Novel multiplex real-time PCR assays reveal a high prevalence of diarrhoeagenic Escherichia coli pathotypes in healthy and diarrhoeal children in the South of Vietnam

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Abstract

Background

Diarrhoeagenic Escherichia coli (DEC) infections are common in children in low-middle income countries (LMICs). However, detecting the various DEC pathotypes is complex as they cannot be differentiated by classical microbiology. We developed four multiplex real-time PCR assays were to detect virulence markers of six DEC pathotypes; specificity was tested using DEC controls and other enteric pathogens. PCR amplicons from the six E. coli pathotypes were purified and amplified to be used to optimize PCR reactions and to calculate reproducibility. After validation, these assays were applied to clinical samples from healthy and diarrhoeal Vietnamese children and associated with clinical data.

Results

The multiplex real-time PCRs were found to be reproducible, and specific. At least one DEC variant was detected in 34.7% (978/2,815) of the faecal samples from diarrhoeal children; EAEC, EIEC and atypical EPEC were most frequent Notably, 41.2% (205/498) of samples from non-diarrhoeal children was positive with a DEC pathotype. In this population, only EIEC, which was detected in 34.3% (99/289) of diarrhoeal samples vs. 0.8% (4/498) non-diarrhoeal samples ($p<0.001$), was significantly associated with diarrhoea. Multiplex real-time PCR when applied to clinical samples is an efficient and high-throughput approach to DEC pathotypes.

Conclusions

This approach revealed high carriage rates of DEC pathotypes among Vietnamese children. We describe a novel diagnostic approach for DEC, which provides baseline data for future surveillance studies assessing DEC burden in LMICs.

Background

Diarrhoeal illness remains the second-highest cause of mortality and morbidity worldwide [1–3]; the main burden of this disease occurs in children in South Asia, Southeast Asia, and Africa [3]. Among the bacterial pathogens associated with diarrhoea in children, Escherichia coli are repeatedly the most common food borne pathogenic species identified [3–6]. However, identifying diarrhoea-causing E. coli can be complex, as pathogenic variants cannot be delineated from commensal E. coli solely by microbiological culture.

Diarrhoeagenic E. coli (DEC) can generally be divided into six pathotypes (enterotoxigenic E. coli, ETEC; enteroaggregative E. coli, EAEC; enteropathogenic E. coli, EPEC, enteroinvasive E. coli, EIEC; and shiga-toxin producing E. coli, STEC), based on specific virulence markers that are encoded on plasmids and/or chromosomal islands [7]. ETEC, EAEC, and EPEC have all been implicated in causing diarrhoea in young children in low-middle income countries (LMICs) [8–10]. EIEC are virtually indistinguishable from Shigella

spp., which are essentially an independent genus within the broader E. coli population. STEC are commonly associated with food-borne disease outbreaks in developed countries and have higher mortality than other E. coli pathotypes due to sequelae of [11–13]. The epidemiology of STEC in LMICs, particularly in children in Southeast Asia, are not well described.

The proportion of diarrhoeal disease associated with DEC in Vietnam is not well investigated as measuring the prevalence of these pathogens in diarrhoeal cases and non-diarrhoeal controls is laborious and not routinely performed. Of the limited DEC studies conducted in Vietnam an investigation originating in Hanoi detected DEC in 22% of stool sample from diarrhoeal cases and 12% of controls using conventional multiplex PCR [7]. Here, we aimed to develop a set of standardized multiplex real-time PCR assays to identify the various DEC in complex samples in a comparatively short turnaround time. To establish the multiplex real-time PCR assays to identify the six DEC pathotypes we designed new or adapted existing specific primers and probes for nine DEC associated genes. The real-time PCR assays were optimized and then used to determine the prevalence of DEC in children with and without diarrhoea disease in Ho Chi Minh City (HCMC), Vietnam. Lastly, we combined these PCR data with available clinical data to identify clinical features in children infected with differing DEC pathotypes and to determine the potential effect of DEC in the stools of diseased and non-diseased children.

Results

Multiplex real-time PCR assay for detecting diarrhoeagenic Escherichia coli

We firstly validated PCR amplification for ETEC, EAEC, EIEC/Shigella, EPEC, and STEC in monoplex using cloned target sequences and then with genomic DNA extracted from the various E. coli pathovars. The sensitivity of the primer and probe sets was determined by generating a series of standard curves using 10-fold dilutions of control plasmid DNA. The limit of detection for all targets was five copies per reaction, with the exception of aggR which could be detected down to 50 copies per reaction. Each primer and probe set were tested against a panel of commonly isolated pathogens found in stool samples, which included Staphylococcus aureus, Klebsiella pneumoniae, Salmonella spp., Campylobacter coli, Campylobacter jejuni, Shigella sonnei, Shigella flexneri, Enterobacter, Proteus, norovirus, and rotavirus. No amplification was observed in the none E. coli samples.

Ultimately, the PCR assays were multiplexed into four reactions, and the sensitivity, intra-assay and inter-assay CVs across the nine target sequences were calculated for each multiplexed PCR reaction. The Ct values for each target were equivalent between the monoplex and multiplex reactions, confirming that multiplexing did not impact sensitivity. The intra-assay and inter-assay CVs ranged from 0.01 to 1.54% and from 0.01 to 2.12%, respectively (Table 2). The linear regressions of the standard curves were between 0.992–0.999 for all targets tested. The resulting efficiency of the amplification ranged from 90.9 to 105.7%, demonstrating the multiplex real-time PCR assays were well optimized, reproducible, and specific.

Table 2

Reproducibility of the assays on diluted plasmid DNA containing cloned target sequences

Target sequence	Co-efficient of variance (%)	Target concentration							
		5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	5×10^1	5×10^0
eltB	Intra-assay variation [a]	0.03	0.04	0.06	0.10	0.35	0.16	0.27	0.35
	Inter-assay variation [b]	0.07	0.11	0.05	0.23	0.41	0.50	0.38	0.67
estA	Intra-assay variation	0.01	0.05	0.03	0.25	0.19	0.16	0.15	0.06
	Inter-assay variation	0.15	0.03	0.04	0.05	0.52	0.36	0.12	0.76
aggR	Intra-assay variation	0.11	0.08	0.08	0.15	0.20	0.37	0.29	-
	Inter-assay variation	0.06	0.11	0.33	0.03	0.29	0.23	1.05	-
ipaH	Intra-assay variation	0.19	0.17	0.20	0.12	0.12	0.60	0.15	0.11
	Inter-assay variation	0.23	0.28	0.20	0.07	0.42	0.20	0.52	1.42
eae	Intra-assay variation	0.07	0.24	0.29	0.06	0.26	0.76	0.20	0.48
	Inter-assay variation	0.38	0.16	0.06	0.46	0.82	0.64	0.63	0.88
bfpA	Intra-assay variation	0.03	0.09	0.11	0.17	0.50	0.90	0.63	0.90
	Inter-assay variation	0.01	0.11	0.05	0.06	0.18	0.38	0.75	1.23
rfbE	Intra-assay variation	0.03	0.11	0.09	0.42	0.20	0.70	0.98	0.30
	Inter-assay variation	0.02	0.04	0.10	0.23	0.60	1.49	1.29	1.08
stx1	Intra-assay variation	0.13	0.13	0.17	0.21	0.50	0.74	0.97	0.60
	Inter-assay variation	0.22	0.50	0.71	0.98	1.19	1.57	1.95	0.55
stx2	Intra-assay variation	0.11	0.14	0.18	0.17	0.25	1.54	0.81	0.24
	Inter-assay variation	0.12	0.22	0.43	0.47	0.87	1.54	2.12	1.97

[a] Intra-assay variation was calculated by measuring the co-efficient of variance of the Ct value on three concurrently run assays.

[b] Inter-assay variation was calculated by comparing variation in Ct value on three independently run assays

The prevalence of diarrhoeagenic Escherichia coli from faecal specimens of children hospitalized with diarrhoea

Between May 2014 and April 2016, we amassed 2,815 MC sweeps (i.e. faecal samples plated on MC media) from 3,166 children hospitalized with bloody and/or mucoid diarrhoea at three tertiary hospitals in HCMC. A single faecal sample was collected from each child within their first two days of hospital admission for diarrhoea. The majority of patients were male (1,731/2,815; 61.5%), with ages ranging from one month to 15 years (median age 10 months, IQR 6.6–17.1 months).

We employed the four multiplex real-time DEC PCRs on all 2,815 MC sweeps to identify DEC targets potentially associated with clinical infection. At least one PCR amplification associated with a DEC variant was positive in 34.7% (978/2,815) of the MC sweeps from paediatric patients hospitalized with diarrhoea. Among the DEC amplification positive samples, EAEC was the most common variant detected, with aggR amplified in 15.7% (443/2,815) of samples (Table 3). Other commonly amplified DEC targets included EIEC/Shigella and EPEC, which were identified in 12.4% (349/2,815) and 12.2% (343/2,815) of the MC sweep samples, respectively.

Table 3
DEC detected in children hospitalized with diarrhoea
(N = 2,815)

Pathotypes	Target gene	N	%
ETEC		182	6.5
LT-ETEC	elt	167	5.9
ST-ETEC	est	7	0.2
LT-ST-ETEC	elt & est	8	0.3
EAEC	aggR	443	15.7
EIEC/Shigella	ipaH	349	12.4
EPEC		343	12.2
Atypical EPEC	eae	322	11.4
Typical EPEC	eae & bfpA	21	0.7
EHEC/STEC		46	1.6
O157	eae & rfbE_O157	20	0.7
non-eae O157	rfbE_O157	26	0.9
	stx1/stx2	4	0.1
Negative		1,837	65.3

Within the EPEC pathotype, atypical EPEC positive samples (eae positive, bfpA negative) were more prevalent than typical EPEC positive samples (eae positive, bfpA positive); 93.9% (322/343) vs. 6.1% (21/343), respectively. ETEC was detected in 6% (182/2,815) of samples, with only a limited number of these samples (8.2%; 15/182) producing an amplicon for heat stable toxin (estAFour diarrhoeal patients harboured samples containing the Shiga toxin-producing genes (stx1/stx2). Among the four cases associated with an STEC positive sample, one was positive for eae and one was positive for both eae and rfbE_O157. Of the two STEC cases that were amplification positive for eae and rfbE_O157, one was additionally positive for eltB (ETEC), the other was positive for aggR (EAEC).

Clinical manifestations of diarrhoeagenic Escherichia coli mono-infection

To investigate clinical syndromes associated with the various DEC in Vietnam, clinical data associated with the patients were accessed and compared between pathotype groups (Table S2). Patient samples from which multiple DEC pathogens were amplified were excluded. Notably, ~ 70% of those with an ETEC, EAEC, EPEC, or STEC O157 positive sample were associated with mucoid, non-bloody diarrhoea, whereas EIEC/Shigella was significantly associated with visible bloody diarrhoea (39.7%, 46/116, $p < 0.001$, χ^2 test). EAEC was the most commonly identified DEC in mono-infection. This pathotype was more commonly associated with children that had wasting or severe wasting (13.5%, 23/170; $p = 0.013$, Fisher's exact test) than the other DEC variants. Whilst EHEC_O157 was identified less frequently than other pathotypes, it was significantly associated with moderate and severe dehydration (40%, 8/20; $p = 0.010$, Fisher's exact test), which commonly required intravenous rehydration therapy.

Generally, we found that infections associated with DEC positive samples were uncomplicated; >90% of patients had improved or recovered after three days and their median hospital stay was five days [IQR 3–7 days]. The use of antimicrobials within this study population was high, with 81.3% (1,513/1,861) of patients receiving empirical antimicrobial treatment prior to any diagnostic testing. Fluoroquinolones, specifically ciprofloxacin, were the most commonly used class of antimicrobials in those with a DEC in their stool (957/1,512, 63.3%).

Diarrhoeagenic Escherichia coli from faecal specimens of diarrhoeal hospitalized children vs. healthy non-diarrhoeal children

Between March 2016 and August 2016, 498 MC sweeps were additionally collected from faecal samples taken from healthy children residing in HCMC and participating in a cohort study [17]. The majority of healthy children were male (269/498; 54.0%), with their age when sampled ranging from 24 months to 5 years (median age 46.4 months, IQR 35.6–52.5 months). In a comparable manner to the diarrhoeal samples, we screened the MC extractions from these healthy children with the multiplex real-time PCRs to detect DEC. At least one pathotype of DEC was detected in 41.2% (205/498) of samples associated with non-diarrhoeal children (Table 4).

Table 4
Direct comparison of DEC detected in samples from children hospitalized with diarrhoea and healthy children

Diarrhoeagenic E. coli	Target gene	Diarrhoea N (%)		Non-diarrhoea N (%)		p value*
Number		319		498		
ETEC		29	(9.1)	42	(8.4)	0.745
LT-ETEC	elt	25	(7.8)	39	(7.8)	0.998
ST-ETEC	est	1	(0.3)	3	(0.6)	1.000
LT-ST-ETEC	elt & est	3	(0.9)	0	(0.0)	0.059
EAEC	aggR	50	(15.7)	89	(17.9)	0.415
EIEC/Shigella	ipaH	93	(29.2)	4	(0.8)	< 0.001
EPEC		39	(12.2)	93	(18.7)	0.015
Atypical EPEC	eae	38	(11.9)	90	(18.1)	0.018
Typical EPEC	eae & bfpA	1	(0.3)	3	(0.6)	1.000
EHEC/STEC		13	(4.1)	20	(4.0)	0.712
O157	eae & rfbE_O157	5	(1.6)	5	(0.6)	0.524
non-eae O157	rfbE_O157	8	(2.5)	18	(3.4)	0.379
	stx1/stx2	0	-	7	(1.4)	0.033
Negative		163	(51.1)	293	(58.8)	0.030

*p value from χ^2 test or Fisher's exact test

To determine the prevalence and distribution of the various DEC in healthy and diarrhoeal children, we compared the data from the healthy children with a subset of the data from matched children in the diarrhoeal study which were between the ages of 2 and 5 years old (319 children; median age 31.5 months, IQR 26.7–38.9 months). The prevalence of ETEC, EAEC, and EHEC_O157 in faecal samples was not significantly different between children with or without diarrhoea (Table 4, Fig. 2). Furthermore, EPEC was detected significantly more frequently in the non-diarrhoeal samples (18.7%, 93/498) than the diarrhoeal samples (11.4%, 33/289) ($p = 0.019$, χ^2 test) (Table 4). The only DEC that was significantly associated with the diarrhoeal samples was EIEC/Shigella, which was detected in 34.3% (99/289) of diarrhoeal samples vs. 0.8% (4/498) non-diarrhoeal samples ($p < 0.001$, Fisher's exact test).

The distribution of DEC co-infection among the cases and the controls was complex and highly variable (Fig. 2). The most common co-infections in the diarrhoeal group were EAEC + EIEC/Shigella (3.8%, 12/319) and EAEC + EIEC/Shigella + ETEC (2.2%, 7/319); whereas EPEC + EAEC (3.4%, 17/498) was more common in the healthy control group. Co-infection with more than one DEC was more likely to be associated with diarrhoeal disease than with healthy controls (16.3%, 52/319 vs. 9.6%, 48/498, $p = 0.005$, χ^2 test). However, due to the predominant presence of EIEC/Shigella in the diarrhoeal group, EIEC/Shigella infection was a potential confounder.

To disaggregate the potential confounding effect of EIEC/Shigella, we performed binary univariate and multivariate logistic regression to identify variables and DEC that were associated with diarrhoeal disease in children aged 24–60 months (Table 5). In the univariate model, co-infection with ETEC, mono-infection with EIEC/Shigella, co-infection with EIEC/Shigella, and co-infection without EPEC, EHEC_O157, and STEC were significantly associated with diarrhoea. However, after controlling for confounders, only mono or co-infection with EIEC/Shigella and wasting were determined to be significantly associated with diarrhoea. Conversely, mono-infection with ETEC, EAEC, and obesity were significantly more common in the non-diarrhoeal children.

Table 5

Univariate and multivariate analysis of DEC mono-infection and co-infection associated with diarrhoeal disease among children from 24–60 months of age using binary logistic regression model

Variable	Univariate Model			Multivariate Model		
	Odds Ratio	95% Confidence Interval	P-value[c]	Odds Ratio	95% Confidence Interval	P-value[d]
Types of infection [a]						
Mono-infection with ETEC	0.39	0.15–1.05	0.062	0.32	0.11–0.94	0.037
Co-infection with ETEC	2.27	1.21–4.27	0.011			
Co-infection without ETEC	1.74	1.00–3.02	0.051			
Mono-infection with EAEC	0.51	0.28–0.93	0.028	0.45	0.24–0.86	0.015
Co-infection with EAEC	1.75	1.06–2.89	0.030			
Co-infection without EAEC	2.55	1.19–5.46	0.016			
Mono-infection with EIEC	52.13	12.57–216.21	< 0.001	49.66	11.90–207.24	< 0.001
Co-infection with EIEC	31.46	7.47–132.47	< 0.001	35.60	8.28–153.12	< 0.001
Co-infection without EIEC	0.66	0.37–1.20	0.173	0.63	0.34–1.18	0.149
Mono-infection with EPEC	0.55	0.32–0.95	0.031	0.59	0.34–1.02	0.057
Co-infection with EPEC	1.22	0.66–2.25	0.525			

[a] Odds Ratio with the reference was non-infection status

[b] Odds Ratio with the reference was normal growth status

[c] P-value considered significant when $p < 0.01$

[d] P-value considered significant when $p < 0.05$

Variable	Univariate Model			Multivariate Model		
	Odds Ratio	95% Confidence Interval	P-value[c]	Odds Ratio	95% Confidence Interval	P-value[d]
Co-infection without EPEC	2.97	1.65–5.34	< 0.001			
Mono-infection with EHEC	0.90	0.33–2.44	0.834	0.96	0.35–2.64	0.937
Co-infection with EHEC	1.14	0.44–3.01	0.785			
Co-infection without EHEC	2.19	1.36–3.52	0.001			
Mono-infection with STEC	-					
Co-infection with STEC	-					
Co-infection without STEC	2.17	1.39–3.40	0.001			
Gender						
Female	0.81	0.61–1.07	0.142	0.65	0.47–0.91	0.013
Growth [b]						
Obese	0.57	0.35–0.92	0.022	0.51	0.29–0.89	0.019
Overweight	0.88	0.57–1.36	0.560	1.00	0.62–1.62	0.998
Risk of overweight	0.68	0.46–1.01	0.053	0.83	0.54–1.27	0.390
Wasted	19.13	2.48–147.54	0.005	19.58	2.43–157.92	0.005

[a] Odds Ratio with the reference was non-infection status

[b] Odds Ratio with the reference was normal growth status

[c] P-value considered significant when $p < 0.01$

[d] P-value considered significant when $p < 0.05$

Discussion

Here, we developed and applied an efficient and robust collection of real-time PCR assays for identifying DEC in MC sweeps isolated from stool samples from a collection of healthy and diarrhoeal children. This approach, in comparison to the traditional method, is straightforward, cost-effective and has a comparatively short turn-around time [28]. Ultimately, the four multiplex real-time PCR assays could detect ten target sequences corresponding with six pathotypes of DEC, which permitted detection of these pathogens with a high degree of accuracy and utility. However, there are some limitations with this our approach. As has been indicated throughout the manuscript, we were unable to differentiate between EIEC and Shigella spp., as the invasion plasmid antigen H (ipaH) and the uidA (the internal control gene for E. coli) are present in both Shigella and EIEC [19]. Further limitations of this approach are associated with issues of how pathotypes such as EPEC, EHEC, and STEC are defined. Through bacterial genomics, we know that organisms lacking either eae or stx or both may still belong to the EHEC group [9, 10]. In addition, the stx genes have been found in other pathotypes of E. coli [11, 12]. Therefore, it is impossible to definitively assign an E. coli to a DEC pathotype without genome sequencing. However, pathotyping DEC through detecting virulence genes remains useful for assessing the potential prevalence of the various pathogenic forms of E. coli in any given population. In addition to the methodological constraints of the study, as we did not enrol healthy children under the age of two years, we could not evaluate associations with diarrhoea in the more vulnerable younger age groups.

While ETEC is the most common DEC internationally, the prevalence of ETEC in this setting was found to be considerably lower than other regions [9,28]. This result is probably due to the study inclusion criteria, as only children presenting with bloody and/or mucoid diarrhoeal illness were enrolled, whereas ETEC is most commonly associated with watery diarrhoea [7]. Here, LT-ETEC were more prevalent than ST-ETEC, which is consistent with earlier studies on ETEC infections in children. However, in these previous studies the association between LT-ETEC infection and diarrhoea was weak [6, 13, 14]. In contrast, in the Global Enteric Multicentre Study (GEMS), ST-ETEC but not LT-ETEC was attributed as a major cause of diarrhoea in all age groups [33]. To determine whether ST-ETEC is an important pathogen in Vietnam, it will be necessary to carry out additional studies focusing on children presenting with watery diarrhoea.

EAEC was the most commonly detected pathotype in children with diarrhoea in this study, which is again consistent with earlier studies that reported high detection rates of EAEC compared to other DEC in Vietnam [14,34]. Several articles have raised the possibility that not all EAEC are pathogenic, and that variants within this group may have different propensities to cause disease [35–40]. However, several outbreaks and human volunteer studies have unequivocally shown that some EAEC can cause disease [35–40]. Here, one third of EAEC mono-infections required antimicrobial IV treatment (i.e. the third generation cephalosporins or imipenem; data not shown) associated with a more severe disease presentation. Notably, samples from three children in this study generated positive PCR amplicons for both EAEC and stx. These cases may represent mixed infections of EAEC and STEC, or potentially hybrid organisms, such as those associated with an extensive outbreak in Europe in 2011 [32]. Although EAEC was not associated with diarrhoea in children within the 24-60-month age group in this study, it was the most commonly detected pathotype from children with wasting. This observation is consistent with the

findings of the recent the MAL-ED study, which reported that EAEC infection is associated with growth i shortfall, irrespective of disease [41].

EPEC was the most common DEC gene target amplified from faecal samples of diarrhoea and non-diarrhoea children. The overwhelming majority of the amplicons generated from both healthy and diseased cohorts were associated with aEPEC. These data are again consistent with EPEC literature, which suggests that typical EPEC is commonly identified in the African continent [28,42], while atypical EPEC tends to predominate in other regions [16]. A case-control study conducted in seven LMICs found that typical EPEC infections were significantly associated with mortality in children under 5 years [6]. The high prevalence of atypical EPEC positive samples in our study group (24–60 months of age) may be partially associated with colonization in the first year of life, as asymptomatic infection with ETEC, EAEC, and EPEC have previously been associated with weaning and the termination of breastfeeding [17].

STEC O157 cause severe diarrhoea and are associated with a high mortality rate in food-borne outbreaks in western countries [18]. EHEC_O157 in this setting had a low prevalence and more than half the positive samples were positive for the rfbE_O157 gene alone, which suggests these are likely to be of lower pathogenicity. Only two samples that tested positive for EHEC_O157 also produced amplicons for the Shiga-toxin gene (stx2), suggesting that O157-STEC is not a significant cause of gastrointestinal symptoms in this location. In the age matched comparison, STEC were isolated from children in the healthy group only. This observation is consistent with data originating in Indonesia, where STEC was detected significantly more frequently in non-diarrhoeal children[19].

In previous studies, co-infection with more than one DEC (or with other enteric pathogens) was found to be significantly associated with diarrhoea [20–24]. Here we found that co-infection with DEC was not associated with diarrhoea and was also common in healthy children. Notably, only co-infection with EIEC/Shigella was significantly associated with diarrhoeal disease. However, as EIEC/Shigella infection alone was highly significantly associated with diarrhoeal illness, the contribution of other DEC to disease in EIEC/Shigella infection is unclear. In a multivariate logistic regression model, DEC co-infection in the absence of EIEC/Shigella was not associated with diarrhoea. This suggests that EIEC/Shigella is the most important cause of DEC mediated moderate-to-severe diarrhoea in this setting.

Conclusions

Multiplex real-time PCR is an efficient method for detecting the six major pathotypes of DEC in a collection of clinical samples. Our new methodology may prove to be more sensitive and cost effective than picking individual colonies and PCR screening. Using this approach, we found a high prevalence of DEC in the stools of both healthy and diarrhoeal children in Vietnam. EAEC and atypical EPEC were the most commonly detected DEC in both groups; whereas, EIEC/Shigella was the only DEC significantly associated with diarrhoeal disease. This study provides new methodology and baseline data for further clinical, epidemiological, and genomic studies in Vietnam and across Southeast Asia and shows that DEC are highly prevalent but not generally associated with diarrhoeal disease in Vietnam.

Methods

Study design

Children aged ≤ 15 years with diarrhoeal illness admitted to one of the three collaborating tertiary hospitals in HCMC, Vietnam from May 2014 to April 2016 were eligible for enrolment. Those with diarrhoeal illness (cases) were defined as ≥ 3 passages of loose stools within 24-hour period along with at least one loose stool containing blood and/or mucus [15]. We excluded children if they had suspected or confirmed intussusception at the time of enrolment [16]. Controls were healthy children between the age of 12–60 months enrolled in diarrhoeal disease cohort in District 8 in HCMC from 2014 to 2016 [17]. The enrolled children attended HVH for routine health check every six months. An anal swab of healthy child was collected by study nurses at these routine visits.

Primer and probe design

The selected target genes for each pathotype were: ETEC, eltB (heat-labile toxin) and/or estA (heat-stable toxin); EAEC, aggR (transport regulator gene [18]); EIEC, ipaH (secreted protein encoded on pINV [19]); EPEC, eae (encoding the intimin adherence gene [7]) and bfpA (encoding a structural component of the bundle forming pilus [20]); STEC, stx1 and/or stx2 (Shiga toxins [7]); and rfbE_O157 (encoding the lipopolysaccharide O157 antigen, the most common STEC serogroup in regions where surveillance data is available). The uidA gene, which encodes beta-glucuronidase and is present in all E. coli, was used as an internal control to monitor both DNA extraction and PCR amplification. A flowchart of the combined assay strategy is shown in Fig. 1.

We classified the DEC amplification results using the following approach; ETEC positive samples were divided into LT-ETEC (eltB positive only); ST-ETEC (estA positive only); and LT-ST-ETEC (eltB and estA positive). Amplification of aggR was sufficient for classification as EAEC and a positive amplification for ipaH identified EIEC/Shigella. EPEC positive samples were divided into typical EPEC (carrying both eae and bfpA) and atypical EPEC (the presence of eae only). The STEC pathotype was identified by the presence of stx1 and/or stx2, and the presence or absence of rfb_O157 was used to differentiate between STEC O157 and the non-O157 STEC serogroups. STEC that have the potential to cause HUS carry additional virulence genes, specifically eae and aggR.

The primer and probe sequences for aggR, ipaH, eae, and uidA) were adapted from previous studies [21–24]. Reference sequences were downloaded from GenBank eltB, estA (STh), bfpA, rfbE_O157, stx1 and stx2 (Table S1) and aligned using AlignX (Vector NTI, Invitrogen) to identify conserved regions within the gene sequence. Primer Quest (IDT, USA) was employed to generate primers and probes of amplicon size 100 to 150 bp. To find optimal pairs, candidate primers and probes were analysed for Tm, %GC, hairpin, self-dimer and hetero-dimer using Oligo Analyzer 3.1 (http://sg.idtdna.com/calc/analyzer). Final primer/probe candidates were blasted against PrimerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to

confirm the in-silico specificity of the selected sequences. The selected primer and probe sequences are shown in Table 1.

Table 1

Sequences of primers and probes to detect DEC in this study

Diarrhoeagenic E. coli target	Primer/probe name	Sequence * (5'-3')	Amplicon size (bp)
ETEC	elt_ETEC_F	CTCGGTCAGATATGYGATTCTT	100
	elt_ETEC_R	AACATTTCAGGTCGAAGTCC	
	elt_ETEC_ probe	FAM-TGTGTCCTTCATCCTTTC AATGGCTT-BHQ1	
	est_ETEC_F	GCTAAACCAGYAGRGTCTTCAA	137
	est_ETEC_R	GCAGGATTACAACACAATTCAC	
	est_ETEC_ probe	LCCyan500-AGTRGTCCTGAAA GCATGAATAGTAGCA-BHQ1	
EAEC	aggR_F	CCATTTATCGCAATCAGATTAA	92[28]
	aggR_R	CAAGCATCTACTTTTGATATTCC	
	aggR_probe	FAM-CAGCGATACATTAAGA CGCCTAAAGGA-BHQ1	
EIEC/ Shigella	ipaH_F	AGGTCGCTGCATGGCTGGAA	99[29]
	ipaH_R	CACGGTCCTCACAGCTCTCA	
	ipaH_probe	LCCyan500-AACTCAGTGCCTCT GCGGAGCTTCGACA-BHQ1	
EPEC/ EHEC	eae_F	CATTGATCAGGATTTTTCTGGTGA TA	102[30]
	eae_R	CTCATGCGGAAATAGCCGTTA	
	eae_probe	FAM-ATAGTCTCGCCAGTA TTCGCCACCAATACC-BHQ1	
EPEC	bfpA_EPEC_F	GTCTRTCTTTGATTGAATCKGC	108
	bfpA_EPEC_R	CATTCTGYGMCTTATTGGAATC	
	bfpA_EPEC_probe	LCCyan500-ACCGTTACYGCM GGTGTGATGTTT-BHQ1	
STEC	stx1_EHEC_F	GCATCTGATGAGTTTCCTTCTA	113
	stx1_EHEC_R	GTTCTGCGCATCAGAATTG	

*R (A/G), Y(C/T), S (G/C), W (A/T), M (A/C), K (G/T) according to International Union of Pure and Applied Chemistry (IUPAC)

Probe detection format (Roche Light Cycler II 480) as followed FAM: 498–580; LCCyan500: 440–488; CY5: 618–660; LC Red 610: 533–610; BlackBerry→ Quencher: BHQ1, BHQ2

Diarrhoeagenic E. coli target	Primer/probe name	Sequence * (5'-3')	Amplicon size (bp)
	stx1_EHEC_probe	FAM-AAGAGKCCGTGGGA TTACGCACAAT-BHQ1	
	stx2_EHEC_F	ACRACGGACAGCAGYTATWC	111
	stx2_EHEC_R	GAACTCCATTAAMKCCAGATA	
	stx2_EHEC_probe	LC Red 610-ATGCAAATCAGTCGTCA CTCACTGGT-BHQ1	
EHEC O157	rfbE_O157_F	CAAGTCCACAAGGAAAGTAAAG	111
	rfbE_O157_R	GAGTTTATCTGCAAGGTGATTC	
	rfbE_O157_probe	LCCyan500-AACTCAGTGCCTCT GCGGAGCTTCGACA-BHQ1	
Internal control	uidA_F	GTGTGATATCTACCCGCTTCGC	82[31]
	uidA_R	AGAACGGTTTGTGGTTAATCAGGA	
	uidA_probe	CY5-TCGGCATCCGGTCAGTGGCAGT-BHQ2	
*R (A/G), Y(C/T), S (G/C), W (A/T), M (A/C), K (G/T) according to International Union of Pure and Applied Chemistry (IUPAC)			
Probe detection format (Roche Light Cycler II 480) as followed FAM: 498–580; LCCyan500: 440–488; CY5: 618–660; LC Red 610: 533–610; BlackBerry→ Quencher: BHQ1, BHQ2			

Construction of control plasmids

PCR amplicons were generated for each of the 10 target genes and ligated into pCR™ 2.1-TOPO→ (Invitrogen, Applied Biosystem, UK). Purified plasmids were used as template to optimize PCR reactions and measure assay reproducibility. Plasmid concentrations (ng/µl) were quantified using a Nanodrop spectrophotometer (Thermo-Scientific, UK), and converted to copy number using the URI Genomics and Sequencing Center online tool (http://cels.uri.edu/gsc/cndna.html).

Real-time PCR

Multiplex real-time PCR reactions were performed in a 25 µl reaction mixture containing a final concentration of 1X buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 3.5 mM of $MgCl_2$, 0.2 µM of each forward and reverse primers, 0.08 µM of each probe and 1U of Hotstart Taq polymerase (QIAGEN, Germany). Five µl of DNA template was used for each PCR reaction. The real-time PCR cycling conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 95^{o}C for 15 s, then 60^{o}C for 60 s, using the Light Cycler 480 II system (Roche, Germany). The threshold cycle (Ct) value for a positive result was considered to be 38 or less.

Reproducibility and linearity analysis

The precision and reproducibility of the real-time PCR assays were assessed using the co-efficient of variance (CV%), measured by dividing the standard deviations of the Ct values by the mean Ct values for each selected concentration. The Ct values of three replicates assayed simultaneously were compared to measure intra-assay reproducibility. The inter-assay reproducibility was calculated from data generated on three separate days. Linearity was determined by linear regression, using Ct values produced from 10-fold dilutions of control plasmid DNA.

Specimen culture and storage

Diarrhoeal faecal specimens were collected in sterile containers and transported to the laboratory within 24 hours [16]. Anal swabs from non-diarrhoeal children were also transported to the laboratory within 24 hours for processing. Specimens were inoculated onto MacConkey agar (MC, Oxoid), and incubated at 37 °C for 18–24 hours [16]. Following incubation, a sweep of colonies was taken from the entire MC agar plate and suspended in 20% glycerol in Brain Heart Infusion (BHI) broth, before being stored at -80°C.

Crude DNA extraction

Eighty µl of the stored colony sweep suspension was centrifuged at 4,000 rpm for 10 minutes, and the pellet was resuspended in 80 µl of molecular grade water (Sigma). The resulting suspension was mixed by gently pipetting up and down, before being boiled at 96 °C for 10 minutes and cooled to room temperature. The lysate was centrifuged at 4,000 rpm for 10 minutes to remove cellular debris, and 5 µl of supernatant was subjected to the real-time PCR assays.

Data collection and statistical analysis

Data were exported into Microsoft Excel (Microsoft, USA), and analysed using Stata v11 (StataCorp, College Station TX, USA). Descriptive comparisons between groups were conducted using non-parametric tests including χ^2 test or Fisher's exact test for categorical variables and the Kruskal-Wallis test for continuous data. Growth status of participating patients were assessed using the WHO global database on growth and nutrition and Prevention and Management of Obesity for Children and Adolescents-Healthcare guidelines using the macro package of Stata v11 developed by WHO [25,26]. Due to the age difference between diarrhoeal and non-diarrhoeal groups, the comparative analyses were performed between all the children in healthy group and the subset children in the diarrhoea group that were aged 24–60 months. Logistic regression was performed to determine the associations with diarrhoea using each type of infection considered as an independent variable. Infections were classified as mono-infection of each pathotype of DEC or co-infections of each specific pathotype and other pathotypes. The types of co-infection were repeated due to multi pathotype co-infection; hence the p-value for univariate model was considered significant when $p < 0.01$. Multivariable logistic regression models were performed and incorporated mono-infections, each specific type of co-infection, gender and growth status with diarrhoea and non-diarrhoea as binary outcomes (performed on Stata v11, StataCorp, College Station TX, USA). For the latter, a p-value of < 0.05 was considered significant. The figure for mixed-infections (Fig. 2)

was generated using the UpSetR package and restructured manually to generate the side by side bar graphs for comparing two groups [27].

Abbreviations

Diarrhoeagenic Escherichia coli (DEC)

Low-middle income countries (LMICs)

Enterotoxigenic E. coli (ETEC)

Enteroaggregative E. coli (EAEC)

Enteropathogenic E. coli (EPEC)

Enteroinvasive E. coli (EIEC)

Shiga-toxin producing E. coli (STEC)

Haemolytic uraemic syndrome (HUS)

Ho Chi Minh City (HCMC)

Global Enteric Multicentre Study (GEMS)

co-efficient of variance (CV%)

MacConkey agar (MC)

Declarations

Ethical approval and consent to participate
Ethical approval for this study was obtained from the OxTREC (OxTREC No. 1045-13 for cases and OxTREC No 1058-13 for controls) as well as from local partners, which included the Institutional Review Boards at Children's Hospital 1, Children's Hospital 2, The Hospital for Tropical Diseases, and Hung Vuong Hospital (HVH). An informed consent form signed by a parent or guardian was required for enrolment.

Consent for publication
All authors have seen and approved the final version of this manuscript for publication.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

Authors declare no competing interests

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Authors' contributions
Conceptualization: SB

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Provided samples: HLP, TTHC, NMN, LLV, CJ

Methodology: LTPT, HTT, LTQN, JIC, PVM, HLP, TTHC, NMN, LLV, CJ, IO, EH

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Review and editing: SB, CJ, EH

Read and approved final version of manuscript: VTD, LTPT, HTT, LTQN, JIC, PVM, HLP, TTHC, NMN, LLV, CJ, IO, EH, SB

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Figures

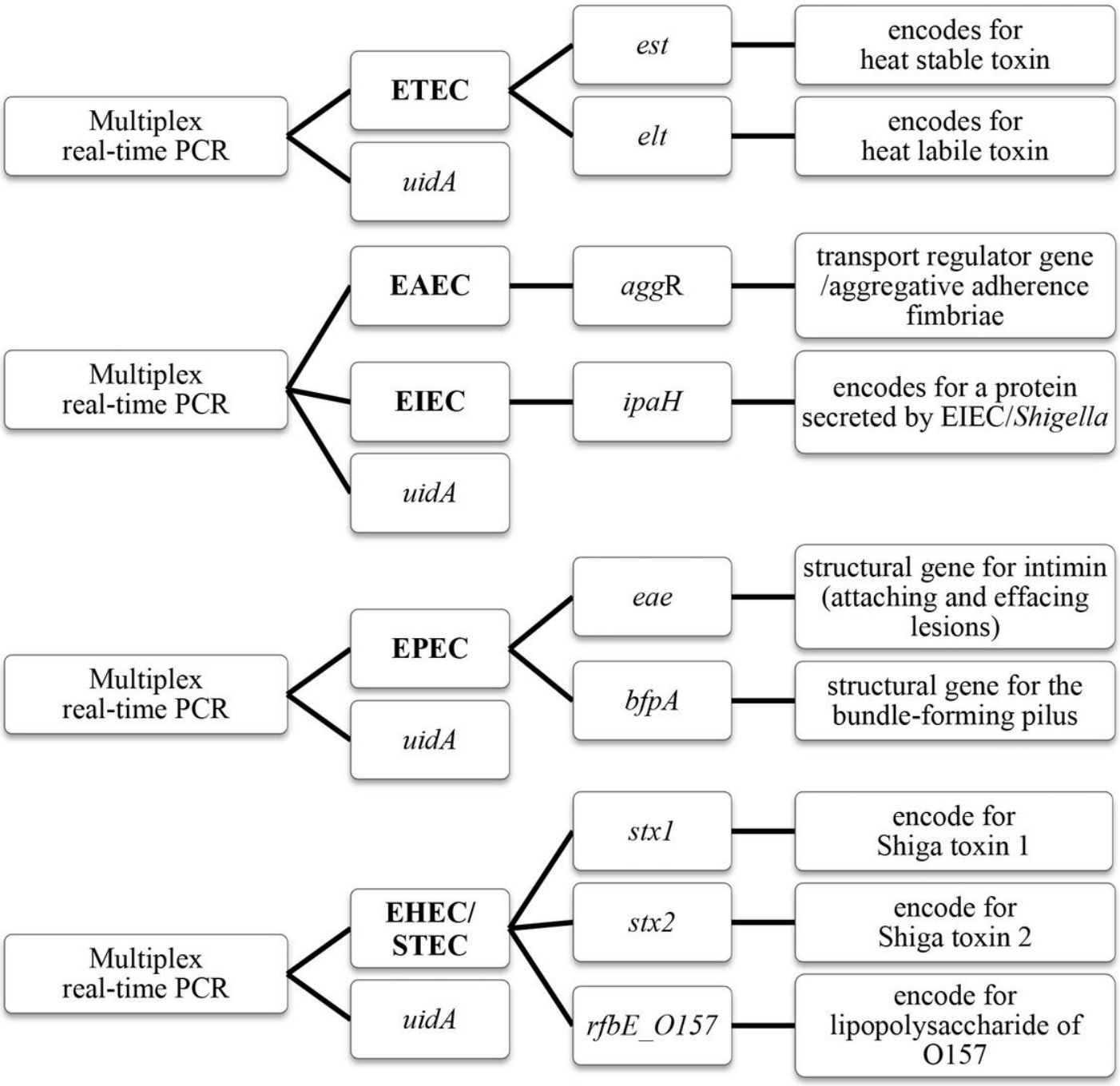

Figure 1

Multiplex PCR strategy Flowchart showing the four multiplex real-time PCR assays for detecting target sequences of DEC; uidA (gene encoded for β-glucuronidase and presented in all E. coli) was selected as an internal control.

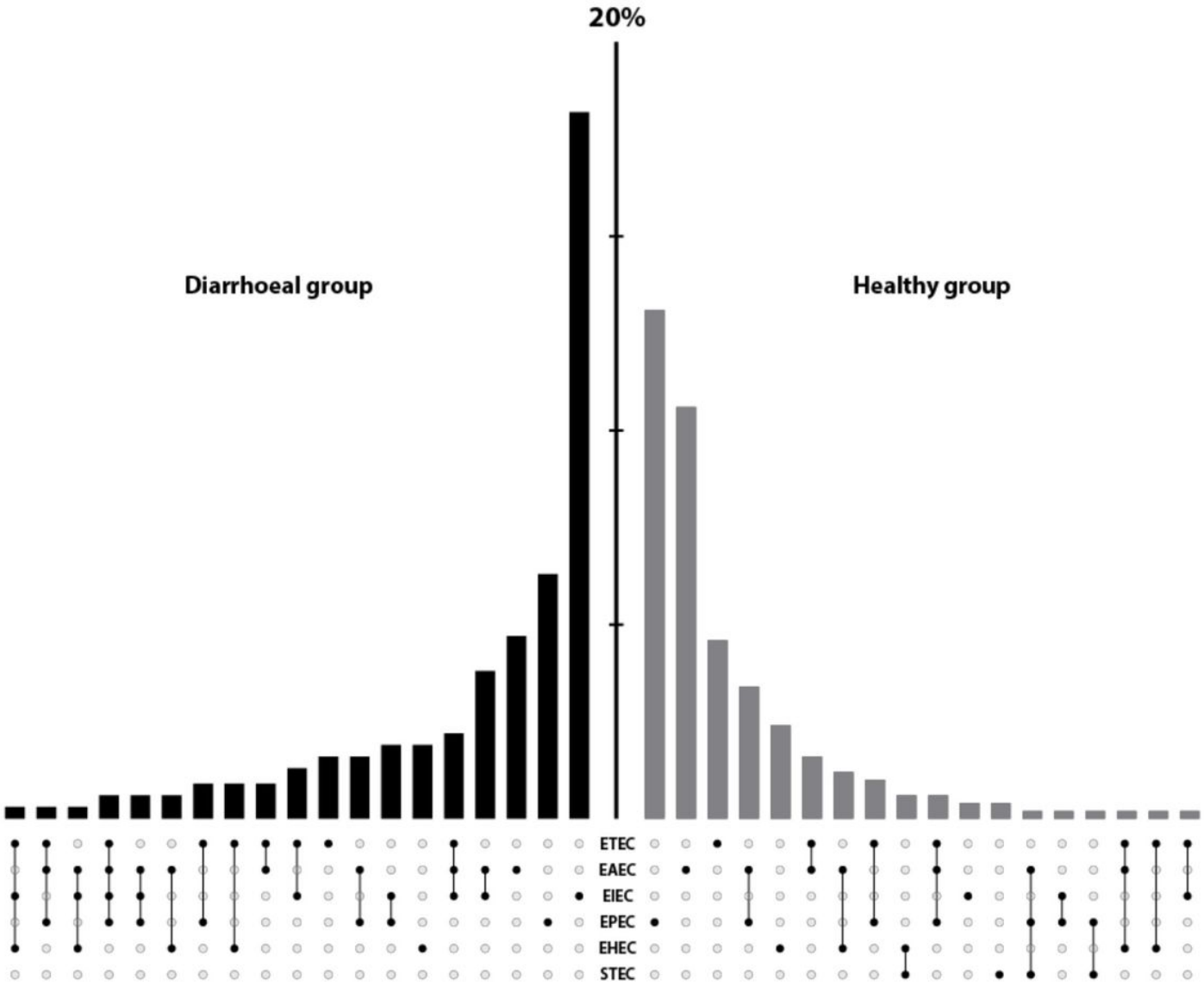

Figure 2

Mono- and co-infection with DEC in the 24-60-month-old healthy and diarrhoeal and healthy childrenBar chart demonstrating the proportion of cases and controls with each combination of DEC pathotypes, with the most frequently isolated DEC pairings located near the centre, the scale (y axis) in 5% increments. The dots and lines between dots at the base of the chart show the co-infection status for six pathotypes of DEC. DEC co-infection patterns among diarrhoea group (N = 319; black) and healthy group (N = 498; grey).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.pdf