ETEC.

program, no primer secondary structures (hairpin and dimers) formation was revealed. The programs developed in our institution allowed us to design mPCR primers with desirable characteristics to be used in a multiplex PCR. Currently, we are developing the platform to make available to users the corresponding programs for single or mPCR design, which is simple and easy.

Once the primers were designed, we proceeded to follow the protocol proposed by Henegariu et al. (1997), the distribution shown in Table 1 was performed thinking it might facilitate diagnoses as well as the identification of the microorganism by observing the sample type and looking for the bacteria mainly involved. This shows that *in vitro* study (*in silico*) is very important, because the primer design is a critical step in all types of PCR methods to ensure specificity and efficient amplification of a target sequence (Kalendar et al., 2011). In this case, the primers designed are able to yield the specific desirable amplicons, as predicted with the *in silico* study.

All the virulence genes were amplified, in the case of the different *Salmonella* serovars employed, the *invA* gene amplified in three serovars (Typhi, Choleraesuis and Typhimurium); the *spvC* gene only amplified in two serovars (Choleraesuis and Typhimurium). The *ipaH* gene amplified in the four species of *Shigella* and EIEC, but the sole presence of *ipaH* (plasmid gene) is not an absolute indicator of virulence, and *set1A* and *set1B* (chromosomal genes) can help in the identification of virulent strains (Lin et al., 2005). *set1A* and *set1B* amplified in *S. flexneri* (genes highly conserved in *S. flexneri* 2a) (Lin et al., 2005), but we also found that *set1B* amplified in *S. boydii* and EIEC.

After standardizing the mPCR sets, the specificity was evaluated by sequencing amplified PCR products and they were shown to be specific, after the bioinformatics study by using the Blast Platform. Moreover, primers designed for the specific detection of bacteria were used to amplify DNA from other bacteria, without obtaining amplicons of the desired size, proving again that the primers are specific. Sensitivity was evaluated using 10-fold dilutions of template DNA extracted from type strains and bacterial suspensions adjusted to 0.5 of the McFarland nephelometer corresponding to 1.5×10^8 CFU/ml (data not shown).

Currently, we are determining the sensitivity and specificity of the methods directly on clinical samples. Sensitivity of the mPCR sets is evaluated by using dilutions from the stool inoculated with type strains, according to the excreted bacterial concentrations (Feachem et al., 1983) and specificity is determined by observing the size of the bands and sequencing. Furthermore, we are using adjuvants to remove inhibitors that may be found in clinical samples. Optimization of the parameters by using clinical samples should provide a basic approach to some of the common problems of multiplex PCR, such as spurious amplification products, uneven or no amplification of some target sequences, and difficulties in reproducing some results. It is important to perform these assays because they are essential for the evaluation and validation of new multiplex PCR for application in the clinical area (Markoulatos et al., 2002).

As the type of diarrhea produced by each enteropathogen microorganism is known, we believe it is a good strategy to carry out the identification quickly, especially of bacteria whose search is not carried out routinely. We consider also that with this methodology we can determine the actual prevalence of the microorganisms under study, know if mixed infections exist, and identify the genera and species most prevalent in our population.

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