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In-House Validation of Rapid Detection PCRs for Bacterial Pathogens Causing Infant Diarrhea

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Abstract

In Argentina, conventional culture methods for the isolation of diarrheal bacteria continue to be the most widely used form of diagnosis in many clinical laboratories. In this work we validated 11 in-house real-time polymerase chain reactions (PCRs) assays for the specific and rapid detection of *Salmonella* spp., *Shigella* spp., enteroinvasive *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, Shiga toxin-producing *E. coli*, *E. coli* O157, *Cronobacter sakazakii*, *Campylobacter jejuni*, *Campylobacter coli*, *Vibrio cholera and Clostridium difficile*. The sensitivity of the assays was less than 10^2 CFU/ml for all the studied pathogens; selectivity and specificity were 100% in all cases and robustness was optimal. These PCR methods could be used to accurately detect the main bacterial causes of infant gastroenteritis.

Keywords: RT-PCR; Validation; Infant; Diarrhea; Bacteria; Detection

Introduction

Worldwide, bacterial diarrhea causes high morbidity and mortality rates, particularly in developing countries. Diarrhea is associated with significant economic and social costs. In Argentina, although more than 300000 episodes of acute diarrhea were notified in the period 2014-2016 [1], disease underreporting is significant. The reasons for under-registration are varied, including some issues associated with laboratory diagnosis. Nowadays, conventional culture methods for isolating these bacteria are the most widely used form of diagnosis in many clinical laboratories of Argentina, even though their processing time precludes the design of patient therapy and control strategies in order to prevent dissemination. Therefore, the development of rapid techniques for the detection of pathogens causing diarrhoea is highly relevant. When dealing with qualitative analytical methods, the following performance parameters must be considered: sensitivity (limit of detection, LOD), selectivity/specificity (inclusivity/exclusivity) and robustness [2]. Although several polymerase chain reaction (PCR)-based methods to detect bacteria causing diarrhea has been developed elsewhere [3-5], none of the protocols have been validated with Argentinean indigenous and regional strains. The objective of this work was to validate 11 in-house real time-PCRs for the rapid detection of 12 pathogenic bacteria that cause infant diarrhoea.

In-House Validation of Rapid Detection for Bacterial Pathogens

Primers and TaqMan® probes previously described in the literature were tested for the detection of specific genes from *Salmonella* spp. (*ttrRSBCA* locus) [6], *Shigella* spp. and enteroinvasive *E. coli* (EIEC) (*ipaH*) [7], enteropathogenic *E. coli* (EPEC) (*eae*) [8], enterotoxigenic *E. coli* (ETEC) (*eltA*) [4], Shiga toxin-producing *E. coli* (STEC) (stx_1 and stx_2) [9], *E. coli* O157 ($rfbE_{O157}$) [9], *Cronobacter sakazakii* (MMS operon) [10], *Campylobacter jejuni*/*C. coli* (*cadF*) [3], *Vibrio cholerae* (*toxR*) [4] and *Clostridium difficile* (*tcdB*) [4] by real-time PCR.

The following parameters were determined using pure bacterial cultures and for each target gene: a) working range (LOD), with one strain in cell suspensions ranging from 10^1 to 10^5 colony forming units (CFU)/ml; b) inclusivity, with ten positive strains (eight for *C. sakazakii*) at a concentration 10X above the LOD previously determined; c) exclusivity, with nine negative strains (the rest of the pathogens besides the target one) at a concentration of 10^6 CFU/ml; and d) robustness, with five positive and five negative strains at a concentration of 10^6 CFU/ml, changing the following variables: test day, equipment and laboratory place. The strains used to determine LOD, inclusivity, exclusivity and robustness are listed in Table 1. All strains excepting those from *C. jejuni*/*C. coli*, *V. cholera*, *C. difficile*; *C. sakazakii* and ETEC belong to the strain collection of IGEVET (Instituto de Genética Veterinaria "Ing. Fernando Noel Dulout", UNLP-CONICET LA PLATA), La Plata, Buenos Aires, Argentina. PCR standardization of *C. jejuni*/*C. coli*, *V. cholerae* and *C. difficile* was performed using quantified DNA from pure strains kindly provided by Virginia Zbrun, María Inés Caffer and José Di Conza, respectively.

DNA was extracted from bacterial cultures grown in brain heart infusion (Biokar, Zac de Ther, France) during the exponential growth phase. Bacterial concentration was then confirmed by plating on plate count agar (Britania, Argentina). The boiling protocol described by Leotta et al. [11] was used to perform all DNA extractions. Extracted DNA was then serially diluted in PCR-grade water to reach the desirable concentration.

Singleplex RT-PCRs were performed with a StepOne Plus thermocycler (ABI) in a 25-µl reaction mixture containing 12.5 µl of PB-L master mix (Bio-Logical Products, Argentina), 0.4 µM of each forward and reverse primer, 0.2 µM of each 6-carboxyfluorescein (FAM)-labeled TaqMan probe (IDT, Biodynamics, Argentina) and 5 µL of the template DNA, under the following conditions: initial denaturation at 95°C for 10 min, and 40 two steps cycles of 95°C for 15

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Bacteria	species/serotype	Source	N	Genotype	LOD
Vibrio	*V. cholerae* O1	Human	9	*toxR*	*
	V. cholerae no O1 no O139	Human	1	*toxR*	-
Shigella	*S. boydii*	ATCC349583	1	*ipaH*	*
	S. flexneri ser2	Human	1	*ipaH*	-
	S. flexneri	Human	1	*ipaH*	-
	Shigella spp.	Human	3	*ipaH*	-
	S. flexneri ser1	Human	1	*ipaH*	-
	S. dysenteriae ser1	Human	1	*ipaH*	-
	S. sonnei	Human	1	*ipaH*	-
	S. flexneri	ATCC12022	1	*ipaH*	-
ETEC		Animal	10	*eltA*	*
EPEC	O157: H7	Human	4	*eae*	*
	O111: HNM	Human	1	*eae*	-
	O145: HNM	Human	1	*eae*	-
	O103: H25	Human	1	*eae*	-
	O157: H7	Food	1	*eae*	-
	O109: H25	Food	1	*eae*	-
	O103: H21	Food	1	*eae*	-
STEC	O157:H7	Human	2	*stx*2 /*stx*1	*
	O157:H7	EDL933	1	*stx*2 /*stx*1	-
	O145:HNM	Human	1	*stx*2	-
	O8:H2	Human	1	*stx*2	-
	O121:H19	Human	1	*stx*2	-
	O130:H21	Food	1	*stx*2 /*stx*1	-
	O157:H7	Food	1	*stx*2 /*stx*1	-
	O178:H19	Food	1	*stx*2	-
	O113:H21	Human	1	*stx*2	-
	O111:HNM	Human	1	*stx*1	-
	O13:H16	Animal	1	*stx*1	-
	O128ab:H2	Human	1	*stx*1	-
	O91:H21	Food	1	*stx*1	-
	O103:H21	Food	1	*stx*1	-
O157	O157:H7	Human	7	*rfbE*O157	*
	O157:H7	EDL933	1	*rfbE*O157	-
	O157:H7	Food	2	*rfbE*O157	-
Salmonella enteritidis	Dublin	Animal	1	*ttr*	-
	Meleagridis	Food	1	*ttr*	-
	Derby	Food	1	*ttr*	-
	Senftenberg	Food	1	*ttr*	-
	Typhimurium	Animal	1	*ttr*	-
	Mbandaka	Animal	1	*ttr*	-
	Enteritidis	ATCC13076	1	*ttr*	-
	Newport	Food	1	*ttr*	-
	Saintpaul	Food	1	*ttr*	-
	Pullorum	ATCC13036	1	*ttr*	-
Campylobacter	*C. jejuni*	Animal	5	*cadF*	*
	C. coli	Animal	5	*cadF*	-
Clostridium	*C. difficile*	ATCC43593	1	*tcdB*	*
	C. difficile	Human	9	*tcdB*	-
Cronobacter	*C. sakazakii*	Food	6	MMS	*
	C. sakazakii	ATCC51329	1	MMS	-
	C. sakazakii	ATCC57329	1	MMS	-
*Strains used to determine the limit of detection (LOD)					

Table 1: Strains used to determine LOD, inclusivity, exclusivity and robustness.

s and 59°C for 1 min. External positive (the one corresponding to each target gene) and negative (nuclease-free water) controls were included in each run. A fluorescent signal 10-fold higher than the standard deviation of the mean baseline emission was indicative of a positive detection. The cutoff values (threshold cycles) were set above the highest FAM end-point fluorescence signals of the negative samples.

The LOD was found to be less than 10^2 CFU/ml, depending on the pathogen. Inclusivity and exclusivity were 100% in all cases, and robustness was optimal when modifying the different variables mentioned previously (Table 2).

Pathogen	Target gene	LOD[a] (CFU/ml)	Inclusivity %	Exclusivity %
V. cholerae	*toxR*	10^1	100	100
C. jejuni/*C. coli*	*cadF*	10^1	100	100
Shigella spp./EIEC	*ipaH*	10^1	100	100
E. coli $_{O157}$	*rfbE*O157	10^1	100	100
ETEC	*eltA*	10^1	100	100
EPEC	*eae*	10^1	100	100
STEC	*stx*1	10^2	100	100
-	*stx*2	10^2	100	100
Salmonella spp.	*ttr*	10^2	100	100
C. difficile	*tcdB*	10^2	100	100
C. sakazakii	MMS	10^1	100	100
[a] Limit of detection (LOD)				

Table 2: RT-PCRs limit of detection, inclusivity and exclusivity.

Discussion and Conclusion

In conclusion, we achieve assays with 100% selectivity, no false positive results for any target, and high selectivity, accuracy and detection probability. The assays were robust against pipetting, equipment and temperature variability.

The LOD of our assays (10^1 to 10^2 CFU/ml) was below the analytical sensitivity of other reported RT-PCR-based methods [3,4,6,10] and the bacterial burden often reported in symptomatic patients, as measured by culture methods (10^3 to 10^9 CFU/g stool for bacteria). Despite preliminary assays showed consistent results applying the RT-PCRs with DNA extracted from stool specimens, a validation assay in this kind of matrix is required.

We believe that 11 different simplex real-time PCR assays represent an easier and more reliable strategy (simultaneous detection and PCR product verification) than conventional multiplex PCR (limitations in the ability to resolve many fragments in agarose and potential non-specific products generation). It is important to point out that all the target genes/microorganisms were standardized with the same master mix quantities and cycling conditions so that clinical applications of the PCR assay panel described here will offer a substantially shorter turnaround time compared with other described PCRs and conventional cultures, and if adopted may contribute to improve diarrhea diagnosis, patient management, dissemination control and epidemiological surveillance.

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