Improved Detection of Bacterial Pathogens in Patients Presenting with Gastroenteritis by Use of the EntericBio Real-Time Gastro Panel I Assay

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In this study, we evaluated the use of EntericBio real-time Gastro Panel I (Serosep, Limerick, Ireland) for routine use in a clinical microbiology laboratory for simultaneous detection of Campylobacter jejuni, coli, and lari, Shiga toxin-producing Escherichia coli (STEC), Salmonella spp., and Shigella spp. in feces. This system differs from its predecessor (the EntericBio Panel II system, Serosep) in that it allows real-time detection of pathogens directly from feces, without pre-enrichment. It also specifically detects Campylobacter jejuni, coli, and lari rather than all Campylobacter species, as is the case with the previous system. A total of 528 samples from patients presenting with acute gastroenteritis were screened prospectively with this assay, and results were compared with those of the current method, which combines screening the samples with a molecular assay (the EntericBio Panel II assay) and retrospective culture of the specimens in which the target was detected. Discrepancy analysis was conducted using culture and molecular methods. The real-time assay produced 84 positive results, specifically, Campylobacter spp. (n = 44); Stx1 and/or Stx2 (n = 35); Shigella spp. (n = 3); and Salmonella spp. (n = 6). Of these, 4 samples represented coinfections with Campylobacter spp. and STEC. The real-time assay showed an increased detection rate for pathogens, apart from Salmonella spp.

Four Campylobacter-positive and 6 Stx-positive results remained unconfirmed by any other method used. The isolation rates for PCR-positive samples were as follows: Campylobacter spp., 80%; STEC, 45.7%; Salmonella spp., 100%; and Shigella spp., 66.7%. The sensitivity, specificity, positive predictive value, negative predictive value, and efficiency were 100%, 97.8%, 88.1%, 100%, and 98.1%, respectively.

A
cute infectious gastroenteritis is common and has important economic and social consequences for both communities and health systems due to its high rate of occurrence (1). Worldwide, it is associated with a high incidence of morbidity and mortality (2), particularly among children (3).

Most clinical microbiology laboratories still use traditional, culture-based diagnostic techniques for routine detection of bacterial enteric pathogens. These are both time-consuming and laborious and, in addition, have a prolonged delay in the reporting of results. This can have a significant impact on institutions such as hospitals or nursing homes, where early detection and prevention of disease spread is crucial. Molecular detection of pathogens has, however, been shown to be faster and more sensitive than traditional culture (4, 5). It is especially important where culture-based pathogen detection is problematic or lacks sensitivity. The best example of this is the genus Campylobacter, certain members of which, such as C. jejuni, can remain viable but noncultivable (6), therefore producing false-negative results if detection is based solely on a culture. Moreover, some species which can cause gastroenteritis, such as C. upsaliensis, are unlikely to be correctly isolated and identified on common media (7). Furthermore, the exact role of some fastidious Campylobacter spp. (such as C. ureolyticus or C. concisus) in gastroenteritis is still under investigation despite their being detected in large proportions of samples from patients with gastroenteritis (8, 9).

Here, we present a validation study of EntericBio real-time Gastro Panel I (Serosep, Limerick, Ireland), a commercial real-time PCR system (CE marked for in vitro use) for simultaneous detection of Campylobacter jejuni, coli, and lari, Shiga toxin-producing Escherichia coli (STEC) (through detection of Stx1 and Stx2), Shigella spp., and Salmonella spp. directly from patients’ feces.

MATERIALS AND METHODS

Patient samples. Between 17 August 2012 and 3 October 2012, a total of 528 feces samples were submitted to the Clinical Microbiology Laboratory in Cork University Hospital, Ireland, for routine investigation for enteric bacteria. All samples were from patients presenting with symptoms of gastroenteritis. The samples were screened prospectively with the EntericBio real-time Gastro Panel I (Serosep, Limerick, Ireland) assay in parallel with routine sample testing.

Routine laboratory detection. Routine feces analysis in this laboratory is based on simultaneous molecular detection of four major enteric pathogens, Campylobacter spp., Shiga toxin-producing E. coli (STEC), Shigella spp., and Salmonella spp., using the EntericBio Panel II (Serosep) system followed by confirmation of the positive results by culture and/or by the National Reference Laboratory. The details of the EntericBio Panel II system were previously described by Koziel et al. (10). Briefly, the EntericBio Panel II system combines overnight enrichment of the fecal sample with DNA extraction (through sonication), multiplex PCR, and detection of the targets by hybridization and color development. Samples identified as positive with the EntericBio Panel II system for any of the four targets were subject to further investigation as shown in Table 1. All positive DNA extracts were stored at −20°C for further analysis.
**TABLE 1** Routine confirmation of positive results obtained with the EntericBio Panel II system

<table>
<thead>
<tr>
<th>Organism(s)</th>
<th>Culture methoda</th>
<th>Identification method</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter spp.</em></td>
<td>CCDA (W11015, Lip, Ireland); microaerophilic environment at 42°C for 48 h</td>
<td>Typical macroscopic and microscopic appearance</td>
</tr>
<tr>
<td>STEC</td>
<td>N/A</td>
<td>Confirmation by National <em>E. coli</em> Reference Laboratory, Cherry Orchard, Dublin, Ireland</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>DCA (CM0227; Oxoid) and XLD (Lab 32; LabM) agars; incubation 24–48 h at 37°C</td>
<td>API20E, serological methods; confirmation by National <em>Salmonella</em>, <em>Shigella</em> &amp; <em>Listeria</em> Reference Laboratory, Galway, Ireland</td>
</tr>
<tr>
<td><em>Shigella spp.</em></td>
<td>DCA and XLD; incubation 24–48 h at 37°C</td>
<td>API20E, serological methods; confirmation by National <em>Salmonella</em>, <em>Shigella</em> &amp; <em>Listeria</em> Reference Laboratory, Galway, Ireland</td>
</tr>
</tbody>
</table>

a CCDA, cefoperzone charcoal desoxycholate agar; DCA, desoxycholate citrate agar, XLD, xylose lysine desoxycholate agar; N/A, not applicable.

**EntericBio real-time Gastro Panel I assay.** The system allows multiplex detection of the following pathogens: *Campylobacter spp.*, STEC (detectors of *stx1* and *stx2* genes), *Salmonella spp.*, and *Shigella spp.* It differs from the previous version of the system in that it specifically detects *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* species whereas the EntericBio Panel II system detects all *Campylobacter spp.* According to the manufacturer, the limits of detection of the assay are 47 *Salmonella* cell equivalents, 14 *Shigella* cell equivalents, 11 *Campylobacter* cell equivalents, 13 *Stx1* equivalents, and 19 *Stx2* cell equivalents (probability ≥ 95% by Probit analysis). All aspects of the assays were conducted in accordance with the manufacturer’s instructions.

**Specimen preparation.** Fecal samples were tested directly without pre-enrichment, approximately 1 day after arrival in the laboratory, in parallel with the routine testing. After arrival in the laboratory, and prior to testing, all samples were stored at 4°C. Samples were inoculated onto an EntericBio Sample Processing Solution (S.P.S.) tube (Serocept) with a FLOQSwab (Copan, Brescia, Italy), and the suspension was heated in a heating block (QBD4; Grant, Shepreth, United Kingdom) at 97°C for 30 min to liberate the bacterial DNA. Samples were allowed to cool before being pipetted onto real-time PCR strips. The real-time PCRs were prepared in 20 μl by pipetting 15 μl of EntericBio resuspension buffer (Serocept) and 5 μl of sample suspension (from the top) onto PCR strip parts A (containing reagents for detection of *Salmonella* and *Shigella* spp.) and B (Campylobacter spp. and Stx1 and Stx2), as supplied by the manufacturer. Pipetting of the resuspension buffer and samples was performed using an automatic pipetting station (epMotion 5070; Eppendorf, Stevenage, United Kingdom). The strips were then shaken to mix all the reagents for 1 min at 1,500 rpm in a plate shaker (MixMate; Eppendorf) and centrifuged for 30 s at 1,500 rpm (Centrifuge 5430; Eppendorf) before real-time amplification. The remaining volume of each extracted sample was stored at −20°C for further analysis.

**Controls.** Positive and negative process controls and a kit (positive) control were included in every run to assess the efficiency of DNA extraction and amplification. The positive process control was prepared by adding a mixture of either *Campylobacter jejuni* (ATCC 29428) and *E. coli* O157 positive for type 1 and type 2 Shiga toxin (Stx1 and Stx2) (confirmed wild-type isolate) or *Shigella sonnei* and *Salmonella enterica* serovar Typhimurium (confirmed wild-type isolates) onto a S.P.S. tube and heating it along with the samples. The negative process control consisted of an uninoculated S.P.S. tube. The positive kit control for all targets, consisting of a mixture of genomic DNAs isolated from *Salmonella enterica* (ATCC 29428) and *E. coli* O157:H7 (Stx1 and Stx2 positive), *Shigella flexneri*, and *Campylobacter jejuni*, was supplied by the manufacturer, and this was included in each run to assess the performance of the kit during PCR amplification and detection. Additionally, synthetic chimeric plasmid was included as an internal amplification control (IAC) in each reaction mix (as one of the components of the lyophilized reagents) to assess possible real-time PCR inhibition.

**Real-time assay and data interpretation.** Real-time amplification was carried out in a LightCycler 480 II instrument (Roche, West Sussex, United Kingdom). The amplification conditions were as follows: 45 cycles of 95°C for 10 s and 66°C for 30 s followed by 1 cycle of 40°C for 10 s. Fluorescence measurements were taken at the end of the annealing step. Data were acquired and analyzed with LightCycler 480 software v.1.2.1.62 (Roche). The crossing point (Cp) was determined by the software using the Second Derivative Maximum method. Since the assay employs hydrolysis probes for each pathogen target, no melting curve analysis was performed. The distinction of the individual pathogen targets in strips A and B is based on target-specific probes labeled with different fluorophores with distinct excitation and emission spectra. The results were interpreted on the basis of the presence or absence of the sigmoidal amplification curve, i.e., the increase in fluorescence signal for each pathogen target. Moreover, the presence of IAC amplicons in both strips (A and B) signified that an individual test was valid. A real-time PCR run was considered valid when the process control and kit control were positive and the negative control was negative.

**Confirmation of results.** In cases where discrepant results were obtained between the two systems, alternative culture and molecular confirmation methods were used.

Culture of all additional positive samples obtained with the EntericBio real-time Gastro Panel I assay was attempted in accordance with methods outlined in Table 1. When the results could not be confirmed by culture, molecular methods (conventional PCR followed by real-time PCR) were employed for confirmation. All PCR products (conventional and real time) were electrophoresed on 2% agarose gels (Sigma-Aldrich, Wicklow, Ireland) stained with ethidium bromide and visualized under UV light using a high-performance UV transilluminator (UVP, Cambridge, United Kingdom).

All conventional PCRs in this study were performed in a thermal cycler (model 2720; Applied BioSystems, Warrington, United Kingdom) in a 25-μl reaction volume containing 2 μl of DNA template, 1 μl of HotStarTaq Plus DNA polymerase (203603; Qiagen, West Sussex, United Kingdom), 2.5 μl of 10× CoralLoad PCR buffer, and 0.5 μl of 25 mmol/liter MgCl2 (provided with HotStarTaq Plus DNA polymerase), 4 μl of a mixture of deoxyribonucleotide triphosphates (dNTPs) (Sigma-Aldrich Ireland, Arklow, Ireland) (1.25 mmol/liter of each dNTP), and 1 μl of each of the relevant primers described later (Eurofins MWG Operon, London, United Kingdom) (25 pmol/μl). The cycling conditions were as follows: initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 1 min (target-specific temperature), extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

All real-time PCR procedures were performed using a LightCycler 480 instrument (Roche) and a LightCycler 480 SYBR green 1 Master kit (catalog no. 04707516001; Roche) and the primer set described for the relevant conventional PCR. PCR preparation and cycling conditions were as suggested in the manufacturer’s instructions, and the annealing temperatures were set as for conventional PCR.

**Campylobacter spp.** For additional *Campylobacter*-positive results obtained with both the EntericBio real-time Gastro Panel I and the EntericBio Panel II assays, the remaining DNA aliquots were tested with...
previously published species-specific primers for the presence of *C. jejuni*, *C. coli*, and *C. lari* (12) by both conventional and real-time PCR. In cases where these were negative, *Campylobacter*-specific 16S rRNA primers (13) were used for confirmation of the additional positives followed by sequencing of 816-bp PCR amplicons (Eurofins MWG Operon, Germany) with the same set of primers and sequence analysis with the BLAST algorithm on the NCBI database. Annealing temperatures for *Campylobacter* confirmation tests were set at 62°C, 60°C, 53°C, and 60°C for *C. jejuni*, *C. coli*, *C. lari*, and *Campylobacter* 16S rRNA-specific PCR, respectively. Additionally, for identification of *Campylobacter*-positive stool samples to the species level, *C. ureolyticus*-specific PCR (8) was employed, since a high prevalence of this bacterium in patients with gastroenteritis was reported in previous studies (10, 14).

**Stx1 and/or Stx2.** Additional Stx1- and/or Stx2-positive stool samples were incubated in EntericBio enrichment broth (Serosep) for 24 h at 37°C, and then 1 μl was spread on a MacConkey agar plate (CM0007; Oxoid, Cambridge, United Kingdom) and incubated for 24 h at 37°C. Five presumptive *E. coli* colonies were selected, and a colony PCR for detection of the presence of *stx*1 and *stx*2 genes using the uniplex PCR method described by Paton and Paton (15) was performed. Original stored DNAs of samples which failed to be confirmed by the method described above were tested with an alternative molecular method by both conventional and real-time PCR, using Paton and Paton primers (15) and the methods described above.

**Shigella spp.** Discrepant Shigella results were confirmed either by culture (as described in Table 1) or by an alternative molecular method using *ipaH*-specific primers (16) for samples that failed to grow. PCR amplicons of the correct size were then sequenced to confirm the presence of the *Shigella ipaH* gene.

### RESULTS

**Molecular detection of pathogens.** The results of the comparative prospective study of 528 feces samples using the EntericBio Panel II and EntericBio real-time Gastro Panel I systems are summarized in Table 2.

**Confirmation of positive results.** Any discrepant results between the two systems obtained during the study were investigated further by first using the culture methods previously outlined in Table 1 followed by alternative molecular methods (conventional PCR, real-time PCR, sequencing, and BLAST analysis). The data from the analysis of the discrepant results are summarized in Table 3.

**Campylobacter-positive samples.** A total of 40 samples tested positive for *Campylobacter* spp. with both systems. The EntericBio real-time Gastro Panel I assay detected an additional 4 samples positive for *Campylobacter* spp., and 7 samples which tested positive for *Campylobacter* spp. with the EntericBio Panel II assay were negative with the real-time assay. None of the additional positives was cultured on cefoperzone charcoal desoxycholate agar (CCDA) plates using the methods outlined in Table 1; therefore, all were tested using alternative molecular methods. The seven additional positives not detected by the real-time assay were

### TABLE 3 Summary of confirmation of discrepant results obtained with both systems

<table>
<thead>
<tr>
<th>Organism(s)</th>
<th>Conventional PCR</th>
<th>Real-time PCR</th>
<th>BLAST analysis</th>
<th>Total</th>
<th>Conventional PCR</th>
<th>Real-time PCR</th>
<th>BLAST analysis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em> spp.</td>
<td>0</td>
<td>7</td>
<td>N/A</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Stx1/Stx2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>N/A</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shigella</em> spp.</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>N/A</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Culture of *Campylobacter* - and *Shigella*-positive samples was performed as described in Table 1. Stx-positive cultures were grown on MacConkey agar, and presumptive colonies were tested for the presence of *stx* with PCR.

b Seven additional *Campylobacter*-positive samples were detected by the EntericBio Panel II assay only. All were confirmed to be *C. ureolyticus*.

c Four additional *Campylobacter*-positive samples were detected by the EntericBio Real-Time Gastro Panel I assay. These were not confirmed by any of the additional assays employed and were therefore deemed false positives.
tested for the presence of *C. jejuni*, *C. coli*, and *C. lari* (the *Campylobacter* spp. specifically detected by the real-time assay) to assess the specificity of the assay. None of them tested positive, and *Campylobacter*-specific 16S rRNA PCR was therefore used to identify the species present in these samples. Sequence and BLAST analysis revealed that all of these 16S rRNA sequences were highly similar to the *C. ureolyticus* 16S sequences deposited in the database. This was later confirmed by testing the samples for the *C. ureolyticus*-specific hsp60 gene (8). These samples were therefore deemed true negatives. The additional four samples which tested positive with the EntericBio real-time Gastro Panel I system were not confirmed by any of the employed molecular methods and were therefore regarded as false positives.

The isolation rate for *Campylobacter* spp. detected by the molecular screening was high; 35 samples were cultured on CCDA plates, accounting for 80% and 74% of all *Campylobacter*-positive samples detected by the EntericBio real-time Gastro Panel I and the EntericBio Panel II assays, respectively. A total of 9 samples that were positive with the real-time assay failed to grow on CCDA agar; 4 of those were later determined false positives, as the presence of Campylobacter spp. could not be confirmed.

All of the *Campylobacter*-positive stool samples obtained with both systems were identified to the species level with the alternative PCR described previously, and the results revealed that 51.1% (*n = 24*) were positive for *C. jejuni*, 14.9% (*n = 7*) were positive for *C. ureolyticus* (detected only by the EntericBio Panel II assay), and 6.4% (*n = 3*) were positive for *C. coli*. Six samples (12.8%) were part of mixed infections with *C. jejuni* and *C. ureolyticus*. Analysis of the 16S rRNA of 6 samples (12.8%) was positive for *C. jejuni* and/or *C. coli*, but the exact species could not have been determined due to the high similarity of the 16S rRNAs of these two species. The species of one *Campylobacter*-positive sample remained unidentified. The seven samples where *C. ureolyticus* was detected as the only *Campylobacter* species present were the previously mentioned samples detected only by the EntericBio Panel II system. The EntericBio Real-Time Gastro Panel I assay did not detect these samples. Samples positive for *C. ureolyticus* (*n = 6*) were detected only by the real-time assay when they were part of the coinfection with *C. jejuni*.

Since none of the tested feces samples was positive for *C. lari*, additional tests were carried out to investigate the ability of the EntericBio real-time Gastro Panel I assay to detect this *Campylobacter* sp. Ten feces samples which previously tested negative for *Campylobacter* spp. were inoculated into S.P.S. tubes, and each was spiked with *C. lari* subsp. *lari* DSM 11375 DNA at final concentrations of 0.1 ng/µL, 25 pg/µL, and 2.5 pg/µL. Following this, the samples were processed in accordance with the manufacturer’s instructions for routine testing. All spiked samples (for all *C. lari* concentrations) were reported as *Campylobacter* positive by the EntericBio real-time Gastro Panel I assay.

### Table 4: Serotype and Shiga toxin profiles for 35 Stx-positive samples detected with the EntericBio real-time Gastro Panel I system

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>No. of positive samples</th>
<th>Stx1</th>
<th>Stx1 + Stx2</th>
<th>Stx2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>O26</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>O111</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>O145</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Not isolated</td>
<td>2</td>
<td>5</td>
<td>6*</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Not confirmed</td>
<td>2</td>
<td>0</td>
<td>4*</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

* One sample was from a patient with previous O26 (Stx1/Stx2) infection.

### Table 5: Overall performance of the EntericBio real-time Gastro Panel I and the EntericBio Panel II assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>% sensitivity</th>
<th>% specificity</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>% efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>EntericBio Panel II</td>
<td>92.7</td>
<td>100</td>
<td>100</td>
<td>98.7</td>
<td>98.9</td>
</tr>
<tr>
<td>EntericBio real-time Gastro Panel I</td>
<td>100</td>
<td>97.8</td>
<td>88.1</td>
<td>100</td>
<td>98.1</td>
</tr>
</tbody>
</table>

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*Stx1- and/or Stx2-positive samples.* A total of 24 samples tested positive for the presence of Stx1 and/or Stx2 using the method currently in place in our laboratory. A total of 11 additional positive results were obtained with the EntericBio real-time Gastro Panel I assay; however, 6 of these could not be confirmed by the alternative PCR methods employed and were therefore treated as false positives for the purpose of this study. Sixteen STEC isolates were collected from the samples by the National *E. coli* Reference Laboratory, and the presence of Shiga toxin was confirmed with alternative molecular methods in 13 of the culture-negative samples. The serotypes and Shiga toxin profiles of the positives detected in this study are outlined in Table 4.

### Salmonella-positive samples.
Six fecal samples tested positive for the presence of *Salmonella* spp. with both systems. All of these were confirmed by culture using the methods outlined in Table 1. *S. enterica* Typhimurium (*n = 2*) and *S. enterica* Enteritidis (*n = 2*) were the most commonly isolated serotypes. One sample was positive for *S. enterica* Dublin, and one was determined to be *S. enterica* Overschie.

### Shigella-positive samples.
Two *Shigella* spp. were detected with the EntericBio Panel II assay, and one additional sample tested positive with the EntericBio real-time Gastro Panel I assay. The two positive results were confirmed by culture as *Shigella sonnei* and *Shigella dysenteriae*. The additional positive result obtained with the real-time assay did not yield a positive culture using the methods previously outlined, but the presence of the *ipaH* target was confirmed with the alternative molecular assay, and the sequence analysis revealed 99% identity to the *Shigella flexneri* *ipaH* gene sequence. This result was therefore classified as a true positive, but this information was not used clinically as this was a validation study only.

The performance of the EntericBio real-time Gastro Panel I assay was compared with the performance of the EntericBio Panel II assay in this study. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency for both systems are presented in Table 5.

These did not vary substantially for the individual pathogens detected by the EntericBio real-time Gastro Panel I assay, with sensitivity, specificity, PPV, NPV, and efficiency of 100%, 99.2%, 90.9%, 100%, and 99.2%, respectively, for *Campylobacter* spp. and 100%, 99.8%, 82.9%, 100%, and 98.9%, respectively, for *Stx1/Stx2*.

The system reported 10 positive results which could not be confirmed by any of the culture and molecular methods employed, and they were treated as false positives for the purpose of...
this study. Reaction inhibition was observed in 4 of 532 samples tested (0.75%), and these samples were excluded from the study, as the inhibition was present even on re-extracting the sample. All of the inhibited samples tested negative for the screened pathogens with the EntericBio Panel II system.

**DISCUSSION**

The purpose of this study was to investigate the possibility of improving the detection of major bacterial pathogens causing gastroenteritis by implementing the EntericBio real-time Gastro Panel I assay for routine sample screening. The sensitivity of pathogen detection directly from feces samples, without prior overnight enrichment, was also assessed. The improved sensitivity of retrospective culture, guided by molecular detection, compared to that of traditional culture methods has already been demonstrated by previous studies (4, 5). However, prior to this study, the system used for routine detection of enteric pathogens in our laboratory required overnight sample enrichment, before DNA extraction and detection, in addition to retrospective culture of the positive results. The turnaround time for reporting was a minimum of 24 h for negative samples and between 72 and 120 h for positive samples. The EntericBio real-time Gastro Panel I assay is technically capable of generating results within 3 h of sample receipt in the laboratory, though feces samples are processed in batches for reasons of economy. This shorter turnaround time for positive samples with the EntericBio real-time Gastro Panel I assay has clear implications for more prompt clinical management and infection prevention and control as well as for the public health management of cases of infectious gastroenteritis.

Of all the *Campylobacter* spp. detected using the real-time system, 80% were confirmed by culture on CCDA plates. The difficulty in culturing campylobacters may largely be attributed to the occurrence of isolates in a viable but noncultivable state. Cells in this state cannot be cultured on artificial media; however, they can remain virulent and retain the capability of invading intestinal cells (6). Therefore, the molecular detection of pathogenic *Campylobacter* spp. may still be of significant clinical importance for patients despite the failure to culture these organisms. The discrepancy in the isolation rates between the two systems resulted mostly from the fact that the detection of *Campylobacter* spp. with the EntericBio Panel II assay was based on *Campylobacter* genus detection. Consequently, we were unable to grow *Campylobacter* spp. (such as *C. ureolyticus* or *C. concisus*) which have more fashidious requirements for growth conditions than those provided by the routine culture methods in most clinical laboratories. However, the virulence characteristics and the role of these and some other *Campylobacter* spp. in gastroenteritis have yet to be elucidated; therefore, their clinical importance for diagnostic laboratories remains to be established.

As a result of this study, we discontinued culturing of *Campylobacter*-positive samples and report them solely on the basis of real-time detection, which has decreased the time to reporting of these positive results to a maximum of 24 h. The overall number of *Campylobacter*-positive samples remains high (more than 8% of all samples tested), and this reflects the situation observed in Ireland (17) and the European Union, where *Campylobacter* infections are estimated to cause 9.2 million infections a year with a significant financial cost (€2.4 billion per annum) (18). Interestingly, in the current study, coinfections with *Campylobacter jejuni* and *C. coli* and STEC were observed in four samples.

During the course of this study, 35 (6.6%) samples tested positive for Stx1 and/or Stx2, making STECs the second most common bacterial cause of gastroenteritis. The reported number of STEC cases in Ireland in 2011 was 283, and the incident rate of STEC infections in Ireland tends to be quite high relative to other European countries (17). However, only 45.7% of the detected positives were confirmed by culture. The isolation rates reported by other groups range from 17% to 18% (5) and from 39.5% (19) to 88.9% (20). However, it is important to mention the possibility of stx genes being occasionally present in members of *Enterobacteriaceae* other than *E. coli*, such as *Citrobacter freundii* (21) or *Enterobacter cloacae* (22). Even though PCR detection of stx genes does not allow one to distinguish STECs from other organisms which can occasionally carry these genes, some reports suggest they might be equally important from the clinical perspective, as they have been found to be associated with cases of gastroenteritis and hemolytic uremic syndrome (21, 22).

A large subset of our isolates were O157 serotypes (56.3%); however, non-O157 serotypes were also detected in a substantial proportion of the samples tested—O26 (18.8%) and both O111 and O145 (12.5% each). Other studies reported higher proportions of non-O157 STECs of 55% (20) and up to 80% (23). In 2011, non-O157 STEC infections were responsible for 26.5% of STEC infections in Ireland (17), with some regional variation observed between clinical laboratories, most likely resulting from various routine diagnostic methods for this pathogen. As many of STEC cases in Ireland have a seasonal distribution with a peak incidence in late summer (17), the true prevalence was difficult to assess over the relatively short time this study was conducted.

In many diagnostic laboratories, the routine culture isolation of STECs is biased toward the O157 serotype; therefore, the other emerging STEC serotypes of clinical importance are likely to be underreported. The isolation of the O157 serotype is based on the inability of most strains of this serotype to ferment sorbitol, which facilitated the development of differential media, such as sorbitol MacConkey (SMAC) agar. Unfortunately, non-O157 STEC serogroups lack such a distinct phenotypic feature which would help in their isolation from other, non-stx-positive strains. Some selective chromogenic media for STECs, such as CHROMagar STEC (CHROMagar Microbiology, Paris, France), have been developed and were shown to be successful in isolating a large proportion (75%) of STEC strains; however, not all STEC serotypes, including sorbitol-fermenting O157 strains, were grown successfully on this medium (24). Therefore, PCR methods based on detection of stx genes offer quick and sensitive detection of STECs in fecal samples and are particularly suited to routine diagnosis in many clinical laboratories nowadays. The EntericBio real-time Gastro Panel I assay proved to be more sensitive in STEC detection by reporting 21% more stx-confirmed samples than the previous version of the system. Five samples positive for stx were missed by the previous version of the system. However, these samples did not produce STEC on culture but the presence of an stx gene(s) in them was confirmed by alternative PCR. The difficulty in growing non-O157 STEC serotypes has already been discussed.

The overall percentage of *Shigella*-positive samples detected in our study is quite low (0.6%), and others found higher proportions of *Shigella* spp. at 1.4% (5) and 23.4% (25) with different real-time assays. The difference in the proportions of *Shigella*—
positive samples detected in our study is, most likely, a result of a low prevalence of this pathogen in Ireland. According to the national data published for 2011, only 42 cases were reported nationwide and an ongoing decrease has been observed since 2008 (17).

The detection of Shigella spp. was improved with the EntericBio real-time Gastro Panel I system, which detected one additional positive sample directly from feces. However, this sample failed to yield Shigella spp. on culture. It is likely that the traditional culture methods do not offer the same sensitivity as the real-time assay, which can be particularly important in the detection of pathogens such as Shigella spp., for which an infectious dose could be as low as 10 cells (26). However, the ipaH target in this detection was confirmed by sequencing to be 99% similar to the Shigella flexneri ipaH gene. The enriched DNA extract (used for the EntericBio Panel II assay) was also retested with the EntericBio real-time Gastro Panel I assay and was positive for the Shigella ipaH target. Moreover, the clinical details supplied for this patient (foreign traveler, bloody diarrhea) also suggested possible shigellosis. It is also important to mention the possibility of the ipaH plasmid being present in enteroinvasive E. coli species (EIEC), which would not be differentiated by this assay from Shigella spp. However, the importance of such a differentiation is of epidemiological rather than clinical significance, since infections by the two pathogens can result in very similar disease states. Finally, we note that the detection of Salmonella spp. was the same in both systems.

In summary, this system showed improved detection of pathogens, although the degrees of improvement differed for individual bacteria.

The overall performance of the real-time assay was good, giving sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency of 100%, 97.8%, 88.1%, 100%, and 98.1%, respectively, and a low level of sample-related inhibition.

These parameters did not vary substantially for individual pathogens, with sensitivity, specificity, PPV, NPV, and efficiency of 100%, 99.2%, 90.9%, 100%, and 99.2%, respectively, for Campylobacter spp. and 100%, 99.8%, 82.9%, 100%, and 98.9%, respectively, for stx1/stx2.

Overall, 10 positive results reported by the real-time system could not be confirmed. The crossing point (Cp) values were low (Cp ≥ 31.28) in the unconfirmed samples, which might suggest a low level of pathogens in the specimen. This explanation may be supported by the fact that two unconfirmed samples, which tested positive for stx with the EntericBio real-time Gastro Panel I assay, were from patients who tested positive for the presence of STEC with a sample submitted to our laboratory on a different occasion. Moreover, 4 unconfirmed Campylobacter-positive samples and 3 stx-positive samples were negative with the EntericBio real-time Gastro Panel I assay when retested from fresh samples. Further studies would be required to assess the clinical relevance of the weak real-time positive results.

We have also investigated the sensitivity of pathogen detection by the EntericBio real-time Gastro Panel I assay directly from feces samples, without pre-enrichment, as well as following the overnight enrichment. Enriched samples prepared for the EntericBio Panel II assay were used for this purpose—both were tested prospectively with the EntericBio real-time Gastro Panel I assay. The enrichment did not seem to improve the detection of pathogens, and no additional positives were detected with the EntericBio real-time Gastro Panel I assay following the enrichment of the samples. However, the enrichment seemed to eliminate the weak positive samples, which could not be confirmed; none of the four extra Campylobacter spp. detected directly from the feces was detected by the real-time assay after enrichment, and only 3 of 6 unconfirmed stx-positive samples were detected in the enriched samples. This result might be due, as previously mentioned, to low levels of pathogens in these samples, which were diluted with the enrichment below the level of detection for the EntericBio real-time Gastro Panel I assay.

The EntericBio real-time Gastro Panel I system offers a rapid and sensitive CE-marked method for detection of major gastrointestinal pathogens in the clinical laboratory setting and facilitates prompt reporting of results. It is also 15% less expensive than the EntericBio Panel II assay. The major disadvantage of PCR-based systems is, as ever, the inability to differentiate between results reflecting the presence of a living organism and possible artifact DNA from previous infections or dead organisms. Therefore, traditional culture techniques remain necessary for this reason and also for epidemiological purposes and to obtain antimicrobial sensitivities.

Nevertheless, the high negative predictive value and sensitivity with fast result reporting compared to culture and the EntericBio Panel II assay make the EntericBio real-time Gastro Panel I system well suited for routine screening of large numbers of feces samples from symptomatic patients or outbreak-related contact patient investigations, and it is for these reasons that this method has been adopted for routine use in our laboratory.

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