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Enteroaggregative *Escherichia coli* quantification in children stool samples using quantitative PCR

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Abstract

Enteroaggregative *Escherichia coli* (EAEC) is a common cause of infectious diarrhea, especially in children living in poor-resource countries. In this article, we present a SYBR Green-based real-time polymerase chain reaction (qPCR) method for quantitative detection of EAEC in DNA directly extracted from human stool samples. In order to test the proposed qPCR system, we examined specificity, sensitivity, repeatability, and also the degree of DNA extraction efficiency using EAEC strain 042 spiked into EAEC-free stool sample. The specificity of this assay was proved using 6 strains of EAEC, 7 strains of other *E. coli* types, and one strain of *Shigella*. The detection limit of qPCR was 67 CFU/reaction. In naturally infected stool samples, we found EAEC in quantities varying from 6.7×10^5 to 2×10^9 CFU/g of feces. We could not detect any reduction after stool DNA extraction for the amounts of 10^7 and 10^6 CFU/mL of spiked EAEC. This qPCR assay is simple, rapid, reproducible, sensitive, specific, and allows rapid EAEC quantification to be used in a variety of further EAEC studies. This new quantitative method provides a relatively simple means to quantify EAEC, which will likely be key to understanding the pathophysiology and impact of EAEC infection.

Keywords

qPCR; Enteroaggregative *Escherichia coli*; DNA stool samples; childhood diarrhea

INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) represents an emerging type of diarrheagenic *E. coli* (DEC) worldwide, and children are especially vulnerable to this infection (1). Even in the absence of diarrhea, EAEC colonization warrants investigation, since it may cause chronic intestinal inflammation leading to childhood malnutrition and growth impairment (2). Moreover, the recent description of a large-scale outbreak of hemolytic uremic

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syndrome and bloody diarrhea in Germany in the spring of 2011 has demonstrated the importance of DEC with EAEC features as an enteric pathogen (3).

Nataro and colleagues (1987) described that EAEC differ from other *E. coli* pathotypes by its “stacked brick-like” pattern of adherence *in vitro*, when co-cultured with human laryngeal carcinoma (HEp-2) cells (4). This microscopic adhesion pattern for diagnosis requires testing random colonies from stool culture and biochemical tests, and must be interpreted by experienced microbiologists. This cumbersome analysis is inefficient if large numbers of colonies from stool samples must be analyzed. This inefficiency poses an obstacle, especially for epidemiologic studies (1). Therefore, molecular biology assays based on the detection of virulence genes have been extensively used to identify EAEC (5-9).

We hypothesized that the bacterial load found in the fecal material could complement the importance of detecting virulence factors of EAEC. For this purpose, we developed a SYBR Green-based real-time polymerase chain reaction (qPCR) protocol to detect and to quantify the colony forming units (CFU) of EAEC per gram of fecal material. For EAEC detection, we used PCR assays to amplify two specific bacterial DNA sequences: *aaiC* (*aggR*-activated island C) is responsible for encoding a type VI secretion system and it is located in a conserved chromosomal sequence in EAEC DNA (10); and *aatA* (EAEC ABC transporter A) comprises a DNA fragment from the EAEC virulence plasmid that encodes an outer membrane protein of the ABC transporter complex (11, 12). Since plasmids may vary in gene content, and they have the potential to be transferred to unrelated bacteria (13), we chose to quantify EAEC using the chromosomal gene (*aaiC*) as the qPCR target. With this work, we expect to show a relevant method to further testing: i) food and water for contamination with EAEC, ii) the shedding of microorganisms in animal models, and iii) clinical infections, helping to accurately measure the quantity of EAEC present in fecal material that may be important in determining asymptomatic colonization, acute or persistent diarrhea, or growth outcomes.

MATERIALS AND METHODS

Bacterial strains, cultured specimens and DNA extraction

EAEC strain 042 (O44:H18), isolated from an infant with diarrhea in Lima, Peru, in 1983 (14), was used as positive control, and also to optimize the amplification conditions. Five additional EAEC strains - JM221 (O92:H33, Mexico), 55989 (O104:H4, Central African Republic), H223-1 (O101:H38, Peru), H194-2 (O175:H27, Peru), and 239-1 (ORough:H21, Thailand) - were used (5). Moreover, other *E. coli* pathotypes (Enterotoxigenic *E. coli* - ETEC H10407, ETEC 1392-752A; Enteropathogenic *E. coli* - EPEC 2348/69, EPEC 149-6084; Enterohemorrhagic *E. coli* - EHEC O157:H7) and commensals *E. coli* (HB101, Nissle 1917, and HS) were selected and used in the qPCR to assess the specificity of the designed primers. MacConkey agar (BD, Sparks, MD, USA) was used to culture all *E. coli* strains prior to DNA extraction. We also tested *Shigella flexneri* cultured on SS agar (BD, Sparks, MD, USA). *Shigella* was added to our specificity tests, since this bacterium presents a great genetic similarity to *E. coli* (15). Boiling in water was used for DNA extraction for all colonies, as follows: 2-3 colonies/mL of autoclaved MilliQ water were boiled for 10 minutes; after centrifugation (956 x g for 10 minutes), the supernatant containing at least 30 ng of DNA was used as template for PCR.

DNA extraction directly from stool samples

The tested samples were part of a study approved by the local and national Ethical Committee in Brazil and by the University of Virginia Institutional Review Board. Briefly, the stool samples were collected from children living in a poor urban community in

Fortaleza, Ceara, Brazil. A consent form was read and signed by the guardians of each child. The samples were collected at each household and transported in iced boxes within 4 hours to the microbiology laboratory. The samples were kept in the freezer at -80°C until the DNA extraction was performed. *QIAamp DNA stool Mini kit* (Qiagen, Valencia, CA, USA) was used for genomic DNA extraction directly from 200 mg or 200 μL of stool samples. For enhancing the pathogen's DNA extraction we made two improvements in the original protocol: i) a vigorous homogenization of the samples with 300 mg of 0.1 mm glass beads (BioSpec, Bartlesville, OK, USA) using a MiniBeadBeater (BioSpec, Bartlesville, OK, USA), and ii) an incubation at 95°C in lysis buffer.

All samples were quantified for DNA using a Nanodrop200 (ThermoScientific, Wilmington, DE, USA). The purity of DNA extracted was determined by measuring the 260–280 nm absorbance ratio (A_{260}/A_{280}). DNA obtained was kept at -20°C until the qPCR tests were performed. We used DNA extracted from 14 stool samples tested by conventional PCR for the presence of the EAEC using *aaiC* and *aatA* genes, as described in Table 1. In order to certify the quality of the extraction procedures, we performed an internal control PCR. This reaction amplifies an encoding *E. coli* 16S rRNA, *rrs* – a detectable gene where fecal coliform can be found (16). Primers and amplification cycling conditions for this PCR reaction were described in Table 1. All primers were chosen after analysis with The Basic Local Alignment Search Tool (BLAST, NCBI, Bethesda, MD, USA). No apparent homology with other nucleotide sequences within GenBank (National Center for Biotechnology Information – NCBI, Bethesda, MD, USA) was detected. DNA extracted from a commensal strain (HS) of *E. coli* was used as positive control.

SYBR Green-based real-time polymerase chain reaction (qPCR)

Individual qPCR reactions consisted of a total volume of 25 μL /reaction, with 12.5 μL of iQ SYBR Green Supermix (Bio-Rad, San Diego, CA, USA), 2 μL of DNA extracted from colonies or samples, 200 nM of *aaiC*-q primers and ultra-pure water. The cycling conditions for *aaiC* amplification were also described in Table 1. To determine sensitivity of the qPCR reactions, standard curves were performed using DNA extracted from a 10-fold dilution series of EAEC 042, as briefly described: from a 2 colonies/mL of sterile phosphate buffer solution (PBS) as diluent, so called “mother tube” (MT), we made 1:100, 1:10 000 and 1: 1 000 000 dilutions, in order to estimate the number of CFU presented on MT. Following the later procedure, we cultured duplicated samples of 1 μL or 10 μL from each dilution, using a flame-sterilized L-shaped glass rod, on MacConkey agar (BD, Sparks, MD, USA) plates. After 18-24 h of incubation at $37 \pm 2^{\circ}\text{C}$, counts were reported as CFU/mL. Meanwhile, the MT was kept at 4°C overnight to avoid bacterial growth. The DNA extraction was done from the MT and its 10-fold dilution series. Boiling water method was used to DNA extraction.

To quantify the CFU/mg of stool sample tested, the mean quantitative cycle (C_q) obtained by each sample (tested duplicated in each batch) were extrapolated to the standard curve constructed. With this information, we were able to calculate the amount of CFU/reaction (2 μL of template) and so the CFU/g of stool samples, the final form of the expression of the results. Every experiment batch presented a negative control (ultra-pure water). Positive control was DNA from EAEC 042 strain.

Amplification efficiency and Limit of Detection (LoD)

The efficiency's curves were determined three times, in non-consecutive days of experiments. For this purpose, we used 2 μL of each template - generated from the DNA extraction of the MT and its consecutive 10-fold dilutions. We performed the qPCR amplifications from DNA extracted of different concentrations to reach our amplification

efficiency curves. These data were given according to the curve efficiency performance: slope, R^2 , and efficiency parameters.

We determined the qualitative LoD points using the diluted samples that were no longer contributing appropriately to a high efficiency curve, repeating 10 times the qPCR amplification reaction. LoD was set at the concentration where amplification was reached before 35 cycles at 95% of the time.

Evaluation of pathogen detection in spiked sample

In order to evaluate if some loss of detection occurred when stool samples were submitted to the DNA extraction protocol, we spiked EAEC 042 in stool samples with no amplification of *aaiC* or *aatA* genes. The concentrations of EAEC spiked ranged from 1.5×10^7 to 1.5×10^4 CFU/mL, and each concentration was spiked in two different aliquots (200 mg) of fecal material. DNA extraction of those spiked samples was performed, using *QIAamp DNA stool Mini kit* (Qiagen, Valencia, CA, USA), as described above. A new sensitivity curve was done using the DNA extracted from the same pure culture used for spiking to ensure that Cq values obtained were extrapolated to standard curves established in a similar matrix. The concentrations of EAEC in the spiked samples were calculated from this new sensitivity curve.

Melting curves and agarose gel electrophoresis analysis

To ensure the specificity of the PCR product, we conducted a melting curve analysis, in which the reaction temperature was subsequently increased by 0.5 °C every 20 s, beginning at 60 °C and ending at 95 °C. Throughout the curve construction process, the changes in fluorescence were measured, and the data obtained with the iQ5 Optical System software (version 2.0, Bio-Rad, San Diego, CA, USA) were processed in order to verify if a single peak was obtained. In addition, to make sure that the amplified products showed the predicted size, a 2% ethidium bromide staining agarose gel was performed and analyzed by *ChemiDoc XRS™* (Bio-Rad, San Diego, CA, USA).

RESULTS

Specificity

Table 2 shows the specificity of the *aaiC*-q primers. All EAEC strains were positive for *aaiC*, with a melting point at 79 °C (Fig. 1D). Single bands of the expected amplicon were seen. No other tested strain generated an amplicon.

Sensitivity

The plots of DNA concentration (DNA extracted from EAEC strain 042 pure culture) versus Cq value are depicted in Fig. 1. Efficiency curves, generated in three non-consecutive days, were linear over five orders of magnitude from approximately 10^6 to 10^2 CFU/reaction (R^2 values > 0.99). Across this range, the standard deviation of the triplicates was less than 10% of the mean. The slopes of our obtained standard curves were within 5.8% of the -3.33 value expected for perfect doubling in each cycle (ranging from -3.387 to -3.526).

The qualitative LoD was given using more diluted concentrations of templates less than 6.72×10^2 - the last concentration that contributed to an acceptable value of the amplification efficiency curve. The LoD point was 67 CFU/reaction, obtained from samples ranging from 6.72×10^1 to 6.72×10^{-3} CFU/ reaction (Table 3).

Quantification of EAEC on stool samples

Our pre-tested samples, which were positive for *aaiC* and *aatA* genes, were also positive at the proposed qPCR protocol. The results for these samples are listed in Table 4. The negative samples did not reach any *aaiC* amplification, besides its qualities were assured since *rrs* did amplify.

DNA isolation efficiency

The linear ranges found for spiked fecal samples presented quite similar ranges from pure culture of the reference strain for ranges of 10^7 and 10^6 CFU/reaction. A 10-fold decay on detection for concentrations 10^5 and 10^4 was observed, as shown in Table 5. The sensitivity curve used for calculation of EAEC in those samples was linear from 1.5×10^8 to 1.5×10^4 CFU/mL, presented efficiency of 96.0%, R^2 of 0.999, and slope -3.42 .

DISCUSSION

In the present work, we developed a protocol capable of quantifying EAEC in limited stool aliquots, as little as 200mg. Despite the importance of EAEC as enteric pathogen (17), its diagnosis still represents an obstacle to a complete understanding of its impact on human health.

Compared to traditional culture, the use of PCR-based methods for detection of a variety of enteric pathogens are quite promising since they bring significant improvements in speed, sensitivity, and reliability (18). Real time PCR protocols further extend the advantages of conventional PCR procedures, since they offer even more speed and sensitivity, accompanied by a reduction of carry-over contamination risk (19), and provide the capability of quantifying the target pathogen in many sources like food, feces and water (20, 21). Taniuchi and colleagues (2012) evaluated the performance of testing DNA obtained from stool, compared to colony pools picked from MacConkey agar and stool enriched on Gram-negative (GN) broth in a multiplex PCR-based luminex assay for DEC pathotypes diagnosis (9). The authors showed that stool DNA yielded higher sensitivity than colony pools, a finding that supports the choice of this method in the present study.

The samples were first assessed by conventional PCR, using two EAEC specific genes that included the recently described chromosomal gene *aaiC*, and the plasmid sequence *aatA*. Both genes have been used for EAEC diagnostic purpose (6, 9, 22). Due to the heterogeneity of EAEC pathotype, there is not a single gene or a set of them which would allow the detection of all EAEC strains (1). The classification of EAEC in “typical” and “atypical”, according to the presence of *aggR* (aggregative adherence regulator) regulon (23), resulted in the development of several PCR assays based on the detection of *aggR* in combination with other EAEC plasmid sequences (24-26). Those additional sequences showed not to be restricted to EAEC (27, 28). Although exclusively found among EAEC strains, *aggR* gene belongs to a variable region of the EAEC genome (10), an area frequently subject to horizontal transmission (13). Thus, *aggR* may be more useful as a marker of EAEC virulence than as a diagnostic gene. The likely transfer of DNA mobile elements was recently confirmed by the European outbreak of diarrhea, which was caused by an EAEC strain that acquired a lambdoid prophage carrying the gene for the Shiga toxin (3). Therefore we selected a conserved chromosomal sequence (*aaiC*) as target for the EAEC quantification.

After demonstrating the high specificity of this protocol for EAEC, assessed by consistent negative results with other pathogenic and non-pathogenic *E. coli* strains and with a *Shigella* strain, we evaluated the sensitivity performance. The standard curve obtained from several dilutions of DNA from EAEC strain 042 pure culture detected linearly from 6.7×10^2 to 6.7

$\times 10^6$ CFU/reaction. Also, EAEC could be detected qualitatively to the limit of 67 CFU/reaction. Since the concentrations of EAEC in our local stool samples naturally infected varied from 6.7×10^5 to 2×10^9 CFU/g of feces, corresponding to 1.35×10^3 to 4.06×10^6 CFU/reaction, our detection limits should be very useful to enumerate EAEC in samples obtained from even asymptomatic children, where lower loads of the microorganism might be expected. These limits are also consistent with other works using qPCR amplification from pure cultures of other DEC pathotypes (29-31), and other bacteria (32, 33).

The DNA isolation efficiency of EAEC 042 was evaluated by a spiking protocol. The results showed a decreasing efficiency of the recovery of EAEC 042 from spiked samples: the ranges of spiked EAEC 042 of 10^7 and 10^6 UFC/200 mg of stool were readily recovered, with a mean of detection efficiency ranging from 88-113%. At 10^4 - 10^5 UFC/mL spiked material, some loss of detection (around 50%) was noticed. A number of studies of qPCR protocols designed for bacterial quantification showed considerable variation in DNA isolation efficiency; however, many of these focused on food or water contamination, which involves an enrichment step, thus hampering comparative assessments (34-36). Using fecal material, Kralic and colleagues (2011) described a non-linearly increasing efficiency of *Mycobacterium avium* subsp. *paratuberculosis* recovery, reaching 31.8% isolation efficiency at 10^8 cells/gram of spiked fecal material, 53.1% isolation efficiency for 10^4 cells/gram, and finally reaching 101.4% isolation efficiency for 10^2 cells/gram (21). On the other hand, Ireng and colleagues (2009) observed a DNA extraction efficiency of 80% for the same microorganism (37). Further experiments are needed in order to confirm our findings, and to evaluate the factors that could be involved in this loss of detection found here.

Efficiency, linear range, and sensitive detection limits, are essential parameters in characterizing a qPCR test performance (38). The presented protocol achieved high quality values for those essential parameters with consistent linearity of the standard curve, good patterns of repeatability and reproducibility, with excellent specificity.

In conclusion, the proposed protocol is suitable for quantification of EAEC in large epidemiological studies, in order to determine if quantity of CFU/g of fecal material could elucidate associations with the severity or morbidity of overt diarrhea, inflammation or growth failure associated with EAEC infections, especially in childhood population. Together with other approaches that could be investigated, such as determining the microorganism's range of potential genetic "virulence" factors and assessment of its related expressed proteins, the present study offers a promising tool to enable a better understanding the manifestations and pathophysiology of EAEC infection.

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Figure 1.

A, B, and C: qPCR standard curves relating Cq value to DNA concentration for *aaiC-q* specific primers tested with different concentrations of DNA extracted from EAEC strain 042. The construction of the curves was done in three different and non-consecutive days (A, B, and C). The slopes values given by each curve was: -3.482 , -3.526 , and -3.387 respectively. **D:** Melting curves relating to *aaiC-q* DNA amplifications reached by the following EAEC strain's DNA: 042, JM221, 55989, H-223-1, H194-2, and 239-1.

Table 1

Nucleotide sequence of primers, size of amplicons, and PCR conditions of the genes used.

Gene	Reaction	Nucleotide sequence (5'-3')	GenBank (NCBI) sequence number	Amplicon size (bp)	Annealing conditions*	Reference
<i>aaiC</i>	PCR	ATTGTCTCTCAGGCATTTCAC ACGACACCCCTGATAACAA	FN554766.1	215	20" at 57°C	This work
<i>aaiA</i>		CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	AY351860	630	20" at 57°C	8
<i>ITS</i>		CCCCCTGGACGAAGACTGAC ACCGCTGGCAACAAAGGATA	JN547259.1	401	20" at 63°C	16
<i>aaiC-q</i>	qPCR	CTTCTGCTCTTAGCAGGGAGTTTG AAGCGTGAAATGCCTGAGGA	FN554766.1	123	20" at 60°C	This work

PCR: common PCR, qPCR: quantitative PCR.

* Every cycle begins with a denaturing step (20 sec. at 95 °C) and finishes with an extension of 45 s. to 1 min. at 72 °C.

Table 2Specificity of *aaiC* gene detection for the tested strains.

Bacteria	Strain	Cq value	Melting curve (°C)
EAEC	042	14.09 ± 0.06	79.5
	239-1	14.62 ± 0.06	79.0
	55989	16.36 ± 0.42	79.5
	H223-1	17.46 ± 1.67	79.5
	H194	20.17 ± 0.25	79.0
	JM221	18.35 ± 0.30	79.0
EPEC	2348/69	-	-
	149-6084	-	-
ETEC	1392-752A	-	-
	H10407	-	-
EHEC	H157:O7	-	-
Commensals <i>E. coli</i>	HS	-	-
	Nissle 1917	-	-
	HB101	-	-
<i>Shigella flexneri</i>	-	-	-

EAEC – Enteraggregative *Escherichia coli*; EPEC – Enteropathogenic *Escherichia coli*; ETEC – Enterotoxigenic *Escherichia coli*; EHEC – Enterohemorrhagic *Escherichia coli*.

Table 3

Detection and quantification limits of the qPCR assay performed with the 10-fold serial dilutions of Enterotoxigenic *Escherichia coli* (EAEC) strain 042 culture.

Tubes	EAEC 042 CFU/mL	EAEC 042 CFU/reaction (2 μ L)	Cq value $\pm$ St. dev.	Signal ratio
1	3.36×10^9	6.72×10^6	12.87 ± 0.22	9/9
2	3.36×10^8	6.72×10^5	16.25 ± 0.31	8/8
3	3.36×10^7	6.72×10^4	20.54 ± 0.39	8/8
4	3.36×10^6	6.72×10^3	23.61 ± 0.39	9/9
5	3.36×10^5	6.72×10^2	26.98 ± 0.30	9/9
6	3.36×10^4	6.72×10^1	32.19 ± 0.56	7/7
7	3.36×10^3	6.72×10^0	36.54 ± 1.42	3/12
8	3.36×10^2	6.72×10^{-1}	33.90 ± 2.75	2/13
9	3.36×10^1	6.72×10^{-2}	35.25 ± 0.32	2/13
10	3.36×10^0	6.72×10^{-3}	33.63 ± 1.89	5/10

Table 4Quantification of EAEC in stool samples based in *aaIC* gene detection.

Sample Identification	Calculated EAEC 042 CFU/reaction $\pm$ St. dev.)	Extrapolated EAEC 042 CFU/200mg of stool (CFU/200mg)	Extrapolated EAEC 042 CFU/g of stool (CFU/g)
20907	$2.60 \times 10^4 \pm 0.34$	2.60×10^6	1.30×10^7
32003	$1.03 \times 10^4 \pm 0.03$	1.03×10^6	5.01×10^6
15011	$1.45 \times 10^6 \pm 0.17$	1.45×10^8	7.25×10^8
20004	$1.35 \times 10^3 \pm 0.17$	1.35×10^5	6.70×10^5
10507	$6.15 \times 10^4 \pm 0.44$	6.15×10^6	3.07×10^7
18210	$3.12 \times 10^3 \pm 0.43$	3.12×10^5	1.56×10^6
22903	$1.18 \times 10^4 \pm 0.20$	1.18×10^6	5.90×10^6
31303	$7.20 \times 10^3 \pm 1.15$	7.20×10^5	3.60×10^6
31503	$4.03 \times 10^6 \pm 0.31$	4.03×10^8	2.01×10^9

Table 5

Evaluation of the efficiency of EAEC 042 DNA isolation procedure from human faeces using the *q-aaiC* quantitative PCR (qPCR) assay.

Tubes	Theoretical input of EAEC 042 [*]	Experimental output of EAEC 042 by <i>q-aaiC</i> qPCR [†]	Number of replicates	Mean EAEC 042 isolation efficiency ^{**} (%)
1	1.50×10^7	$1.32 \times 10^7 \pm 0.05$	4	88%
2	1.50×10^6	$1.70 \times 10^6 \pm 0.02$	2	113%
3	1.50×10^5	$6.72 \times 10^4 \pm 2.62$	4	45%
4	1.50×10^4	$8.28 \times 10^3 \pm 0.18$	4	55%

^{*} Number of CFU of EAEC 042 used for spiking of 200mg of human faeces.

[†] Number of CFU of EAEC 042 recovery after DNA isolation by *q-aaiC* qPCR.

^{**} Considering the theoretical input corresponding to 100%.