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Original research article

Design of an internal amplification control for a duplex PCR used in the detection of Shiga toxin producing *Escherichia coli* in pediatric feces

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ABSTRACT

A conventional PCR targeted directly to the detection of Shiga toxin-producing *Escherichia coli* (STEC) in diarrheal stools of symptomatic patients may require the introduction of internal controls to detect false negative results. In the present study, we designed a competitive internal amplification control (IAC) to be included in a well-known PCR protocol used to amplify the *stx1* and *stx2* genes from STEC isolates. The IAC was introduced in the PCR reaction and amplified when *E. coli* O157:H7 cultures and contaminated pediatric feces were assayed. When STEC concentration was 10^3 CFU ml^{-1} in pure culture and 10^4 CFU g^{-1} in contaminated stools, the IAC at concentration of 0.143 pg μl^{-1} in the PCR reaction mixture was co-amplified with the *stx2* sequence, producing bands of 279 and 349 bp, respectively. These STEC values were considered the detection limits of the duplex PCR. The specific detection of STEC by duplex PCR including IAC might be achieved directly on pediatric feces when the pathogen load reaches concentrations of at least 10^4 CFU g^{-1}.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a foodborne enteropathogen that causes bloody and non-bloody diarrhea, hemorrhagic colitis (HC), and life-threatening hemolytic uremic syndrome (HUS). The severity of STEC clinical manifestations is related to the production of one or more Shiga toxins (Stx1, Stx2 and Stx2 variants), but other virulence-associated factors such as intimin, enterohaemolysin, and an autoagglutinating adhesion factor may contribute to this pathogenesis [23]. Although the O157 serotype of *E. coli* has been frequently isolated from patients with HUS, several studies suggest that up to 50% of STEC illness is caused by non-O157 serotypes, of which there exist over a hundred [4]. In Argentina, STEC-HUS is endemic, with 300–500 new cases recorded per year, being responsible for 10–17 cases per 100,000 children under five-year-old [2,24].

Early detection of STEC in clinical samples is crucial for determining the most appropriate treatment as well as effective epidemiological control measures. Culture methods are widely used for detection and identification of STEC in human stools, but they may fail to discriminate Shiga-toxin producing strains from non-producing ones [8]. In contrast, laboratory molecular techniques can provide rapid and reliable results regarding the pathogenic potential of isolates, and therefore, contribute to the early implementation of adequate therapies [9]. Several polymerase chain reaction (PCR) strategies mainly targeted at virulence genes such as *stx1*, *stx2*, *eae*, *ehxA* [5] and the O-antigen encoding $rfbE_{O157:H7}$ gene [19] have been used to characterize STEC isolates [17,19,26] or to detect STEC directly on clinical stool samples [3,7,10,16]. In Argentina, reference microbiological laboratories use a conventional PCR method targeting $stx1/stx2/rfbE_{O157:H7}$ genes in order to

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characterize *E. coli* strains after isolation on Sorbitol Mac Conkey agar or CHROMagar O157 [17]. However, PCR may yield false negative results when applied to complex matrices due to the presence of substances such as heme, bilirubins, bile salts, and complex carbohydrates [13] that inhibit the amplification reaction [12]. False negative results in PCR can be attributed to expired reagents, poor techniques, or equipment failure [18,25]; therefore, the introduction of an internal amplification control (IAC) in the PCR reaction mix is mandatory [14,15,21].

An IAC is a non-target DNA sequence included in the same sample tube, which is co-amplified simultaneously with the target DNA sequence. Thus, in a reaction with an IAC, a control signal will always be produced, even though there may not be target sequences [14]. IACs can be used in competitive and noncompetitive reactions and the efficiency of each PCR reaction can be monitored in both cases. In competitive reactions, the target DNA and the IAC are co-amplified with one common set of primers under the same conditions and in the same tube. When the target DNA is in a proportionally greater amount than the IAC, the DNA bands can be observed but the IAC bands cannot. When neither of them are amplified, it means that inhibition of the PCR has occurred, making the result of this sample false [14]. In conventional PCR, IAC and target DNA products can be identified by their different molecular masses, which are detectable by agarose gel electrophoresis.

In order to the rapid detection of STEC in stool samples of pediatric patients in our region, this study aimed to (i) develop an overlapping PCR strategy in order to design a competitive IAC, (ii) include the designed IAC in a conventional *stx1* and *stx2* PCR protocol widely used in the identification and characterization of STEC isolates in Argentina, and (iii) determine the IAC performance in a duplex PCR applied to pediatric stools artificially contaminated with STEC.

2. Materials and methods

2.1. Bacterial strains

Two STEC strains were assayed in this study: *E. coli* O157:H7 *stx1*$^-$/*stx2*$^+$, a local strain isolated from stools of a 16-month-old girl, who had been admitted to a hospital in San Luis city, Argentina, and diagnosed with HUS, and the reference *E. coli* O157:H7 EDL933 Sor-/βglu-/E-Hly+, biotype C, *stx1*$^+$/*stx2*$^+$, *eae*$^+$ strain. Both strains were used to assess the detection limit of *stx1* and *stx2* genes by a duplex PCR from pure STEC cultures. Only the local STEC strain was used to contaminate the pediatric stools. Both strains produced non-sorbitol fermenting colonies on Sorbitol Mac Conkey agar (SMAC, Britania Lab, Buenos Aires, Argentina) and positive agglutination against O157 antiserum (National Institute of Infectious Diseases, Buenos Aires). The production of Shiga toxins was confirmed by cytotoxicity assays on Vero cells. *E. coli* ATCC 25922 was used as a negative control. Organisms were maintained at 4 °C on trypticase soy agar (TSA, Britania Lab) slants. Prior to each experiment, inocula were transferred to trypticase soy broth (TSB, Britania Lab) and incubated for 24 h at 37 °C.

2.2. Detection limit of target DNA by a duplex PCR from STEC cultures

One colony of each strain was picked from SMAC, inoculated in 50 ml of EC broth (Merck, Buenos Aires, Argentina) and incubated for 18 h at 37 °C. The inoculum concentration was standardized at OD_{600} 0.2 (Metrolab VD 40 Spectrophotometer, Lab. Rodriguez Corswant, Bernal, Argentina) and estimated by plating onto Plate Count Agar (PCA, Britania Lab) in $1.6 \times 10^8 \pm 3.3 \times 10^7$ CFU ml^{-1} (local strain), and $2.0 \times 10^8 \pm 1.5 \times 10^7$ CFU ml^{-1} (reference strain). Serial decimal dilutions of each strain (10^7 to 10^0) were prepared in sterile ultrapure water. These dilutions were used for DNA extraction and subsequently duplex PCR was performed. The detection limit was reported as colony forming units per milliliter of inoculum (CFU ml^{-1}) or bacterial number per 25 μl in the PCR microtube.

2.3. Detection limit of target DNA by a duplex PCR from STEC-contaminated stools

For each experiment, a pool of five STEC culture-negative stool samples collected from 3-month to 3-year-old children with diarrhea admitted at the local public hospital was used for preparing STEC contaminated samples. Briefly, stools were collected in sterile plastic containers and stored at 4 °C for up to 2 h before processing. Equal portions of each sample were placed into a sterile glass container and mixed with a sterile rod. One-gram amounts were subsequently distributed in sterile flasks before STEC inoculation.

One colony of the local *E. coli* O157:H7 strain was transferred from SMAC to EC broth and incubated for 18 h at 37 °C. The OD_{600} was 0.85–0.9 and the bacterial counts on PCA averaged $1 \times 10^9 \pm 3 \times 10^8$ CFU ml^{-1}. One ml-aliquots of undiluted and serially diluted bacterial suspensions (10^9, 10^7, 10^5, 10^4 and 10^3 CFU ml^{-1}) were added to the one-gram portions of stool samples, and each inoculated sample was submerged into nine milliliters of EC broth and incubated at 37 °C with orbital shaking at 50 rpm (Orbit shaker, Lab Line Instruments, Melrose Park, Ill., USA). One-ml aliquots of contaminated stool samples were removed for DNA extraction at 0, 3, 6, 18, and 24 h of incubation. DNA decimal dilutions were prepared in ultrapure distilled water. Then, undiluted and 10-fold diluted DNA suspensions were assayed by duplex PCR. The detection limit was reported as CFUs per gram of stools (CFU g^{-1}) or bacterial number per 25 μl in the PCR microtube.

2.4. Counts of STEC in artificially contaminated stools after 0, 3, 6, 18 and 24 h enrichments

For enumeration of STEC, one-ml aliquots of stools artificially contaminated with 10^9, 10^7, 10^5, 10^4 and 10^3 CFU ml^{-1} of STEC in EC broth were removed at 0, 3, 6, 18 and 24 h and serially 10-fold diluted. Volumes of 0.1 ml of each dilution were spread onto Rainbow Agar O157 plates (Biolog Inc., Hayward, CA, USA) and incubated at 37 °C for 24 h. Suspect black or grey colonies were selected for counting and subjected to Gram staining and classical biochemical tests for *E. coli*. Counts were reported as $\log_{10}$ CFU g^{-1} of stools.

2.5. Duplex PCR

The boiling method for DNA extraction and the PCR protocol proposed by Leotta et al. [17] with minor modifications were used in this study. Modifications consisted of the removal of the primer pair targeted to the O-antigen encoding $rfbE_{O157:H7}$ gene, thus turning a multiplex PCR into a duplex PCR. Primer pairs used were: Stx1a.

5′- GAAGAGTCCGTGGGATTACG -3′ and Stx1b 5′-AGCGATGCAGCTATTAATAA-3′ for *stx1* (130 bp amplicon) [20], and Stx2a 5′-TTAACCACACCCCACCGGGCAGT-3′ and Stx2b 5′-GCTCTGGATGCATCTCTGGT-3′ for *stx2* (349 bp amplicon) [28]. The duplex PCR was performed in a 25-μl final volume containing 2 μl of DNA template, 1x PCR buffer, 0.1 mM (each) deoxynucleoside triphosphates, 2 pmol μl^{-1} *stx1* primers, 0.4 pmol μl^{-1} *stx2* primers, 1.5 mM $MgCl_2$, 0.02 U μl^{-1} Taq polymerase (PBL, Quilmes, Argentina), and ultrapure water. DNA templates were heated at 94 °C for 5 min and subsequently amplified for 30 cycles, each

consisting of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 3 min (Techne TC-512 thermal cycler, Bibby Scientific US, Burlington, NJ, USA). PCR products were detected by electrophoresis in 2% agarose gel stained with Gel Red Acid Gel Stain® (Biotium, Hayward, CA, USA). A 100 bp DNA ladder (PBL) was included as a molecular mass reference.

2.6. Construction of an internal amplification control (IAC) for duplex PCR

An overlapping PCR strategy based on the deletion of an internal 70 bp region from a 349 bp amplicon corresponding to the subunit A of the *stx2* gene in *E. coli* O157:H7 EDL933 (GenBank Access: FN182287.1, nucleotides 188–536) was used for the IAC construction (Fig. 1). The 349 bp amplicon was cloned into the pCR2.1 TOPO® vector (Invitrogen, Buenos Aires, Argentina) and used as template for the two following amplifications: PCR1, which amplified the 1–144 nt region using the primers Stx2a and StxΔ70Rev.

5′-tggtataactgAAGTATTTGTTGCCGTATTAACGAA-3′ (this work), and PCR2, which amplified the 215–349 nt region using primers Stx2b and StxΔ70For.

5′-caacaaatacttCAGTTATACCACTCTGCAACGT-3' (this work). Lowercase letters on the primer StxΔ70Rev (11 bases) indicate the complementary region to the 5′-end of the second half of *stx2* region starting at nucleotide 215 (red box in Fig. 1A), and the underlined capital letters in this primer correspond to the hybridization region to the 3′-end of the first half of *stx2* (blue box in Fig. 1A). Lowercase letters on the primer StxΔ70For (12 bases) indicate the complementary region to the 3′-end of the first half of *stx2* ending in nucleotide 144 (blue box in Fig. 1B), while the underlined capital letters in this primer correspond to the hybridization region to the 3′-end of the second half of *stx2* (red box in Fig. 1B). The 3'-ends of PCR1 and PCR2 products yielded a 23 nt overlapping region. The amplicons obtained in PCR1 and PCR2 were used as template in a third PCR round including the outer Stx2a and Stx2b primers to generate the 279 bp IAC (Fig. 1C). Cycling conditions in PCR1, PCR2 and PCR3 were those used in the duplex PCR except for the annealing temperature in PCR3, which was decreased to 53 °C. The IAC was cloned into the pCR2.1 TOPO® vector, quantified (Qubit® fluorometer, Invitrogen, USA) and used in the duplex PCR.

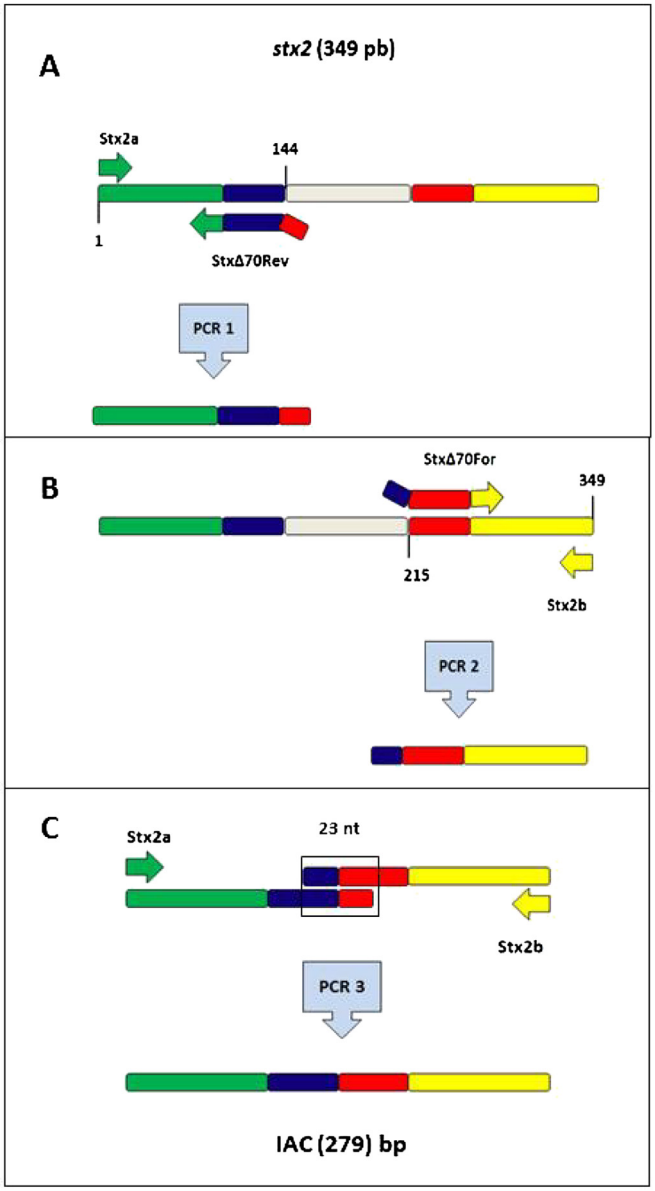

Fig. 1. Scheme of the overlapping PCR strategy employed for construction of IAC. A) PCR amplification of nucleotides 1–144 from the original 349 bp amplicon. B) PCR amplification of nucleotides 215–349 from the original 349 bp amplicon. C) Reverse primer from PCR 1 (A) and forward primer from PCR 2 (B) harbor complementary regions. When both amplicons are combined in the presence of outer primers, an IAC is obtained by deletion of a 70 bp fragment (nucleotides 145 to 214).

2.7. Detection limit of IAC

Serial dilutions ($1–10^{-8}$) of the pCR2.1-IAC vector were prepared in TE 1X buffer (10 mM Tris (Sigma Aldrich, St. Louis, MO, USA), 1 mM EDTA (Sigma), pH 8.0) and used as templates to assess the detection limit by duplex PCR using the primer pair Stx2a-Stx2b. The amplicons were electrophoresed and visualized as described above. The detection limit was reported as DNA pg μl^{-1} or DNA copy number/25 μl PCR microtube.

2.8. Inclusion of IAC in the reaction mix of duplex PCR

A reaction mixture was prepared containing all compounds for duplex PCR plus 2 μl of the IAC at the concentration corresponding to its detection limit. Two microliters of undiluted DNA extracted from the local and reference STEC strains cultured in EC broth or two microliters of undiluted or ten-fold diluted DNA from STEC-contaminated stools were added. The duplex PCR was performed according to the cycling program described above. All experiments were performed in triplicate on different days.

2.9. Statistical analysis

Mean values in counts from three replicates were subjected to analysis of variance by GraphPad Prism 5.0 software (GraphPad, San Diego, CA) to determine if significant differences ($P \leq 0.05$) in STEC populations existed between enrichment times.

3. Results

3.1. Counts of STEC in artificially contaminated stools after 0, 3, 6, 18 and 24 h enrichments

Pediatric stools were artificially contaminated with STEC, enriched in EC broth and analyzed by counting onto Rainbow Agar O157 at 0, 3, 6, 18 and 24 h enrichment. As Table 1 shows, STEC counts at 0 h reached 8.29 ± 0.12, 6.42 ± 0.23, and 4.95 ± 0.20

Table 1
STEC counts in artificially contaminated stools after EC-enrichment at different times.

STEC counts in contaminated stools (log_{10} CFU g^{-1} mean ± SD)[a]				
STEC concentrations used in stool contamination (CFU ml^{-1})				
Enrichment time (h)	10^9	10^7	10^5	10^4
0	$8.29^A \pm 0.12$	$6.42^A \pm 0.23$	$4.95^A \pm 0.20$	ND
3	$8.43^{AB} \pm 0.25$	$6.84^{AB} \pm 0.31$	$5.26^{AB} \pm 0.18$	ND
6	$8.66^B \pm 0.30$	$7.15^B \pm 0.26$	$5.47^B \pm 0.25$	ND
18	$9.79^C \pm 0.18$	$8.77^C \pm 0.50$	ND	ND
24	$9.35^D \pm 0.26$	$8.54^C \pm 0.42$	ND	ND

ND: no detected.
[a] Values followed by different capital letters indicate statistically significant differences ($p < 0.05$).

log_{10} CFU g^{-1} when samples were inoculated with 10^9, 10^7 and 10^5 CFU ml^{-1} of STEC suspensions, respectively. Significant increases from 1.55 to 2.35 log_{10} in 18-h STEC counts compared to 0-h STEC counts yielded 9.79 ± 0.18 and 8.77 ± 0.50 CFU g^{-1} in stools contaminated at levels of 10^9 and 10^7 CFU ml^{-1} ($p \leq 0.05$). At 24 h, the STEC counts in these samples decreased by 0.44 and 0.23 log_{10}, respectively. When the pathogen inoculum in stools was 10^5 CFU ml^{-1}, counts of 4.95 ± 0.20, 5.26 ± 0.18 and 5.47 ± 0.25 CFU g^{-1} were obtained at 0, 3 and 6 h enrichment; however, no STEC characteristic colonies were observed subsequently. STEC was not detected in stools inoculated with 10^4 CFU ml^{-1} at any enrichment time.

3.2. Detection limit of stx1 and stx2 from STEC cultures and STEC contaminated stools by using duplex PCR without IAC

The detection limits of target DNA extracted from *E. coli* O157:H7 cultures and contaminated stools were determined before including the IAC in the PCR reaction mixture. The detection limits of the *stx2* (349 bp) and *stx1* (130 bp) genes were 10^3 CFU ml^{-1} or 13 CFU/25 μl PCR microtube when DNA of both local (Fig. 2A) and reference STEC (Fig. 2B) strains in EC broth was assayed by duplex PCR.

By this technique, the *stx2* amplification was observed in undiluted DNA extracted from STEC contaminated stools at levels of 10^9 and 10^7 CFU ml^{-1} at 0, 3, 6, 18 and 24 h enrichment (Fig. 3). No *stx2* amplification was observed in undiluted DNA obtained from STEC contaminated stools at level of 10^5 CFU ml^{-1} at any time. However, when 10-fold diluted DNA from contaminated stools at 10^5, 10^4 and 10^3 CFU ml^{-1} was assayed, the STEC detection limit corresponded to 10^4 CFU ml^{-1} at 18 h (Fig. 3).

3.3. Determination of the IAC detection limit

Serial dilutions of IAC assayed by duplex PCR showed a detection limit of 0.143 pg μl^{-1} in the PCR reaction mixture, corresponding to 3.15×10^4 DNA copies/25 μl PCR reaction (Fig. 4).

3.4. Duplex PCR including IAC

The IAC and the target DNA were introduced in the same PCR reaction. DNA was extracted separately from local and reference STEC cultures which had previously been adjusted to concentrations of 10^4 to 10^1 CFU ml^{-1}. Co-amplification of IAC and *stx2* was observed when both local and reference STEC strains were assayed at 10^3 CFU ml^{-1} (Fig. 5A and B, respectively). At lower STEC concentrations, IAC was amplified but *stx2* was not. Unhindered amplification of the *stx1* gene (130 bp) for the reference STEC strain was observed at 10^4 CFU ml^{-1} (Fig. 5 B, lane 8). IAC was not amplified when the reference strain concentration in culture was 10^9 CFU ml^{-1}; yet, bands *stx1* and *stx2* were visible (Fig. 5B, lane 12).

Undiluted DNA extracted from STEC contaminated stools at levels of 10^5, 10^7 and 10^9 CFU ml^{-1} was assayed by duplex PCR including IAC (Fig. 6). The IAC amplification but no *stx2* amplification was observed at STEC levels of 10^5 CFU ml^{-1} in stools. IAC was co-amplified with *stx2* from stools contaminated at 10^7 CFU ml^{-1}, and *stx2* amplification but no IAC amplification was observed in 10^9 CFU ml^{-1} contaminated stools. When 10-fold diluted DNA from STEC contaminated stools at levels of 10^4, 10^3, 10^2, 10^1 and 10^0 CFU ml^{-1} was assayed, the STEC detection limit corresponded to stools contaminated with 10^4 CFU ml^{-1} (Fig. 6). At this concentration, IAC was co-amplified with the *stx2* gene; at lower concentrations, only the IAC amplification was observed. Nonspecific amplifications were not observed.

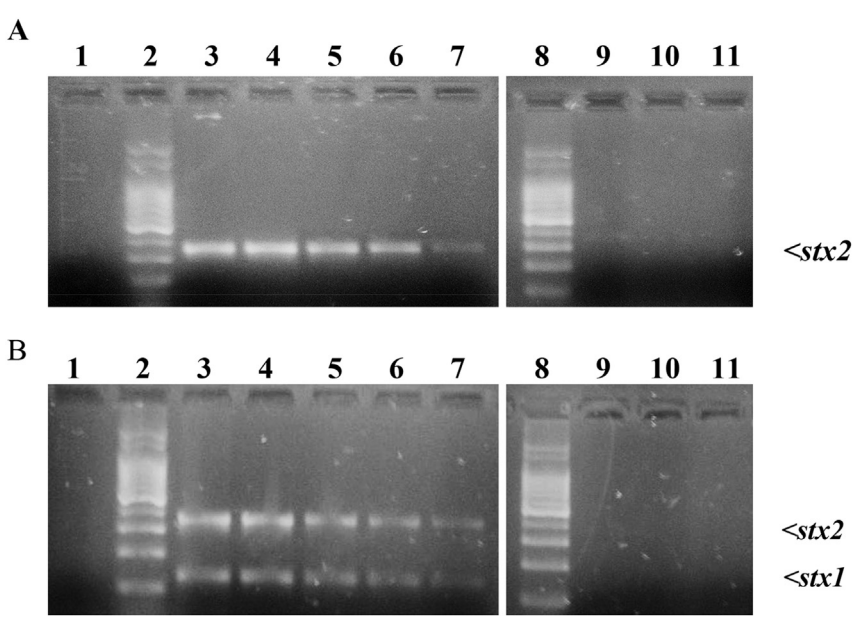

Fig. 2. Detection limits by duplex PCR of local (A) and reference (B) STEC strains cultured in EC broth. Lane 1: *E. coli* ATCC 25922 (negative control); lanes 2 and 8: 100 bp DNA marker; lanes 3–7 and 9–11: 10^7–10^0 CFU ml^{-1} (*stx1*, 130 bp; *stx2*, 349 bp).

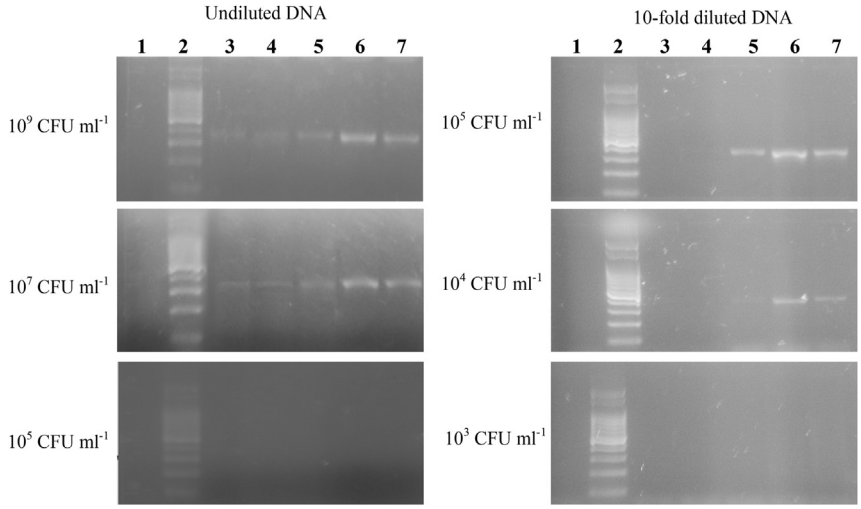

Fig. 3. Detection limits by duplex PCR in undiluted and 10-fold diluted DNA of STEC contaminated stool samples (local strain *stx2*$^+$, 349 bp). Lane 1: negative control; lane 2: 100 bp DNA marker; lanes 3–7: 0, 3, 6, 18 and 24 h enrichment, respectively.

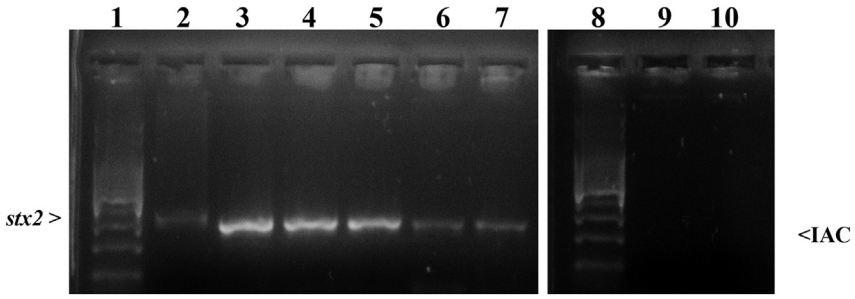

Fig. 4. Gel electrophoresis showing the detection limit of IAC (279 bp) at 0.143 pg μl^{-1} in the PCR reaction mixture. Lanes 1 and 8: 100 bp DNA marker; lane 2: local STEC strain (*stx2*, 349 bp); lanes 3–7 and 9: IAC (3.15×10^8 to 3.15×10^3 copies/25 μl PCR reaction); lane 10: negative control.

4. Discussion

A conventional multiplex PCR targeted to *stx1*, *stx2* and $rfbE_{O157:H7}$ genes [17] is successfully applied in Argentina for characterizing STEC isolates. This protocol can detect those *stx* variants that cause severe disease in humans except for stx_{2e} and stx_{2f} types [6]. In this study, the protocol was slightly modified to detect *stx1* and *stx2* genes in pediatric diarrheal stools. In addition, an IAC was designed to detect false negative results. A 349 bp sequence of *stx2* gene [28] was selected to construct a competitive IAC because the *stx2* genotype is the most frequently identified (90.3%) in STEC strains recovered from symptomatic patients in our country [22]. In addition, Stx 2 is 100–1000-fold more potent than Stx1 [11].

IAC concentration is a critical factor in PCR. Abdulmawjood et al. [1] constructed a 200 bp IAC to detect *E. coli* O157 from culture using a PCR targeted to the *rfbE* gene and obtained a detection limit of 1.12×10^4 target DNA copies/reaction when the IAC was included at a concentration of 52.3 fg μl^{-1}. Wieczorek and Osek [27] estimated an optimal IAC concentration of 1 pg μl^{-1} to detect STEC from cultures by PCR. In our work, the detection limit of the IAC was determined to be 143 fg μl^{-1}. When the concentration of target DNA was much higher than the IAC concentration, the former was preferentially amplified. Conversely, if no target DNA was present, only the IAC was amplified. When both concentrations were similar, amplicons corresponding to the IAC and the target DNA were observed in the same gel lane.

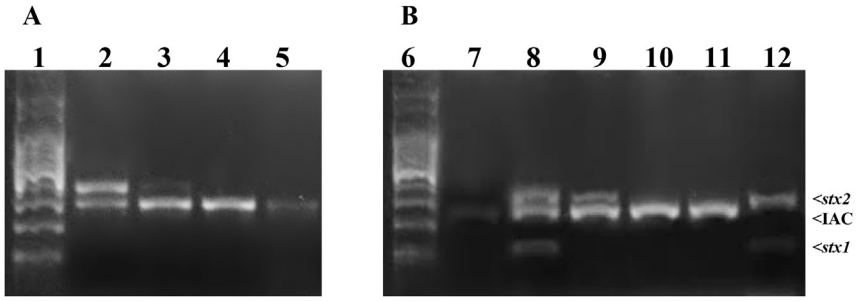

Fig. 5. Introduction of IAC in duplex PCR. A) IAC and DNA of the local STEC strain (*stx1*$^-$/*stx2*$^+$) from 10^4 to 10^1 CFU ml^{-1} in EC broth, lanes 2–5; B) IAC and DNA of the reference STEC strain (*stx1*$^+$/*stx2*$^+$) from 10^4 to 10^1 CFU ml^{-1} in EC broth, lanes 8–11, and at 10^9 CFU ml^{-1}, lane 12. IAC and DNA of *E. coli* ATCC 25922 (negative control), lane 7; 100 bp DNA marker, lanes 1 and 6.

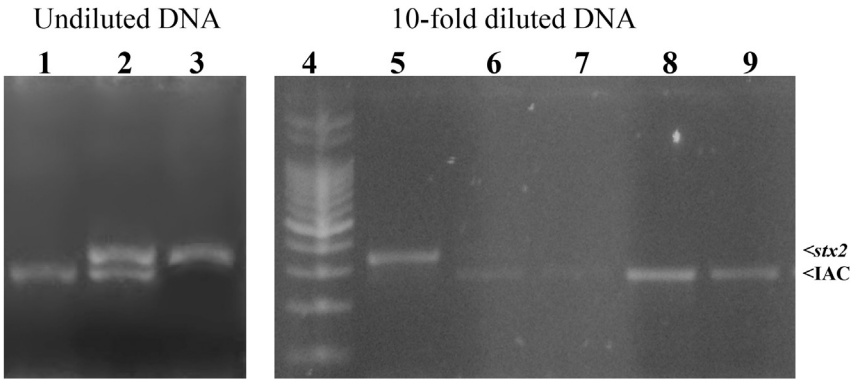

Fig. 6. Duplex PCR for undiluted and 10-fold diluted DNA extracted from STEC contaminated stools at different levels. IAC was included into each PCR reaction mix. Lane 1–3: 10^5, 10^7 and 10^9 CFU ml^{-1}; lane 4: 100 bp DNA marker; lanes 5–9: 10^4 to 10^0 CFU ml^{-1}, respectively.

Since small size products are favored by PCR and are amplified more efficiently than larger products [12], a local *stx1*$^-$/*stx2*$^+$ STEC strain was used for contamination of pediatric stools in order to avoid the expense of dNTPs, $MgCl_2$ or Taq polymerase involved in the amplification of the *stx1* gene. Previously, an enrichment step was performed for assuring DNA detection of viable bacteria. The above mentioned PCR protocol was able to establish the STEC detection limit in experimentally contaminated stools at level of 10^4 CFU ml^{-1} at 18 h enrichment. The *stx2* amplification at this STEC concentration was possible when 10-fold diluted DNA from STEC contaminated stools was used as template in the PCR reaction mix. Even though the target DNA was not amplified at lower concentrations, PCR worked satisfactorily as demonstrated by the IAC amplification. We hypothesize that at lower STEC concentrations in stools, the intestinal microflora might mask the pathogen and interfere in the STEC detection. Brian et al. [3] used 100-fold diluted DNA to detect STEC by PCR in experimentally inoculated human stools. PCR sensitivity observed by these authors corresponded to at least 2000 bacteria per mg of stools (approximately 6.30 log_{10} CFU g^{-1}) and was lower than that observed in our study. The *stx2* detection by duplex PCR including IAC in DNA extracted directly from feces was applied with positive results in the feces of one child admitted at a medical center in San Luis city, one week after the onset of nonhemorrhagic diarrhea without HUS. The STEC count in the child's feces was 10^7 CFU per gram (data not shown). In a similar study, Brian et al. [3] applied PCR to DNA isolated directly from stool samples of children with EHEC infection in the USA, and observed that the feces of one patient with HUS were positive for SLT-II sequences four days after the onset of diarrhea. However, the result was negative three days later, even though low loads of the pathogen may have been present in feces. Neither EHEC counts in these stools nor inclusion of IAC in the PCR protocol were reported by these authors. Chui et al. [4] performed a comparative study of conventional PCR and four real-time PCR assays targeting the *stx1* and *stx2* sequences in order to identify STEC in both enriched stool samples and in a panel of O157 and non-O157 strains; however, no IAC was included in the conventional PCR. Although variable specificity and sensitivity values were observed in enriched stool samples, the effectiveness of molecular methods for the detection of STEC directly from stool enrichment was demonstrated [4].

5. Conclusions

In this study, an IAC designed in our laboratory was applied for the first time in the detection of the *stx2* gene from STEC contaminated stools by a conventional duplex PCR. This protocol could be applied for direct detection of both O157 and non-O157 STEC strains in stools of symptomatic children when the pathogen load reaches concentrations of at least 10^4 CFU g^{-1}. The inclusion of an IAC in the PCR protocol may contribute to detect false negative results when STEC contaminated stools are analyzed. The sensitivity of this duplex PCR was increased when ten-fold diluted DNA was assayed.

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References

[1] A. Abdulmawjood, S. Roth, M. Bülte, Two methods for construction of internal amplification controls for the detection of *Escherichia coli* O157 by polymerase chain reaction, Mol. Cell Probes 16 (2002) 335–339.
[2] J. Antman, L. Geffner, L. Pianciola, M. Rivas, Síndrome urémico hemolítico (SUH) en Argentina, 2010-2013.Extracto del Boletín Integrado de Vigilancia, N° 222-SE 30, Área Vigilancia, Ministerio de Salud de la Nación, 2014. Available in, http://www.msal.gov.ar/zoonosis/images/stories/info-equipos-de-salud/pdf/2014-08_informe-suh.pdf (accessed 21.01.15).
[3] M.J. Brian, M. Frosolono, B.E. Murray, A. Miranda, E.L. Lopez, H.F. Gomez, et al., Polymerase chain reaction for diagnosis of enterohemorrhagic *Escherichia coli* infection and hemolytic-uremic syndrome, J. Clin. Microbiol. 30 (1992) 1801–1806.
[4] L. Chui, M.R. Couturier, T. Chiu, G. Wang, A.B. Olson, R.R. McDonald, et al., Comparison of Shiga Toxin-producing *Escherichia coli* detection methods using clinical stool samples, J. Mol. Diagn. 12 (2010) 469–475, http://dx.doi.org/10.2353/jmoldx.2010.090221.
[5] A. Etcheverría, N.L. Padola, Shiga toxin-producing *Escherichia coli*: factors involved in virulence and cattle colonization, Virulence 4 (2013) 366–372, http://dx.doi.org/10.4161/viru.24642.
[6] L. Galli, G.A. Leotta, M.J. Gugliada, M. Rivas, In silico analysis of the capability of two polimerase chain reaction techniques for *stx* gene detection, Rev. Arg. Microbiol. 40 (2008) 9–12.
[7] A. Gerritzen, J.W. Wittke, D. Wolff, Rapid and sensitive detection of Shiga toxin-producing *Escherichia coli* directly from stool samples by real-time PCR in comparison to culture, enzyme immunoassay and Vero cell cytotoxicity assay, Clin. Lab. 57 (2011) 993–998.
[8] O.G. Gomez Duarte, J. Bai, E. Newell, Detection of *Escherichia coli, Salmonella* spp, *Shigella* spp, *Yersinia enterocolitica, Vibrio cholerae* and *Campylobacter* spp enteropathogens by 3-reaction multiplex polymerase chain reaction, Diagn. Microbiol. Infect. Dis. 63 (2009) 1–9, http://dx.doi.org/10.1016/j.diagmicrobio.2008.09.006.
[9] L.H. Gould, C. Bopp, N. Strockbine, R. Alkinson, V. Baselski, B. Body, et al., Recommendations for Diagnosis of Shiga Toxin-producing Escher*ichia coli* Infections by Clinical Laboratories, 2009. Available in, http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5812a1.htm (accessed 7.02.15).

[10] T.E. Grys, L.M. Sloan, J.E. Rosenblatt, R. Patel, Rapid and sensitive detection of Shiga toxin-producing *Escherichia coli* from nonenriched stool specimens by real-time PCR in comparison to enzyme immunoassay and culture, J. Clin. Microbiol. 47 (2009) 2008–2012, http://dx.doi.org/10.1128/JCM.02013-08.
[11] C.L. Gyles, Shiga toxin-producing *Escherichia coli*: an overview, J. Anim. Sci. 85 (13 Suppl) (2006) E45–E62.
[12] L.J. Hartman, S.R. Coyle, D.A. Norwood, Development of a novel internal positive control for Taqman® based assays, Mol. Cell Probes 19 (2005) 51–59.
[13] J.L. Holland, L. Louie, A.E. Simor, M. Louie, PCR detection of *Escherichia coli* O157:H7 directly from stools: evaluation of commercial extraction methods for purifying fecal DNA, J. Clin. Microbiol. 38 (2000) 4108–4113.
[14] J. Hoorfar, R. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, P. Fach, Practical considerations in design of internal amplification controls for diagnostic PCR assays, J. Clin. Microbiol. 42 (2004) 1863–1868.
[15] E. Kalle, A. Gulevich, C. Rensing, External and semi-internal controls for PCR amplification of homologous sequences in mixed templates, J. Microbiol. Methods 95 (2013) 285–294, http://dx.doi.org/10.1016/j.mimet.2013.09.014.
[16] M.I. Lefterova, K.A. Slater, I. Budvytiene, P.A. Dadone, N. Banaei, A sensitive multiplex, real-time PCR assay for prospective detection of Shiga toxin-producing *Escherichia coli* from stool samples reveals similar incidences but variable severities of non-O157 and O157 infections in Northern California, J. Clin. Microbiol. 51 (2013) 3000–3005, http://dx.doi.org/10.1128/JCM.00991-13.
[17] G.A. Leotta, I. Chinen, S. Epszteyn, E. Miliwebsky, I.C. Melamed, M. Motter, et al., Validation of a multiplex PCR for detection of Shiga toxin-producing *Escherichia coli*, Rev. Arg. Microbiol. 37 (2005) 1–10.
[18] K. Majidzadeh, A. Mohseni, M. Soleimani, Construction and evaluation of a novel internal positive control (IPC) for detection of *Coxiella burnetti* by PCR, Jundishapur J. Microbiol. 7 (1) (2014) e8849, http://dx.doi.org/10.5812/jjm.8849.
[19] A.W. Paton, J.C. Paton, Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1, stx2, eaeA*, enterohemorrhagic *E. coli hlyA, rfb*O111, and *rfb*O157, J. Clin. Microbiol. 36 (1998) 598–602.
[20] D.R. Pollard, W.M. Johnson, H. Lior, S.D. Tyler, K.R. Rozee, Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction, J. Clin. Microbiol. 28 (1990) 540–545.
[21] M. Radhika, M. Saugata, H.S. Murali, H.V. Batra, A novel multiplex PCR for the simultaneous detection of *Salmonella enterica* and *Shigella* species, Braz J. Microbiol. 45 (2014) 667–676.
[22] M. Rivas, E. Miliwebsky, I. Chinen, C.D. Roldán, L. Balbi, B. García, et al., Characterization and epidemiologic subtyping of Shiga toxin–producing *Escherichia coli* strains isolated from hemolytic uremic syndrome and diarrhea cases in Argentina, Foodborne Pathog. Dis. 3 (2006) 88–96.
[23] M. Rivas, N.L. Padola, P.M. Lucchesi, M. Masana, Diarrheagenic *Escherichia coli* in Argentina, in: A.G. Torres (Ed.), Pathogenic *Escherichia coli* in Latin America, first ed., Bentham Science Publishers Ltd, Oak Park, IL, 2010, pp. 142–216.
[24] M.A. Rivero, J.A. Passucci, E.M. Rodriguez, A.E. Parma, Role and clinical course of verotoxigenic *Escherichia coli* infections in childhood acute diarrhea in Argentina, J. Med. Microbiol. 59 (2010) 345–352, http://dx.doi.org/10.1099/jmm.0.015560-0.
[25] S. Thisted Lambertz, A. Ballagi-Podány, R. Lindqvist, A mimic as internal standard to monitor PCR analysis of food-borne pathogens, Lett. Appl. Microbiol. 26 (1998) 9–11.
[26] G. Varela, I. Chinen, P. Gadea, E. Miliwebsky, M.I. Mota, S. González, et al., Detection and characterization of Shiga toxin-producing *Escherichia coli* from clinical cases and food in Uruguay, Rev. Arg. Microbiol. 40 (2008) 93–100.
[27] K. Wieczorek, J. Osek, Development of a PCR internal amplification control for the detection of Shiga toxin- producing *Escherichia coli*, Bull. Vet. Inst. Pulawy 48 (2004) 397–401.
[28] K.A. Ziebell, S.C. Read, R.P. Johnson, C.L. Gyles, Evaluation of PCR and PCR-RFLP protocols for identifying Shiga toxins, Res. Microbiol. 153 (2002) 289–300.