Culture independent real-time PCR reveals extensive polymicrobial infections in hospitalized diarrhoea cases in Kolkata, India

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Abstract

Culture independent identification of diarrhoeal etiologic agents was performed using DNA harvested from diarrhoeal stool specimens with SYBR Green based real-time PCR targeting *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Campylobacter spp.*, *Shigella spp.*, and 3 different pathotypes of diarrhoeagenic *Escherichia coli*. Conventional culture dependent methods detected bacterial enteropathogens in 68 of 122 diarrhoeal stool specimens. Of 68 specimens, 59 (86.8%) had single pathogen while the remaining 9 (13.2%) had polymicrobial infections with multiple pathogens. Reanalysis of the 68 specimens by culture independent real-time PCR methods showed 25 (36.8%) specimens contained single pathogen while 43 (63.2%) specimens contained mixed infections with multiple pathogens. The prevalence of such high level of polymicrobial infections would not have been detected if real-time PCR was not utilized. Culture dependent analysis assigned 54 of the 122 selected archived specimens as 'no known aetiology'. However, reanalysis of these samples by real-time PCR showed presence of single or multiple pathogens among 34 (63%) of these specimens. Estimation of relative pathogen load by real-time PCR in the stool specimens indicated the inability of conventional culture dependent methods to detect the pathogens was related to lower colony forming units of the pathogen as reflected by lower Ct values. Detection of high levels of polymicrobial infection by real-time PCR indicate that in the settings like Kolkata and around, which is endemic for cholera and other enteric diseases, the concept of one pathogen one disease might need to be re-evaluated.
Introduction

Globally, about two billion cases of diarrhoeal diseases occur every year. It is considered as the second leading cause of death in children less than five years old, killing about 1.336 million children every year [1]. India contributes about 77% of the child deaths in southeast Asia and 18% of the global child deaths due to diarrhoea [1]. The irony lies in the fact that most diarrhoeas are treatable and most of the diarrhoeal deaths are preventable. Diarrhoea should thus be attended rapidly and effectively to detect the causal aetiology and to avoid significant morbidity and mortality as well as to prevent secondary transmission.

Polymicrobial infections in diarrhoeal diseases have been reported extensively in countries where sanitation is compromised and where availability of safe drinking water is restricted [2-8]. In some cases, polymicrobial infections have been considered as a major factor contributing to the severity of diarrhoea [4]. Despite using all modern days bioassay based tools, various hospital and community based diarrhoeal surveillance studies have consistently been unable to detect a causal aetiology in about 30% of the specimens [8-12]. This has stressed the need for more sensitive, specific and rapid detection assays for identifying pathogens from diarrhoeal stools.

Culture dependent methods to identify the enteric pathogens as pure culture followed by characterization through various biochemical tests are considered as gold standard. But it takes considerable time to confirm the aetiology. Further to this an enormous number bacterial species that resides in the human gut are yet to be cultured. In spite of being able to culture hundreds of enteric bacteria, 80-90 % of gut flora still remains as unculturable. Culture independent techniques for identifying and to characterize these uncultivable floras are currently being perused. In the post genomic era, culture independent rapid detection assays have been developed of which real-time PCR based assays have gained much interest.
This study is a part of such a trend in identifying enteropathogens directly from stool specimens.

**Materials and methods**

**Archived diarrhoeal stool specimens and DNA extraction**

Stool specimens were collected from hospitalized diarrhoeal patients after obtaining informed consent and the study was approved by the Institutional Ethical Committee. Samples were analyzed by culture dependent methods for the detection of bacterial, viral and parasitic enteropathogens [8]. In brief, diarrheal stool specimens were streaked on selective plates, colonies grew on the plates were tested through limited number of biochemical tests for presumptive identification. Confirmation of the pathogens were done afterwards through pathogen specific tests. The **ompW** PCR were performed for the species confirmation of *V. cholerae*. Strains of *V. parahaemolyticus*, *Shigella* spp and *Salmonella* spp were serotyped using commercially available antisera (Denka Seiken, Tokyo, Japan, BioRad, Marnes-la-Coquette, France). *V. cholerae* O1 strains were serotyped using antisera prepared in NICED.

Three different lactose-fermenting colonies isolated from each sample were picked from MacConkey agar plate and included in the multiplex PCR assay for the detection of different diarrhoeagenic *E. coli* that include enterotoxigenic *E. coli* (ETEC, inclusive of both heat-labile and heat-stable enterotoxin producers), enteropathogenic *E. coli* (typical and atypical EPEC) and enteroaggregative *E. coli* (EAEC). The diarrhoeal stool specimens were stored frozen at -80°C in aliquots of 500 µl each. A total of 122 specimens were selected from the archive of which 68 had aetiologies of bacterial pathogens and remaining 54 were assigned as 'no known pathogen' (Fig. 1). Among the 68 specimens, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Campylobacter* spp., *Shigella* spp., enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC) were identified among 29, 11, 8, 14, 7, 1 and 9 specimens, respectively. Of the 68 specimens, 59 were identified to contain single pathogen and 9 were with mixed pathogens. One aliquot of the selected specimens were thawed and used for DNA extraction by QIAMP DNA stool mini kit (Qiagen, USA). The real-time PCR reanalysis for abovementioned pathogens were performed using 1 µl of DNA solution.

**Bacterial strains and culture condition**
Bacterial strains for *V. cholerae* O1 (N16961 and O395), *V. parahaemolyticus* (KXV139), *Campylobacter* spp. (*C. jejuni* IDH1138, *C. coli* IDH797, *C. fetus* IDH1156), *Shigella* spp. (*S. sonnei* 500228, *S. boydii* 500202, *S. flexneri* ATCC12022), ETEC (500205), EPEC (11044) and EAEC (2075) were used to validate SYBR Green real-time PCR based species specific detection assay. Luria broth (LB) supplemented with 1%, 3% and 0.5% NaCl was used for culturing *V. cholerae*, *V. parahaemolyticus*, and *Shigella* spp., respectively. Diarrheagenic *E. coli* was also cultured in LB supplemented with 0.5% NaCl. *Campylobacter* spp. was cultured for 48 h at 37 °C in brain heart infusion agar plates supplemented with 5% serum under microaerophilic conditions.

**SYBR Green real-time PCR with pure culture**

The 10 pairs of real-time PCR primers used in this study for the detection of *V. cholerae*, *V. parahaemolyticus*, *Campylobacter* spp., *Shigella* spp., ETEC, EAEC and EPEC. *V. cholerae* O1 antigen coding region specific primers were used as described by Hoshino *et al.* [20]. Primers for *V. parahaemolyticus* and *Campylobacter* spp. were used as described by Kurakawa *et al.* [21]. Primers for invasion plasmid antigen H (*ipaH*) were used as specific primers for *Shigella* spp. [22]. One set of ETEC primers (5’-GGCGACAAATTATACCGTGC-3’ and 5’-AAACATATTTGGTGCTGTCGC-3’) specific to labile toxin (*lt*) gene was developed, while another set specific to stable toxin (*st*) were used as described by Fukushima *et al.* [16]. Reverse primers specific to virulence gene *aggR* of EAEC (5’-TCGGAAAAAGAAGCTTACAGCC-3’) and virulence gene *eaeA* of EPEC (5’-CAGAGATCGCGACTGAAGC-3’) were developed and used in combination with respective pathogroup specific forward primers [16]. Validation of the species specific detection of enteropathogens (*V. cholerae*, *V. parahaemolyticus*, *Campylobacter* spp., *Shigella* spp., ETEC, EPEC and EAEC) by real-time PCR was made using boiled lysate as source of DNA template prepared from pure culture and SYBR Green as detecting dye. Boiled lysate was
prepared by suspending one loopful of pure culture in 200 µl of PBS, boiled for 10 min, centrifuged for 5 min at 10000 x g and debris free 1 µl clear supernatant was used directly for real-time PCR. PCR primers were adjusted to a final concentration of 0.6 pmole/ µl in a 20 µl of reaction volume with 1X Power SYBR Green master mix (Applied Biosystems, USA). The real-time PCR was performed in a 7900 HT Fast real-time PCR machine (Applied Biosystems). During pre-PCR, tubes were heated to 50 °C for 2 min followed by 95 °C for 10 min. Subsequently, complete 35 cycles of PCR were performed using 94 °C for 20 s, 55 °C for 20 s and with an extension step of 72 °C for 50 s. Fluorescence signals were measured at the extension step of each of the cycle. Amplicon specificity was established through melting (Tm) curve analysis [23]. The Tm values of the amplicons generated against DNA from each of the included pathogens with respective primers are presented in Fig. 2. Single peak for the amplicons specific to O1 wb of *V. cholerae* O1 (Fig. 2A), 23S rDNA of *V. parahaemolyticus* (B), *lt* of ETEC (C), *st* of ETEC (D), *eaeA* of EPEC (F), 16S rDNA of *Campylobacter* spp. (G) and *epaH* of *Shigella* spp. (H) is evident. The *aggR* amplicon of EAEC, a gave dual peak (Fig. 2 E) which may be considered due to difference in GC content (high G:C content in one area versus another) within the amplicon. Specificity of *aggR* amplification was further confirmed by visualization of single band in the agarose gel electrophoresis. All PCR assays used in this study produced single amplicon when analyzed through agarose gel electrophoresis. Ability to detect specific pathogen was established as this assay could produce single amplicon even with mixed DNA template based on the usage of specific set of primers with similar melt curve generating same Tm as compared to a situation when tested individually with purified DNA template (Fig. 2). For all assays, negative controls were included that comprised of PCR grade water as well as lysates prepared from heterologous organisms.

**Detection and relative quantification of pathogens by real-time PCR**
Bacterial suspensions from pure culture were made and subsequently dilution plating was performed using 10 folds diluted suspensions to estimate number of colony forming units (CFU)/ml of the suspension. From each of the serial dilution tubes as generated for dilution plating, 100 µl of suspension was taken out to prepare boiled lysate and 1 µl of which was used in the real-time PCR. Threshold cycle (Ct) values obtained for each of the dilutions were plotted against normalized CFUs and organism specific standard curve was generated. DNA extracted from diarrheal stool specimens was used directly for detecting enteropathogens and pathogen specific Ct values were recorded. Obtained pathogen specific Ct values were plotted on standard curve for an estimation of the load of the pathogen when present in the diarrheal stool in the form of single or multiple pathogens and expressed as CFU/ml equivalence.

Results

Bacterial enteropathogen detection by real-time PCR

Real-time PCR assay successfully detected *V. cholerae*, *V. parahaemolyticus*, *Campylobacter spp.*, *Shigella spp.*, ETEC, EAEC and EPEC when boiled lysate prepared from respective strains were used. The melt curve analysis of the product obtained in the real-time PCR assay is presented in Fig. 2. Detection of specific melting curve with characteristic $T_m$ for each species confirmed specificity of real-time PCR detection. Amplification was possible only with homologous combinations of pathogen and its primer pairs. A linear relationship was established between the Ct value and number of viable cells included in the assay that ranged between $10^9$ CFU/ml and $10^4$ CFU/ml and such relationship was subsequently utilized to estimate pathogen load equivalence in the stool specimens (Fig. 3).

Application of real-time PCR for pathogen detection in stool specimens and estimation of pathogen load
Of 68 specimens, 59 were previously identified to contain sole pathogen and 9 had mixed pathogens by culture dependent methods (Fig. 1). Reanalysis of the 59 specimens by culture independent real-time PCR showed presence of mixed pathogens in 34 specimens and 25 contained sole pathogen (Fig. 1). In fact, all pathogens detected by culture based assays were also detected in respective specimens by real-time PCR. Detection of additional pathogens through real-time PCR assay resulted in an increase of mixed infections from ca.13% to ca. 50%.

Reanalysis of these 68 specimens by real-time PCR showed matching detection of culture based aetiologies with a pathogen load equivalence ranging between $10^9$ and $10^6$ CFU/ml (Ct values ranged between 13 and 23). Interestingly, Ct value for the pathogens that remained undetected by the culture dependent methods ranged between 25 and 30 that corresponded to pathogen load equivalence ranging between $10^5$ and $10^4$ CFU/ml. A comparative analysis on the pathogen detection among the 68 specimens by real-time PCR against culture dependent methods is presented in Table 1.

The culture independent real-time PCR detection of pathogens was subsequently extended to 54 specimens, which were assigned as "no-known pathogen" by culture dependent methods. The presence of pathogens was detected by real-time PCR in 34 of 54 specimens which were originally assigned as "no-known pathogen" (Fig. 1, Table 2). Of the 34 specimens, 25 and 9 had single and mixed pathogens, respectively. Analysis of pathogen specific Ct values obtained with real-time PCR positive 34 specimens showed pathogen load equivalence that ranged between $10^5$ and $10^4$ CFU/ml equivalence.

**Discussion**

This study was initiated to detect bacterial enteropathogens directly from stool specimens and that targeting pathogen specific virulence genes or rDNA regions. This was an effort to understand the inadequacy, if any, of culture dependent methods in comparison to culture
independent assays. Culture independent real-time PCR based reanalysis of 68 specimens (including sole and mixed pathogens) revealed detection of all aetiologies that were identified by culture dependent methods thereby validating the real-time PCR methods. Interestingly, real-time PCR detected additional pathogens in most of these specimens. In fact, many of the samples, which were reported to contain sole pathogen, were shown to have multiple pathogens following reanalysis by real-time PCR (Fig. 1, Table 1).

The real-time PCR assay revealed an interesting relationship between pathogen load and aetiologies as detected by culture dependent methods. The culture dependent methods based aetiologies was detected in specimens with pathogen load $10^6$ or more CFU/ml. Analysis also revealed $10^4$ CFU/ml equivalence was the limit for detecting pathogens by the real-time PCR assay. This relationship also remained valid with specimens that were identified to have mixed pathogens by culture dependent methods; pathogen load ranged between $10^6$ and $10^7$ CFU/ml equivalence. Approximately one third of hospitalized diarrhoeal cases yielded mixed infections by culture dependent methods as shown in several studies in impoverished settings including recently in Kolkata [3,4,6-8]. While culture dependent methods showed presence of mixed pathogens among 9 (13.2%) cases, real-time PCR based detection increased the percentage of mixed pathogens to 50% (Fig. 1). Comparative analysis of pathogen detection by culture dependent vs. culture independent real-time assay is presented in Fig. 4. It is evident from Fig. 4 that good number of specimens contained multiple pathogens. In fact, in some cases presence of 4 enteropathogens were also detected. Therefore, the real-time PCR based reanalysis established that mixed infections are much higher than previously conceived.

Diarrhoeal surveillance studies have shown that approximately 30% of the specimens do not yield any known aetiologies in diverse geographic settings. A recent study conducted in Kolkata showed that 27.9 % of the stool specimens from hospitalized diarrhoea patients did
not yield any pathogen despite examining the samples for 26 known diarrhoeal pathogens [8]. In this study we therefore extended our analysis to examine 54 specimens that were assigned as 'no known pathogen' by culture dependent methods. However, when examined by real-time PCR 34 of these 54 specimens showed presence of one or more pathogens (Figs. 1 and 4). The density of the pathogens present in these specimens ranged between $10^4$ and $10^5$ CFU/ml equivalence. Existence of pathogen below $10^6$ CFU/ml equivalence, a load below the detection limit, to be considered as basis for under detection of aetiologies by culture dependent assays. This study therefore unequivocally confirmed the ability of culture independent real-time PCR to detect enteric pathogens at lower densities in stool specimens where culture dependent methods failed to detect the same. Considering reanalysis of 122 specimens, real-time PCR detected 102 specimens with one or more pathogens in contrast to 68 specimens with aetiologies by culture dependent methods.

Detection of multiple pathogens in single diarrhoeal stool specimen indicates that the subjects living in this impoverished setting are assaulted by multiple enteric pathogens at any given time. Therefore, the concept of one pathogen one disease might need to be re-evaluated. This study has been carried out with limited number of bacterial enteropathogens. Inclusion of real-time PCR based detection methods for other viral and parasitic pathogens may further enhance the melange of pathogens harboured by subjects living in poorly hygienic conditions. Relative distribution of the pathogens as detected by culture based methods (Table 1 and Fig. 4) should not be construed as true representation of their degree of associations among clinical cases in settings of Kolkata and around as selection of these specimens were only made for a comparative analysis between culture dependent and independent assays.

Polymicrobial infections are common in settings of low resource countries. This is in stark contrast to what is seen in the sanitized developed country settings where the aetiology of diarrhoea is due to single pathogen. As majority of the patients came from low income group
living in poorly hygienic conditions, detection of multiple pathogens in diarrheal stool specimens indicated gross contamination in food and water that they consumed. Synergistic action of microorganisms impacting each other in the polymicrobial infection situations has already been reported for wound infections as well as diarrhoeal cases caused by either EAEC or EPEC [24,25]. Preferential association between enteric pathogens present as mixed infection has also been demonstrated recently [26]. Consumption of grossly contaminated food and water by the majority of the patients living under impoverished conditions lead to infection by multiple pathogens and subsequently to hospitalization. However, the significance of contrasting densities (about 100 folds more of one pathogen as compared to another) of enteric pathogens in mixed infection needs to be addressed in greater detail through a case-control field study to portray actual scenario. Clinical findings of the status of patients having mixed infection will be described in a distinct work. Detection of polymicrobial infections with pathogens in lower densities by the real-time PCR assay raised a concern on likely existence of potentially good number of human carrier. Polymicrobial infections among hospitalized patients thus clearly emphasized the need to pursue more exploratory approach to understand the epidemiological, inter-microbial interactions and clinical implications of the presence of more than one pathogens.

**Acknowledgement**

Part of this study was been presented as Poster in a symposium "Fifty years discovery of cholera toxin: A tribute to SN De", Kolkata, India during October 25-27, 2009.

**Transparency Declaration**

The authors declare no conflict of interest of any nature. The work was supported by fund from Japan Initiative for Global Research Network on Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology of Japan. A.S. and S.S.G is the recipient of Research Assistant Fellowship from the above fund.
Author's Contribution

AS prepared DNA from the faecal samples, performed all Real time PCR assays quantified the load of pathogens present in the stool specimens. SSG, SG, SD, SG and PM isolated the different bacterial pathogens microbiologically from the stool specimens. TK and KN designed some of the primers for this study. AS, AKM, TR, YT, GBN, RKN analyzed the results and wrote the paper. All authors read and approved the final manuscript.

References


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TABLE 1. Real-time PCR based reanalysis of diarrhoeal stool specimens with aetiologies by culture dependent methods

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Number of specimens(^a) with designated pathogen when analyzed by Culture dependent methods(^b)</th>
<th>Number of specimens(^c) with designated pathogen when analyzed by Culture independent methods(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of specimens</td>
<td>Mixed (%)</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>V. cholerae</strong></td>
<td>23 (33)</td>
<td>6 (8.8)</td>
</tr>
<tr>
<td><strong>V. parahaemolyticus</strong></td>
<td>9 (13.2)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td><strong>Campylobacter spp.</strong></td>
<td>3 (4.4)</td>
<td>5 (7.3)</td>
</tr>
<tr>
<td><strong>Shigella spp.</strong></td>
<td>12 (17.6)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td><strong>ETEC</strong></td>
<td>6 (8.8)</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td><strong>EPEC</strong></td>
<td>1 (1.4)</td>
<td>-</td>
</tr>
<tr>
<td><strong>EAEC</strong></td>
<td>5 (7.3)</td>
<td>4 (5.8)</td>
</tr>
</tbody>
</table>

\(^a\)Total number of diarrhoeal stool specimens analyzed were 68 by both culture dependent and culture independent methods

\(^b\)Enteropathogens detection was performed on freshly collected stool specimens following conventional techniques as described [8].

\(^c\)Enteropathogens detection was performed through real-time PCR assay using archived specimens stored at -80 °C.
TABLE 2. Real-time PCR based reanalysis of diarrhoeal stool specimens with ‘No known pathogen’ by culture dependent methods

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Number of specimens(^a) with designated pathogen when analyzed by</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture dependent methods(^b)</td>
<td>Culture independent methods(^c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Single (%)</td>
<td>Mixed (%)</td>
<td>Total</td>
<td>Single (%)</td>
</tr>
<tr>
<td><strong>V. cholerae</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3 (5.5)</td>
</tr>
<tr>
<td><strong>V. parahaemolyticus</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7 (12.9)</td>
</tr>
<tr>
<td><strong>Campylobacter spp.</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Shigella spp.</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4 (7.4)</td>
</tr>
<tr>
<td><strong>ETEC</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9 (16.6)</td>
</tr>
<tr>
<td><strong>EPEC</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>EAEC</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2 (3.7)</td>
</tr>
</tbody>
</table>

Out of 54 specimens 34 were found to contain bacterial pathogen and 20 specimens remained as ‘No known pathogen’

\(^a\)Total number of diarrhoeal stool specimens analyzed were 54 by both culture dependent and culture independent methods

\(^b\)Enteropathogens detection was performed on freshly collected stool specimens following conventional techniques as described [8].

\(^c\)Enteropathogens detection was performed through real-time PCR assay using archived specimens stored at -80 °C.
Diarrheal stool specimens (n=122)

Culture dependent
- Sole (n=59)
- Mixed (n=9)
- No known pathogen (n=54)

Culture independent
- Sole (n=25)
- Mixed (n=34)

- Sole (n=25)
- Mixed (n=9)
- No known pathogen (n=20)

Fig.1 Comparative analysis for detecting pathogens by culture dependent and independent methods
Fig. 2 Melting curve analysis of real-time SYBR Green PCR amplicons of (A) wb of *V. cholerae* O1, (B) 23S rDNA of *V. parahaemolyticus*, (C) *lt* of ETEC, (D) *st* of ETEC, (E) *aggR* of EAEC, (F) *eae* of EPEC, (G) 16S rDNA of *Campylobacter spp.* and (H) *epaH* of *Shigella spp.* Temperature of melting (Tm) of the amplicons specific to 7 different pathogens are indicated. In all the panels bold line (——) represents analysis of PCR amplicons obtained against DNA from each of the abovementioned pathogens used individually with respective primers whereas broken line (-----) represents analysis of PCR amplicons obtained against mixture of 1:100 times diluted DNA (all abovementioned 7 pathogens) with pathogen specific primers.
Fig. 3  Standard curve generated by plotting varying number of *V. cholerae* viable cells against corresponding Ct value obtained in a real-time PCR assay carried out in 20 μl reaction volume with SYBR Green as detecting dye. Estimation of actual counts of viable cells were made by dilution plating method using 10-fold serial dilution of the cells. The average Ct value for duplicate samples obtained per dilution was used for plotting.
Fig. 4 Detection of pathogens (A) in 68 diarrhoeal stool specimens with aetiology by culture dependent and culture independent real-time PCR assays and (B) in 54 diarrheal stool specimens with ‘no known pathogen’ by culture dependent assay but with aetiology when analyzed by real-time PCR assays. White bar (Ⅰ) and black bar (Ⅱ) represents detection of pathogens by culture based assays and culture independent real-time PCR assays, respectively. Number on the side of each bar represents specimens with respective aetiology. Abbreviation: n, total number of specimens analyzed; Vc, *V. cholerae*; Vp, *V. parahaemolyticus*; Cj, *Campylobacter* spp.; and Shig, *Shigella* spp.