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**Original Article**

# Detection of Uncommon Enteric Bacterial Pathogens from Acute Diarrheal Specimens Using SYBR-Green Real Time PCR

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**SUMMARY:** Acute diarrheal disease is a major health problem, and the second most common cause of death in children under 5 years of age. Conventional diagnostic methods are laborious, time consuming, and occasionally inaccurate. We used SYBR-Green real-time PCR for the detection of 10 uncommon bacterial pathogens using fecal specimens from acute diarrheal patients. In the SYBR-Green real-time PCR assay, the products formed were identified based on a melting point temperature curve analysis, and the assay was validated with the respective reference strain. In a retrospective study, we tested 1,184 stool specimens previously examined using conventional culture methods. Enterotoxigenic *Bacteriodes fragilis* was detected in 6.7% of the samples followed by enterotoxigenic *Bacillus cereus* (5.1%), *Clostridium perfringens* (3.9%), and *Aeromonas hydrophila* (3.8%). In the prospective study, *A. hydrophila*, *Staphylococcus aureus*, and *C. perfringens* were predominantly detected in 11 > 5 years of age, using real-time PCR. The real-time PCR assay is comprehensive, rapid, accurate, and well suited for surveillance or diagnostic purposes to detect uncommon bacterial pathogens, and should be useful in initiating appropriate care and thereby reducing patient risk.

## INTRODUCTION

Diarrhea is one of the leading causes of morbidity and mortality in young children, since the annual estimated deaths in developing countries are thought to be more than a million (1,2). Many common etiological agents associated with diarrhea can be identified by conventional culture methods. However, several hospital-based surveillance studies have shown that associated pathogens were not detected in 25–35% of cases (3,4). The procedures used for identifying pathogens vary considerably based on the nature of the infection, the target microorganism, and the laboratory technique (5). However, for several enteric bacterial pathogens, time-consuming traditional culture methods are still in use in many laboratories (6). Real-time PCR is one of the most useful techniques currently used to advance the rapid diagnosis and detection of diarrheal pathogens (7). Depending on the design, this technique has the potential to detect a spectrum of pathogens, including several uncommon enteric bacteria that are difficult to grow/identify by culture methods. In the past, several real-time PCR based assays have been established that allow for the identification of bacterial pathogens (8–11).

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Several studies have shown that real-time PCR is useful in the early detection and quantification of bacterial pathogens, and this may help in the timely initiation of proper clinical care (10,11). The use of real-time PCR provides a great opportunity for the comprehensive and rapid detection of pathogens directly from fecal specimens. Since real-time PCR is useful for the early detection and quantification of bacterial pathogens, it may help in the timely initiation of proper clinical care (12). The aim of the study was to detect uncommon pathogens in stool samples collected from hospitalized acute diarrheal patients, using a SYBR-Green real time PCR assay.

In our previous diarrheal surveillance study, pathogens could not be detected in 28% of the acute diarrheal cases, despite screening for almost 20 common enteric pathogens (4). The importance of other infrequent enteric pathogens is well known, but they are not routinely screened for in developing countries. We have included about 10 uncommon diarrheal pathogens to screen the stool DNA. The target genes used for the real-time PCR are specific for these pathogens and have been intensively investigated in previous studies.

## MATERIALS AND METHODS

Over the period from January to December 2013, 1,184 stool specimens were collected from diarrheal patients of all age groups admitted to the Infectious Diseases Hospital, as well as children younger than 5 years of age who were being treated at the B. C. Roy Memorial Hospital for Children in Kolkata, India. These patients had either watery or bloody diarrhea. Stool

samples were collected using sterile catheter tubes before the administration of any antibiotics, and were transported to the laboratory in sterile McCartney bottles and processed within 2 h. Aliquoted stool samples were stored in a −80°C freezer. In the initial phase of this study, specimens reported as containing no identifiable pathogens were considered for real-time PCR.

Stool specimens were analyzed for all serogroups of *V. cholerae*, *V. parahaemolyticus, V. fluvialis*, diarrheagenic *Escherichia coli* (ETEC, EPEC, EAEC, and EHEC), *Campylobacter* spp. (*C. jejuni*, *C. coli*, and *C. fetus*), *Shigella* spp, and *Salmonella* spp. by standard culture and seological methods (4,13). For uncommon bacterial pathogens, commercially available selective media (Becton Dickinson-Difco, Franklin Lakes, NJ, USA and HiMedia, Mumbai, India) were used for the isolation and identification of bacteria.

Control bacterial strains were procured from the American Type Culture Collection (ATCC). *S. aureus* (ATCC 25923) (14), Enterotoxigenic *Bacillus cereus* (ETBC) (ATCC 14579) (15), *Clostridium perfringens* (ATCC 13124) (16), *C. difficile* (ATCC 9689) (17), *Listeria monocytogenes* (ATCC 35152) (18), *Plesiomonas shigelloides* (ATCC 14029) (12), *Yersinia enterocolitica* (ATCC 23715) (19), Enterotoxigenic *Bacteroides fragilis* (ETBF) (ATCC 25285) (20), *Aeromonas hydrophila* (ATCC 7966) (21), and *Providencia alcalifaciens* (ATCC 27971) (12) were used to validate the SYBR-Green real time PCR-based species-specific detection assay. The reference strains were cultured on non-selective blood agar under aerobic/anaerobic conditions at 37°C for 24 h. Bacterial cells were harvested and suspended in 1 mL of 0.9% NaCl solution, followed by centrifugation at 6,800 × *g* for 5 min. After discarding the supernatants, the cell pellets were used for DNA extraction. Primer pairs with different melting temperatures (*Tms*) for the PCR products were used to amplify the target genes (Table 1).

DNA was extracted from 200 mg of stool using a slightly modified QIAmp Stool Mini Kit protocol (Qiagen, Valencia, CA, USA). Briefly, 1.4 mL of lysis buffer was added to the stool, and then pre-treated by bead-beating using 0.15- mm garnet beads for 2 min, followed by boiling for 10 min before continuing with the manufacturer's extraction protocol. The concentrations of DNA were determined by measuring the optical density at 260 nm with a Nano Drop BioSpectrometer (Eppendorf, Hamburg, Germany). The amount of DNA from positive control strains was adjusted to 20 ng/μL for each run. The DNA was then used immediately for PCR amplification and also stored at −20℃ for future work.

Ten real-time PCR primer pairs were used in this study for the detection of *S. aureus*, ETBC, *C. perfringens*, *C. difficile*, *L. monocytogenes, P. shigelloides*, *Y. enterocolitica*, ETBF, *A. hydrophila,* and *P. alcalifaciens*. The size and *Tms* of the PCR products are shown in Table 1. The specificity and sensitivity of the PCR assay using each primer were confirmed with standard strains as well-known bacterial species. All oligonucleotide primers were synthesized by Sigma (Bangalore, India).

Real-time PCR was carried out using a Roche Light-Cycler 480 (Roche Diagnostics, Penzberg, Germany) using SYBR Green qPCR super mix. Reagents for the real-time PCR assay were purchased from Roche Diagnostics. The Simplex SYBR Green I assay was performed using a SYBR Green master mix (Roche Diagnostics) and was carried out in a 20 μL reaction mixture containing 10 μL of Quantitect PCR master mix (Qiagen), 10 pmol of each of the forward and reverse primers, and 1 μL of nucleic acid sample under the following conditions: an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. The temperature transition rate was 20°C/s except for the annealing step, where the rate was 5°C/s. Fluorescence was measured at the end of each extension step. A melting curve analysis was performed after the last

Table 1. List of the primers used in the real-time PCR assay

| Organism | Target gene | Primer name | Sequence | Product size (bp) | *Tm* | Reference |
|---|---|---|---|---|---|---|
| *Yersinia enterocolitica* | *ail* | AIL-F<br>AIL-R | ATGATAACTGGGGAGTAATAGGTTCG<br>CCCAGTAATCCATAAAGGCTAACATAT | 163 | 79.1 | 19 |
| *Clostridium perfringens* | *cep* | GAP-11-F<br>GAP-12-R | GGTTCATTAATTGAAACTGGTG<br>AACGCCAATCATATAAATTACAGC | 154 | 78.2 | 12 |
| *Providencia alcalifaciens* | *gyrB* | PAG38-F<br>PAG110-R | TCTGCACGGTGTGGGTGTT<br>ACCGTCACGGCGGATTACT | 73 | 79.5 | 12 |
| *Listeria monocytogenes* | *hly* | Lm-hly-F<br>Lm-hly-R | GGGAAATCTGTCTCAGGTGATGT<br>CGATGATTTGAACTTCATCTTTTGC | 106 | 78.3 | 12 |
| *Staphylococcus aureus* | *femB* | FemB-F<br>FemB-R | AATTAACGAAATGGGCAGAAACA<br>TGCGCAACACCCTGAACTT | 93 | 81.6 | 12 |
| *Plesiomonas shigelloides* | *gyrB* | PSG-F64<br>PSG-R313 | TTAACGCCCTGTCGGATAAG<br>TCGAGCAGATGAATCGACAC | 250 | 87.4 | 12 |
| *Aeromonas hydrophila* | *ahh1* | AHH1-F<br>AHH1-R | GCCGAGCGCCCAGAAGGTGAGTT<br>GAGCGGCTGGATGCGGTTGT | 130 | 87.1 | 12 |
| *Enterotoxigenic B. cereus* | *nheB* | SG-F3<br>SG-R3 | GCACTTATGGCAGTATTTGCAGC<br>GCATCTTTTAAGCCTTCTGGTC | 152 | 78.1 | 12 |
| *Clostridium difficile* | *tcdA* | tcdA 441-F<br>tcdA 579-R | TCTACCACTGAAGCATTAC<br>TAGGTACTGTAGGTTTATTG | 158 | 77.4 | 17 |
| *Enterotoxigenic B. fragilis* | *bft* | ETBF-F<br>ETBF-R | GGGACAAGGATTCTACCAGCTTTATA<br>ATTCGGCAATCTCATTCATCATT | 126 | 80.4 | 20 |

amplification cycle by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 60°C, retaining the samples at 45°C for 30 s, followed by slow heating at 0.1°C/s to 85°C. Fluorescence was measured through the slow heating phase. The melting curve analysis was used to identify the specific PCR products derived from each pathogen. A sample was regarded as positive when the LightCycler software determined a crossing point in the qualitative analysis screen. Data was analyzed using the LC480 software (Roche Diagnostics). Color compensation objects were created as described in the LC480 manual.

The sensitivity and specificity of the simplex real-time PCR assay was tested using several different control samples. Bacterial DNA from a pure culture of control samples was diluted using a 10-fold dilution series to estimate the number of CFU/mL in the culture. The threshold cycle (Ct) values obtained for each of the dilutions were plotted against normalized CFUs and an organism-specific standard curve was generated. DNA extracted from diarrheal stool specimens was used directly to detect enteropathogens and pathogen-specific Ct values were recorded. The pathogen-specific Ct values obtained were plotted on a standard curve to estimate the pathogen load present in the diarrheal stool in the form of either single or multiple pathogens and expressed as CFU/mL equivalence.

## RESULTS

In this study, we developed a SYBR-Green based real-time PCR screening system to identify uncommon diarrheal pathogens in stool specimens. This real-time PCR assay can identify virulence genes specific for 10 uncommon diarrheal pathogens. The fluorescent amplification and *Tm* curves for the simplex SYBR-Green PCR products derived from pathogen DNA gave corresponding Ct values ranging from 14 to 27, which corresponded to an equivalent pathogen load ranging between $10^8$ and $10^5$ CFU/mL.

Standard curves were made using the mean Ct values from duplicate 10-fold serial dilutions of DNA that included the target region of amplification and the specificity of the real-time PCR was then evaluated. No cross-amplification or fluorescence signal was observed when the assay was tested using control DNA or any genomic DNA or stool sample infected with different bacterial or protozoan pathogens. The melting curve analysis permitted the distinct identification of pathogens and the *Tm* values for the melting curves were highly reproducible when the assays were conducted in triplicate. The sensitivity of the assay was determined using 10-fold serially diluted genomic DNA samples containing known concentrations of each control DNA. The *Tm* values of PCR the products of stool samples, including each causative pathogen, could be identified based on a *Tm* curve analysis using control bacteria in the same run.

We screened 1,184 specimens collected from January to December 2013. Based on the results of analysis using culture-dependent methods, these samples could be broadly categorized as 'no known pathogen' (571, 48.2%) or 'known pathogen" (613, 51.8%). Using these 1,184 stool DNAs, we further screened for 10 uncommon pathogens namely *L. monocytogenes*, *P. shigelloides*, enterotoxigenic *B. fragilis*, *A. hydrophila*, *P. alcalifaciens*, *S. aureus*, enterotoxigenic *B. cereus, C. perfringens*, *C. difficile,* and *Y. enterocolitica*. The presence of uncommon pathogens could be detected by real-time PCR in 184 (32.2%) of the 571 specimens which had been assigned as containing 'no known pathogen' following our previous protocol. In the total 'no known pathogen' samples, 35 (6.1%) were positive for ETBF, 28 for ETBC (4.9%), 21 for *A. hydrophila* (3.7%), 20 for *C. perfringens* (3.5%), 18 for *P. shigelloides* (3.2%), 17 for *L. monocytogenes* (3.0%), 17 for *C. difficile* (3.0%), 14 for *P. alcalifaciens* (2.5%), 9 for *Y. enterocolitica* (1.6%), and 5 for *S. aureus* (0.9%) (Table 2). In the remaining 613 samples, more than one common pathogen (i.e. mixed pathogens) was detected in 244 (39.8%) samples. In these 244 samples, 45 (7.3%) were positive for ETBF, 32 (5.2%) for ETBC, 24 for (3.9%) *A. hydrophila*, 26 for (4.2%) *C. perfringens*, 21 (3.4%) for *P. shigelloides*, 21 for (3.4%) *L. monocytogenes*, 22 for (3.6%) *C. difficile*, 30 (4.9%) for *P. alcalifaciens*, 5 (0.8%) for *Y. enterocolitica,* and 18 (2.9%) for *S. aureus* (Table 2).

Based on the total number of samples screened, 80 (6.76%) were positive for ETBF, 60 for ETBC (5.07%), 46 for *C. perfringens* (3.89%), 45 for *A. hydrophila* (3.80%), 44 for *P. alcalifaciens* (3.72%), 39 for *P. shigelloides* (3.29%), 39 for *C. difficile* (3.29%), 38 for *L. monocytogenes* (3.21%), 23 for *S. aureus* (1.94%), and 14 for *Y. enterocolitica* (1.18%) (Table 2). As result, the real-time PCR collectively detected uncommon pathogens in 428 (36.1%) of the 1,184 stool specimens.

One hundred and 79 of the 187 patients were exclusively infected by uncommon pathogens (sole infection). The majority of these patients (85.3%) belonged to the older age group (> 5 years old), rather than younger children (< 5 years of age), and had clinical symptoms of watery diarrhea with abdominal pain and some dehydration (Table 3). *A. hydrophila*, *S. aureus*, *C. perfringens*, ETBC, and *Y. enterocolitica* were predominantly detected in older children group. ETBF and *P. alcalifaciens* were found in younger children, but their prevalence was lower compared to that in the older children group (Table 3). With the exception of *L. monocytogenes* and *S. aureus*, most of the other uncommon pathogens were predominantly detected in male patients.

About 244 (39.8%) of the uncommon pathogens were

Table 2. Number of uncommon pathogens present in stool samples by real-time PCR

| Pathogen | Sole pathogen (%) | Mixed with other common pathogen (%) | Total No. of pathogens present |
|---|---|---|---|
| Enterotoxigenic *B. fragilis* | 35 (6.1) | 45 (7.3) | 80 (6.76) |
| Enterotoxigenic *B. cereus* | 28 (4.9) | 32 (5.2) | 60 (5.07) |
| *C. perfringens* | 20 (3.5) | 26 (4.2) | 46 (3.89) |
| *A. hydrophila* | 21 (3.7) | 24 (3.9) | 45 (3.80) |
| *P. alcalifaciens* | 14 (2.5) | 30 (4.9) | 44 (3.72) |
| *P. shigelloides* | 18 (3.2) | 21 (3.4) | 39 (3.29) |
| *C. difficile* | 17 (3.0) | 22 (3.6) | 39 (3.29) |
| *L. monocytogenes* | 17 (3.0) | 21 (3.4) | 38 (3.21) |
| *S. aureus* | 5 (0.9) | 18 (2.9) | 23 (1.94) |
| *Y. enterocolitica* | 9 (1.6) | 5 (0.8) | 14 (1.18) |
| Total | 184 (32.2) | 244 (39.8) | 428 (36.15) |

Table 3. Clinical features of diarrheal patients exclusively infected by uncommon bacterial pathogens

| Organism | Age (%) | | Type of diarrhea (%) | | | Dehydration (%) | | Abdominal pain (%) | Fever (%) | Sex (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ≤ 5 | > 5 | Watery | Liquid | Bloody | Severe | Some | Yes | Yes | Male | Female |
| Enterotoxigenic *B. fragilis* (35) | 9 (26) | 26 (74) | 25 (71) | 8 (23) | 2 (6) | 8 (23) | 27 (77) | 24 (68) | 4 (11) | 17 (49) | 18 (51) |
| Enterotoxigenic *B. cereus* (28) | 2 (7) | 26 (93) | 21 (75) | 7 (25) | — | 4 (14) | 24 (86) | 20 (71) | 6 (21) | 16 (57) | 12 (43) |
| *A. hydrophila* (21) | — | 21 (100) | 17 (81) | 4 (19) | — | 3 (14) | 18 (86) | 16 (76) | 2 (10) | 9 (43) | 12 (57) |
| *C. perfringens* (20) | 1 (5) | 19 (95) | 15 (75) | 5 (25) | — | 4 (20) | 16 (20) | 14 (70) | 2 (10) | 10 (50) | 10 (50) |
| *P. shigelloides* (18) | 4 (22) | 14 (78) | 16 (89) | 2 (11) | — | 3 (17) | 15 (83) | 11 (61) | 2 (11) | 10 (56) | 8 (44) |
| *C. difficile* (17) | 3 (18) | 14 (82) | 15 (88) | 2 (12) | — | 3 (18) | 14 (82) | 12 (71) | — | 9 (53) | 8 (47) |
| *L. monocytogenes* (17) | 2 (12) | 15 (88) | 13 (76) | 3 (18) | 1 (6) | 2 (12) | 14 (88) | 13 (76) | 1 (6) | 6 (35) | 11 (65) |
| *P. alcalifaciens* (14) | 5 (36) | 9 (64) | 10 (71) | 4 (29) | — | 3 (21) | 11 (79) | 9 (64) | 2 (14) | 8 (57) | 6 (43) |
| *Y. enterocolitica* (9) | 1 (11) | 8 (89) | 7 (78) | 2 (22) | — | — | 9 (100) | 6 (67) | 4 (44) | 4 (44) | 5 (56) |
| *S. aureus* (5) | — | 5 (100) | 5 (100) | — | — | 1 (20) | 4 (80) | 5 (100) | — | 2 (40) | 3 (60) |

Table 4. Number of uncommon pathogens present in mixed with other common pathogens

| Organism | *V. cholerae* O1 | *V. parahaemolyticus/ V. fluvialis* | *Shigella* spp. | *Salmonella* spp. | *E. coli* | *Campylo-bacter* spp. | Rota virus | Adeno virus | *Giardia lamblia* | *Crypto-sporidium* |
|---|---|---|---|---|---|---|---|---|---|---|
| Enterotoxigenic *B. fragilis* (45) | 18 (40) | 3 (6) | 1 (2) | — | 9 (20) | 6 (13) | 12 (26) | 4 (9) | 6 (13) | 1 (2) |
| Enterotoxigenic *B. cereus* (32) | 12 (37) | 4 (12) | 2 (4) | 1 (3) | 2 (6) | 4 (12) | 8 (25) | 1 (3) | 3 (9) | 1 (3) |
| *P. alcalifaciens* (30) | 15 (50) | 4 (13) | 1 (3) | — | 3 (10) | 7 (23) | 7 (23) | 2 (6) | 1 (3) | 1 (3) |
| *C. perfringens* (26) | 9 (34) | 3 (11) | 3 (11) | 2 (8) | 2 (8) | 2 (8) | 7 (27) | 2 (8) | 5 (19) | — |
| *A. hydrophila* (24) | 14 (58) | 3 (12) | — | — | 3 (12) | 2 (8) | — | 1 (4) | 5 (29) | 2 (8) |
| *C. difficile* (22) | 10 (45) | 1 (4) | 1 (4) | — | 2 (9) | 1 (4) | 4 (18) | 1 (4) | 2 (9) | 2 (9) |
| *P. shigelloides* (21) | 10 (47) | 3 (14) | 3 (14) | — | 3 (14) | 5 (24) | 3 (14) | — | — | 1 (5) |
| *L. monocytogenes* (21) | 9 (42) | 1 (5) | 1 (5) | — | 3 (14) | 3 (14) | 6 (28) | 2 (10) | 2 (10) | 1 (5) |
| *S. aureus* (18) | 8 (44) | 3 (17) | — | — | 1 (6) | 4 (22) | 8 (44) | 2 (12) | 3 (17) | 2 (12) |
| *Y. enterocolitica* (5) | 1 (20) | 1 (20) | 1 (20) | — | — | — | 2 (40) | 1 (20) | — | — |

detected as co-pathogens in 613 specimens, which were found to contain *V. cholerae*, *V. parahaemolyticus*, *E. coli*, *Shigella* spp., parasites (*Entamoeba histolytica, Giardia lamblia,* and *Cryptosporidium* spp.), or enteric viruses (Rota, Adeno, Sappo, and Noro for viruses) (data not shown). Among the mixed infections, uncommon pathogens with *V. cholerae* were isolated most often (range 34% to 58%), followed by uncommon pathogens with rotavirus (range 14–44%). Details of the mixed infection of uncommon pathogens with other enteric pathogens are shown in Table 4.

In order to confirm the presence of uncommon bacterial pathogens in diarrheal stool specimens, real-time PCR was used to test 250 prospective samples collected over the period from January to March 2015, in parallel with the routine culture method that we used. A total of 42 (16.8%) of these specimens were positive for the 10 uncommon bacterial pathogens by culture methods. Of these 250 specimens, 10 (4%) were positive for *S. aureus* 8 for *L. monocytogenes* (3.2%), 5 for *C. difficile* (2%), 4 for *C. perfringens* (1.6%), 4 for ETBC (1.6%), 3 for (1.2%) each of ETBF, *P. alcalifaciens*, *P. shigelloides*, *Y. enterocolitica,* and 2 for *A. hydrophila* (0.8%).

Real-time PCR analysis of the 250 specimens showed the presence of uncommon pathogens in 88 (35.2%) specimens. As expected, all of the pathogens detected by conventional culture methods were also detected by real-time PCR. The real-time PCR assay resulted in an increase in the detection rate of uncommon enteric pathogens up to 35.2% compared to that by the conventional culture methods (18%) (Table 5). *S. aureus* (6.4%), *L. monocytogenes* (4.8%), *C. perfringens* (4.4%), and ETBF (4.0%) were more frequently detected by real-time PCR compared to that by the culture methods (Table 5).

Table 5. Real-time PCR based re-analysis of diarrheal stool specimens with etiologies by culture-dependent methods ($n$ = 250)

| Pathogen | Culture independent (Real-time PCR) | Culture dependent method |
|---|---|---|
| *S. aureus* | 16 (6.4%) | 10 (4.0%) |
| *L. monocytogenes* | 12 (4.8%) | 8 (3.2%) |
| *C. perfringins* | 11 (4.4%) | 4 (1.6%) |
| Enterotoxigenic *B. fragilis* | 10 (4.0%) | 3 (1.2%) |
| *A. hydrophila* | 9 (3.6%) | 2 (0.8%) |
| *C. difficile* | 7 (2.8%) | 5 (2.0%) |
| *Y. enterocolitica* | 7 (2.8%) | 3 (1.2%) |
| Enterotoxigenic *B. cereus* | 6 (2.4%) | 4 (1.6%) |
| *P. alcalifaciens* | 6 (2.4%) | 3 (1.2%) |
| *P. shigelloides* | 4 (1.6%) | 3 (1.2%) |
| Total | 88 (35.2%) | 45 (18.0%) |

## DISCUSSION

In developing countries, acute diarrheal patients generally have a poly-microbial etiology. This can be correlated with environmental sanitation, hygiene, and poor quality of food or water. In spite of the best coverage of enteric pathogens with multiple assays, several studies have shown that in 25% to 35% of diarrhea cases the etiology remains undetected (4,22). Although *L. monocytogenes* is not known as a diarrheal pathogen, it is recognized as an emerging foodborne opportunistic pathogen, and hence this pathogen was included as one of the ten uncommon pathogens. *C. difficile* is also not known as a diarrheal pathogen unless antimicrobial agents are used for its treatment, although a few reports have shown that *C. difficile* is associated with about 15% diarrhea cases in India (23) and hence, this pathogen was also included as one of the ten uncommon pathogens.

In this study, we have developed a real-time PCR technique to detect uncommon bacterial pathogens in patients with diarrhea. This real-time PCR panel has allowed us to increase the detection rate for enteric pathogens from 18% to 35.2%. Compared to previous studies, our findings showed an increase in the detection rate for bacterial pathogens can be achieved (24). The real-time PCR assay is a rapid, specific and sensitive detection technique capable of detecting several pathogens that can be used for any clinical specimen. Several of these assays have been validated for linearity, limit of detection, precision, as well as sensitivity and specificity and compared to conventional culture and ELISA methods (25). Using real-time PCR, the causative bacteria can be rapidly detected within 3 hr. Almost all the bacterial pathogens can be detected in stool specimens containing $10^3$ to $10^5$ bacteria/g. This is in consideration to the concentration of DNA extracted from the stool specimens and diluted $6 \times 10^4$ fold in the PCR reaction mixture. Generally, the sensitivity of PCR to detect bacteria present in stool samples can be as low as 10 cells in the reaction well (12,26). In this study, we successfully developed a real-time PCR assay for uncommon pathogens from stool specimens by targeting pathogen-specific virulence genes or cDNA regions. The specificity of the target region primer pairs was carefully confirmed by studying various strains of each bacterial species that had been investigated by conventional PCR or real-time PCR methods, as described in other reports (25). In the real time PCR assay, the products formed were identified based on a *Tm* curve analysis. The PCR products generated with each primer pair were based on their *Tm* values (27). Based on *Tm* values, the bacterial pathogens can be detected in stool specimens if they are present in the range of $10^3$ to $10^5$ bacteria/g. The *Tm* values of PCR products derived from the stool samples, including each uncommon bacterial pathogen, can be evaluated along with that of a control bacterial strain in the same run.

In this study, using 1,184 stool specimens collected during 2013, the 10 uncommon pathogens examined could be detected in 428 (36.1%) of these specimens. Two hundred and fifty stool specimens were further examined by real-time PCR to identify the 10 uncommon bacterial pathogens. Forty-five (18%) of specimens were found to be positive for the 10 uncommon bacterial pathogens using culture-based methods. An analysis of the 250 specimens by culture independent real-time PCR showed the presence of pathogens in 88 (35.2%) of these specimens. All the pathogens detected by the culture-based assays were also detected in the respective specimens by real-time PCR, which is in concurrence with our previous study (24). The detection of additional pathogens through the real-time PCR assay resulted in an increase in the detection rate from 18% to 35.2%. The presence of the uncommon pathogens was detected by real-time PCR in 42 of 88 specimens that were originally assigned as containing 'no known pathogen.' An analysis of pathogen-specific Ct values obtained with the 88 real-time PCR-positive specimens showed a pathogen load equivalence that ranged between $10^3$ and $10^5$ CFU/mL. Diarrheal surveillance studies using conventional methods have shown that approximately 30% of specimens do not yield any known etiologies. A study conducted in Kolkata showed that 28% of the stool specimens from hospitalized diarrhea patients did not yield any pathogen, despite examining the samples for about 25 common diarrheal pathogens (4). Our study therefore confirmed the ability of culture-independent real-time PCR to detect uncommon bacterial pathogens, even if they are present in lower numbers in stool specimens. However, 32% of the samples in our study did not have any etiology. Nevertheless, the application of real time-PCR assays will have a good impact on routine diagnostic laboratories, as these bacterial targets could be combined with other enteric pathogens.

The real-time PCR assay represents a major advance in the differential laboratory diagnosis of diarrheal diseases in general. Given the greater performance of the real-time PCR assay developed in this study, we propose its application in routine diagnosis, and in surveillance studies of diarrheal diseases. This method will provide more accurate epidemiological data and a greater understanding of infections by these uncommon bacterial pathogens in humans. Our study could have been strengthened if we had also included non-diarrheal stool samples collected from healthy individuals to prove that the uncommon pathogens included in this study are not reflective of a background infection. Currently, we are working to develop a multiplex real-time PCR assay for the detection of all the uncommon pathogens so that the assay can be performed using one reaction per sample.

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**Conflict of interest** None to declare.

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