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Validation of the EntericBio Panel II® multiplex polymerase chain reaction system for detection of *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and verotoxigenic *E. coli* for use in a clinical diagnostic setting☆

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ABSTRACT

A total of 717 faeces samples were tested prospectively using the EntericBio Panel II® detection system (Serosep, Limerick, Ireland), in parallel with routine laboratory testing, which combines the EntericBio® system with retrospective culture of each specimen where a target is detected. Discrepancy analysis was conducted using molecular methods. The EntericBio Panel II® assay produced 585 negative and 132 positive results, namely, *Campylobacter* spp. ($n = 66$); SLT 1 and/or SLT 2 ($n = 64$); *Salmonella* spp. ($n = 5$); and *Shigella* spp. ($n = 0$). Three samples were positive for more than 1 target. Of these results, 4 *Campylobacter* spp. detections and 4 SLT 1/ SLT 2 detections remained unconfirmed, and the system failed to detect 2 *Campylobacter* spp. targets detected by routine laboratory detection. The sensitivity, specificity, positive predictive value, negative predictive value, and efficiency were calculated to be 98.4%, 98.7%, 93.9%, 99.7%, and 98.6%, respectively.

1. Introduction

Culture-based detection of bacterial enteric pathogens is time consuming and may require up to 96 h to generate a definitive result. However, the use of molecular detection methods in combination with overnight faecal enrichment has the potential to reduce the time to diagnosis by at least 50% (O'Leary et al., 2009).

Furthermore, the molecular detection of bacterial enteric pathogens using, for example, the EntericBio® System (Serosep, Limerick, Ireland), has been shown to be more sensitive than traditional culture methods (O'Leary et al., 2009). However, while the EntericBio® system is capable of detecting *Campylobacter* spp., *E. coli* O157, *Salmonella* spp., and *Shigella* spp. targets, it fails to determine whether or not the *E. coli* O157 detected by this method is toxigenic. In addition, *E. coli* O157 is not the only serotype found among Shiga-like toxin-producing *E. coli* (STEC) and it has been shown that other non-O157 *E. coli* species are a significant cause of infections in humans. Indeed, 41% of STEC cases in Ireland in 2010 were shown to be caused by serotypes other than O157 (HPSC Annual Report, 2010).

The EntericBio Panel II® system was developed to detect STEC, differentiating Shiga-like toxins 1 and 2 (SLT-1 and SLT-2), in addition to *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp.

The purpose of this study was to validate this new system for routine use in the Department of Medical Microbiology at Cork University Hospital, Cork, Ireland.

2. Materials and methods

2.1. Patient samples

A prospective study was conducted between 16 September and 25 October 2011 on 717 anonymised faecal samples from patients presenting with symptoms of gastroenteritis. This study coincided with a known *E. coli* O157 outbreak, and outbreak-related samples were indicated to the investigators.

All samples were tested using the CE-marked EntericBio Panel II® system in parallel with routine sample testing at the Department of Clinical Microbiology of Cork University Hospital which uses the EntericBio® system and is combined with retrospective culture when targets are detected. These results were not made available to the investigators until the validation testing was completed.

2.1.1. Controls

The following control isolates were used in the study: *Campylobacter jejuni* ATCC 29428, and the following wild-type isolates whose identity had been confirmed by a reference laboratory: *Shigella sonnei*, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Enteritidis, and *E. coli* O157 (National *Salmonella*, *Shigella*, & *Listeria*

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Reference Laboratory, Galway, Ireland, or the National *E. coli* Reference Laboratory, Dublin, Ireland).

A positive control for all targets as a mixture of genomic DNA isolated from *Salmonella enterica*, *E. coli* O:157:H7 (producing both SLT 1 and SLT 2), *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 the polymerase chain reaction (PCR) amplification, hybridization, and detection.

2.1.2. Molecular detection with the EntericBio® and EntericBio Panel II® systems

All aspects of the assays were conducted in accordance with the manufacturer's instructions. Faecal samples were processed routinely in the laboratory, using the EntericBio® system, a method that combines overnight enrichment of samples with DNA extraction. It should be noted that aliquots from the same sample of extracted DNA were used for both EntericBio® systems and for the confirmatory assay. Briefly, after DNA extraction, a final PCR reaction volume of 25 μL was prepared, and a positive (process) control, a negative control, and a kit (positive) control were included in each PCR run. Uracil-*N*-glycosylase (UNG) was also included in each reaction mix as one of the components of the lyophilized reagents, in order to prevent carryover PCR contamination of the samples.

Samples were processed in a thermal cycler (Applied BioSystems, 2720 Thermal Cycler, Warrington, UK) as a touchdown PCR protocol, using a starting annealing temperature of 65 °C, decrementing to an annealing temperature of 60 °C, and continuing at that annealing temperature for the remaining 28 cycles. The remaining volume of each extracted sample was stored at −20 °C for further analysis.

Hybridization and colour development were performed in the temperature-controlled AutoProcessor (BeeBlot, Bee Robotics Ltd, UK) using a substrate solution, a wash solution, distilled water, and detection and hybridization solutions. Detection and hybridization solutions were prepared freshly for each run. Nitrocellulose strips (EntericBio Panel II®, Serosep) were placed in the wells in the processing tray along with hybridization solution and denatured PCR products. Samples were processed in the AutoProcessor at 37 °C according to a programme preset by the manufacturer. With this approach, a positive internal control line signified that an individual test was valid. The test results were interpreted on the basis of the presence or absence of a line blot at each of the 5 locations denoting the presence or absence of *Campylobacter* spp., *Shigella* spp., SLT-1, SLT-2, and *Salmonella* spp. in the original sample (Fig. 1). Additionally, the validity of the positive, negative, and kit controls included in each run was assessed to interpret individual results.

2.1.3. Confirmation of the results

In cases when the results obtained with the EntericBio Panel II® version were not confirmed by the EntericBio® system currently in use, alternative molecular confirmation methods were used. For *Campylobacter* spp., the method described by Maher et al. (2003) was used to detect a genus-specific product for the samples which tested *Campylobacter*-positive only with the EntericBio Panel II® system, for which the limit of detection was determined here to be 10 genome copies. The presence of either SLT-1 or SLT- 2 in tested samples was further confirmed with an alternative uniplex molecular assay using primers and reaction conditions described by Paton and Paton (1998), for which the limit of detection was also determined here to be 10 genome copies. PCR products generated with both primer sets were electrophoresed on 2% agarose gels (Sigma-Aldrich, Wicklow, Ireland) stained with ethidium bromide and visualised under UV light using a high-performance ultraviolet transilluminator (UVP, Cambridge, UK). Samples which tested negative with the EntericBio® system, but which were detectable using the EntericBio Panel II®, were also cultured on CHROMagar™ STEC (CHROMagar Microbiology, Paris, France) following enrichment in Todd-Hewitt broth (BD, Co. Clare, Ireland) at 37 °C for 24 h. Mauve colonies were subcultured to MacConkey agar plates for purity (Oxoid, Cambridge, UK; CM0007). DNA from suspect colonies was extracted using the EntericBio® extract tube sonicated for 30 min in a TP 680DH ultrasonic water bath (Elma Hans Schmidbauer GmbH & Co., Singen, Germany). The DNA extract was then tested for the presence of SLT-1 or SLT-2 using the method described above.

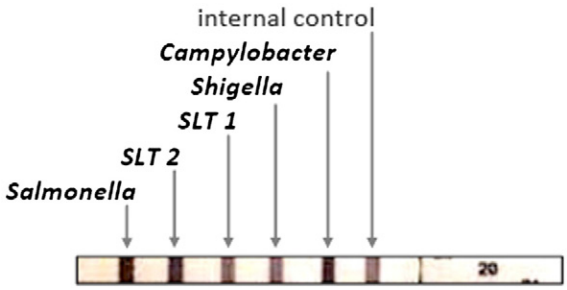

Fig. 1. EntericBio Panel II® hybridization result for all targets (kit control).

Species identification was conducted by using the API 20E system (BioMérieux, France). Serology was performed using the Dryspot *E. coli* Seroscreen kit (Oxoid; DR0300) for the detection of serotypes O26, O91, O103, O111, O128, and O145, followed by confirmation of positive results with the individual O-antisera (Remel, Crossways, UK).

In addition, all samples which tested negative for SLT-1 or SLT-2 by the EntericBio Panel II® system were tested with the alternative molecular method using Paton and Paton (1998) primers in order to determine the negative predictive value (NPV) of the commercial system.

2.1.4. Speciation of Campylobacter-positive samples

A total of 58 DNA *Campylobacter*-confirmed samples were investigated using uniplex species-specific PCR assays for *C. jejuni*, *C. coli* (Linton et al., 1997), *C. lari*, *C. upsaliensis* (Wang et al., 2002), *C. fetus* (Hum et al., 1997), *C. hyointestinalis* (Inglis and Kalischuk, 2003), and *C. ureolyticus* (Bullman et al., 2011a). Four DNA samples were unavailable for speciation, and 4 of the results did not give identification to genus level. All PCR amplifications were performed in a 25-μL reaction volume, containing 2 μL of DNA template, 1 U of HotStarTaq DNA Polymerase (QIAGEN, West Sussex, UK; 203205), 2.5 μL of 10× PCR buffer and 0.5 μL 25 mmol/L of $MgCl_2$ (provided with HotStarTaq DNA Polymerase), 4 μL of dNTPs mixture (1.25 mmol/L of each dNTP; Sigma-Aldrich Ireland, Arklow, Ireland), and 1 μL of each species-specific primer (25 pmol/μL; Eurofins MWG Operon, London, UK). The cycling conditions were initial denaturation for 15 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 1 min and extension at 72 °C for 1 min. Annealing temperature for each primer set was as specified by Bullman et al. (2011b) with the exception of *C. coli* PCR whereby the annealing temperature was set at 60 °C.

Control strains for PCR were as follows: *C. jejuni* subsp. *jejuni* DSM 4688, *C. ureolyticus* DSM 20703, *C. coli* DSM 4689, *C. fetus* subsp. *fetus* DSM 5361, *C. lari* subsp. *lari* DSM 11375, *C. upsaliensis* DSM 5365, and *C. hyointestinalis* subsp. *hyointestinalis* DSM 19053. All control strains were obtained from DSMZ (Germany) and extracted with the QIAamp DNA Mini Kit (QIAGEN, West Sussex, UK; 51304).

A previously described *Campylobacter* genus–specific PCR targeting the 16S rRNA genes was performed on DNA samples negative by species-specific PCR. The PCR amplicons of 816 bp were sequenced using 16S rRNA forward and reverse primers (Eurofins MWG Operon, Germany), and sequences were analysed by BLAST using the NCBI database.

3. Results and discussion

The results of the comparative prospective study of 717 faeces samples using the EntericBio® and the EntericBio Panel II® systems are summarised in Table 1.