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Title: Comparison of the EntericBio® multiplex PCR system with routine culture for the detection of bacterial enteric pathogens.

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Running title: EntericBio® multiplex PCR system for enteric pathogens

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Abstract

The EntericBio system is a multiplex PCR assay for simultaneous detection of *Campylobacter* spp., *Salmonella enterica*, *Shigella* spp. and *E. coli* O157 from faeces. It combines overnight broth enrichment with PCR amplification and detection by hybridisation. An evaluation of this system was conducted by comparing results with routine culture, supplemented with alternative PCR detection methods. In a study of 773 samples, routine culture and the EntericBio system yielded 94.6 and 92.4% negative results, respectively. Forty-two positive results were obtained by culture, all of which were detected by the EntericBio system. This system detected an additional 17 positives (*Campylobacter* spp. n=12; *Shigella* spp. n=1; *E. coli* O157 n=4), of which five results could not be confirmed (*Campylobacter* spp. n=2; *Shigella* spp. n=1; *E. coli* O157 n=2). The target for *Shigella* spp. detected by the EntericBio system is the *ipaH* gene, and the positive *Shigella* spp. result was investigated using sequence analysis and confirmed to be present in *Klebsiella pneumoniae*. The sensitivity, specificity, Positive Predictive Value and Negative Predictive Value were 100%, 99.3%, 91.5% and 100%, respectively. Turnaround times were significantly reduced by the EntericBio system and a result was available between 24 and 32 hours after receipt of the sample in the laboratory. In addition, laboratory waste was significantly reduced with this system. In summary, the EntericBio system proved convenient to use, more sensitive than the conventional culture used in this study, highly specific, and it generated results significantly faster than routine culture for the pathogens tested.

43 Introduction

44 Infectious gastroenteritis is a leading cause of morbidity and mortality worldwide (3) and
45 particularly in developing countries (2). There is also a significant economic and social
46 cost associated with gastroenteritis (14), in addition to increased morbidity and mortality
47 (5).

48 Conventional culture methods remain the norm for isolating bacterial enteric pathogens in
49 clinical laboratories. A major advantage of molecular methods is the reduced time to
50 detection (13). Faster diagnostic outputs allow earlier epidemiological investigations and
51 infection control interventions. Furthermore, the use of molecular methods highlights that
52 conventional methods for the isolation of *Campylobacter* are less sensitive than PCR
53 (15). Molecular methods suitable for use in the detection of faecal enteric pathogens
54 have not been routinely available to clinical laboratories until recently. Here we describe
55 a new multiplex PCR method for clinical diagnostic use, which combines an overnight
56 enrichment step with PCR and hybridization detection using a line-blot assay for the
57 simultaneous detection of *Campylobacter* spp., *Salmonella enterica*, *Shigella* spp. and *E.*
58 *coli* O157 from faeces, and its application in a clinical laboratory.

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66 Materials and Methods

67 **Patient Samples**

68 A total of 773 anonymised faeces samples were tested by both routine culture and the
69 EntericBio system between 16 April and 4 June 2008. All samples were collected from
70 patients with symptoms of gastroenteritis. A history of foreign travel was noted on the
71 specimen container.

72

73 **Controls**

74 The following control isolates were used in the assay: *Campylobacter jejuni* ATCC
75 29428, *Shigella sonnei*, *Salmonella enterica* Typhimurium, Salmonella *enterica*
76 Enteritidis and *E. coli* O157. The non-ATCC strains had been isolated in-house and their
77 identity confirmed at the National *Salmonella* and *Shigella* Reference Laboratory,
78 Galway, Ireland, and the National *E coli* Reference Laboratory, Dublin, Ireland,
79 respectively.

80

81 **Bacterial Culture**

82 Routine culture was performed on all samples to detect *Campylobacter* spp., *Salmonella*
83 *enterica* and *Shigella* spp. and, where the sample was liquid, to detect *E. coli* O157 also,
84 as shown in Table 1.

85

86 **Molecular detection using the EntericBio system.**

87 This CE-marked system (Serosep Ltd., Limerick, Ireland) was used according to the
88 manufacturer's instructions for all samples. The method uses a combination of

enrichment, followed by DNA extraction, PCR amplification and detection of results by means of hybridisation and colour development.

Enrichment of faecal samples was by inoculation of 1.0g of faecal specimen into EntericBio enrichment broth, which equated with the fill line on the broth tube. The broth container was capped and mixed by inversion at least five times before incubating for between 16 and 24h at 37°C.

Following overnight incubation the enriched broths were mixed thoroughly by inversion after removal from the incubator. The broth was allowed to settle for 5min at room temperature to allow large particles to settle. To perform DNA extraction a volume of 200µL of the supernatant from the enriched faecal samples was transferred to an appropriately-labelled EntericBio extract tube (containing a two-phase separation system). Each extract tube was mixed by inversion approximately 20 times, and sonicated for 30min using the Transsonic Digital Sonicator (S – TP 680DH), according to the manufacturer's instructions. The remainder of the enriched EntericBio broths was placed in a freezer at -80°C for storage. These broths were thawed, mixed and subcultured in cases where conventional culture did not yield a matching positive result to that of EntericBio PCR.

PCR Amplification was then carried out using lyophilised PCR strips which were removed from the kit, labelled and placed on a PCR rack. A volume of 14µL of Molecular Biology Grade (MBG) water was pipetted into each PCR tube. From the sonicated EntericBio extract tube, 10µL of the lower phase of the DNA extract solution

was transferred into the pre-labelled PCR tube. A volume of 1µL of Uracil DNA Glycosylase (UNG) (Bioron, Germany 111025) was also added to each PCR tube. A negative control using 24µL of MBG water and 1µL of UNG in a PCR tube was run with each batch of tests. A positive control was also run comprising 5µL of the positive control and 20µL of MBG water.

The PCR tubes were capped firmly and a touchdown PCR programme was run. A pre-PCR incubation at 21°C for 10min was followed by a two-cycle step of 95°C for 30s, 65°C for 30s and 72°C for 30s. This was followed by single cycles using the same step times but using incrementally decreasing annealing temperatures of 64, 63, 62 and 61°C respectively. The programme continued with a further 29 cycles at an annealing temperature of 60°C and was followed by a final extension step of 72°C for 10min. After amplification the PCR tubes were removed from the thermocycler and the samples were denatured by adding 25µL of denaturation solution to each tube, which was then incubated at room temperature for 10min.

Hybridisation and colour development was performed on the EntericBio AutoProcessor (Tecan), using a substrate solution, a wash solution, distilled water and detection and hybridisation solutions.

The hybridization strips were prepared. Using the forceps provided with the kit to remove the hybridization line-blot strips from their storage tube, the strips were placed at the top end of individual channels in the single-use plastic AutoProcessor tray. The entire solution from the PCR tube was transferred into the bottom end of individual channels, taking care not to place the solution onto the strip as the pH would destroy the probes. A

volume of 500µL of hybridisation solution was added to the top end of each channel and the tray was agitated manually to mix both solutions. The tray was placed securely on the AutoProcessor and the hybridization process was initiated. When this process finished after 3h, results were interpreted. A positive internal control line signified that an individual test was valid. Test results were interpreted on the basis of the absence or presence of a line blot at each of the four locations denoting the absence or presence of *Campylobacter* spp., *Shigella* spp., *E. coli* O157 and *Salmonella enterica*, respectively in the original sample (see Figure 1, where it may be seen that for N, the single hybridization line occurs for the internal control and all other targets are negative, and where, for P, hybridization lines are detectable for all four pathogens and the internal control).

Molecular confirmation of results:

In cases where the molecular results were not confirmed by conventional culture (this also included in every case subculture from the incubated EntericBio broth), alternative molecular confirmation methods were used.

For *Shigella* spp., the PCR method of Aranda *et al*. (1) was used to detect the *ipaH* gene. However, in the single case where culture for *Shigella* spp. remained negative, a subculture of the EntericBio broth was made on MacConkey agar (MAST DM140D), and was incubated overnight at 37ºC. Fermentative and non-fermentative colonies were cultured for purity onto further MacConkey agar plates. Following overnight incubation, each colony type had its DNA extracted by a standard boiling method, and this DNA was tested for the presence of the *ipaH* gene. The colony on which the *ipaH* gene was located

was subcultured for purity and re-tested for the presence of the gene, and a species identification was conducted using API 20E (BioMérieux, France).

For *Campylobacter* spp. the PCR method of Linton *et al*., (8) was used. This detects the hippuricase gene of *C. jejuni*, and the method of Maher *et al*., (9) was used to detect a genus-specific product for the samples where positive results were not obtained using the Linton method.

For *E. coli* O157, the method of Müller *et al*., (10) was used to investigate the presence of verotoxin 1 and/or 2 genes among the positive specimens detected by the EntericBio system. Furthermore, each of the faeces samples from which positive results were derived was sent for confirmatory testing to the National *E coli* Reference Laboratory (Dublin, Ireland).

Results

The results of testing 773 clinical samples are shown in Table 2.

All conventional positive culture results were matched by positive results generated by the EntericBio system. However, seventeen additional positive results were generated by the EntericBio system. A single Shigella *ipaH* gene target was detected by the EntericBio system but *Shigella* spp., was not detected by routine culture, or by retrospective culture of the EntericBio broth sample for that patient. The *ipaH* gene was, however, also detected by the method of Aranda *et al*.(1), and this target was located to *Klebsiella pneumoniae*, isolated from the patient's sample. Sequence analysis showed >98% sequence similarity with published sequences of *ipaH* gene amplicons from *Shigella* spp. for short segments flanking the central section of the amplicon which corresponded to sequences of *K. pneumoniae* (data not shown). Ten of the twelve additional positive campylobacter results generated by the EntericBio system gave positive results by hippuricase gene PCR, indicating a species identification of *C. jejuni* in each case. The remaining two positive results remained unconfirmed either by the methods of Linton *et al*. (8) or Maher *et al*. (9) The twelve *E. coli* O157 positive results obtained by EntericBio system, when tested by the National *E. coli* Reference Laboratory generated ten positive results for *E. coli* O157, of which nine were identified as VT2 positive. These remaining two EntericBio results were not confirmed either by the method of Müller *et al*.(10), or by the National *E coli* Reference Laboratory.

204 Discussion

205 Conventional culture has been the gold standard method for detection of bacterial enteric
206 pathogens. The advantages of these methods include identification to species or serovar
207 level, facilitation of outbreak management, and the generation of an antimicrobial
208 susceptibility profile. A disadvantage of using culture is the time taken to generate a
209 result, positive or negative, from a faeces sample. A further disadvantage is the
210 occurrence in some cases of viable but non-culturable *C. jejuni* (11), which makes the
211 recovery of the organism challenging when using culture techniques. In this comparative
212 study between conventional culture techniques and a multiplex PCR method to detect
213 *Campylobacter* spp., *Salmonella enterica*, *Shigella* spp. and *E. coli* O157, the minimum
214 time taken to confirm a positive result for these pathogens using conventional culture
215 ranged between 48 hours for *Campylobacter* spp., and 96 hours for *Salmonella enterica*.
216 The time taken to generate a negative report was 48 hours, or within the next two
217 working days following receipt of the sample. For the EntericBio system, the time taken
218 to generate a report, positive or negative, was between 24 and 32 hours, or within the next
219 working day following receipt of the sample in the laboratory. The advantages of an
220 early laboratory report include the early diagnosis of infection, to allow timely reporting
221 of foodborne diseases and to prevent additional cases in outbreak settings (3).
222 As shown in Table 2, the overall positivity rate for this prospective study was 5.4% using
223 conventional culture techniques and 7.6% using the EntericBio method. For the detection
224 of *Salmonella*, culture and the EntericBio system generated identical results. In the case
225 of *Shigella*, detection by the EntericBio system, which detects the *ipaH* gene, a single
226 unmatched sample yielded a positive result. Using the primer set of Aranda *et al.*(1), the

target was located to *Klebsiella pneumoniae*, which showed 100% similarity with the *ipaH* target primers following sequencing and BLAST analysis to accessions of *Shigella* spp. and containing an internal sequence with >98% similarity to accessions of *K. pneumoniae*. The sample from which this result was obtained was from a patient with gastroenteritis on return from travel in India. The sample did not yield any further pathogens, either bacterial or parasitic. This finding may serve to highlight the transmissibility of virulence factors such as the plasmid-borne *ipaH* gene among members of the Enterobacteriaceae, and suggests the possibility that an effective means to detect a pathogen may originate from first identifying the presence of a virulence gene, followed by the subsequent identification of the organism carrying it.

For *Campylobacter* spp., a total of 30 culture isolations were made from the 773 samples, all of which were detected by the EntericBio system. However, the EntericBio system detected an additional 12 positive results for *Campylobacter*, of which ten were confirmed as *C. jejuni* by the PCR method of Linton *et al*. (8). The PCR method of Maher *et al*. (9) was applied to the remaining two unconfirmed EntericBio positive *Campylobacter* results, but these remained unconfirmed by this method also. These results were thus considered to be false positives. It should be noted that these additional 12 positive results were also subcultured from the EntericBio broths onto Preston agar (incubated at 37 and 42ºC) but did not result in any positive cultures. The finding that currently-used culture-based methods may miss a substantial proportion of *Campylobacter* infections has been noted previously (6). It probably reflects the detection of *Campylobacter* cells in metabolic states that are less amenable to culture on selective media, whether damaged, viable but non-culturable, or dead (7). The clinical

significance of non-culturable *Campylobacter* in human infection remains poorly understood and warrants future investigation. The results of the current study, when compared to the findings of an Irish study by Maher *et al*.(9), show some differences, however. This earlier study compared conventional culture to the detection of *Campylobacter* spp. direct from faeces, using a combination of primers/probe sets with colourimetric membrane-based detection. The study showed molecular detection to be more sensitive than culture also, but the DNA of a variety of species was detected, including *C. concisus*, *C. curvus* and *C. gracilis,* in addition to *C. jejuni.* Those findings are in contrast to a 2007 UK study by Wilson and Aitchison (16), in which combined filtration and culture methods yielded only *C. jejuni*. In the current study, only *C. jejuni* was identified among the non-culturable *Campylobacter* spp., although the molecular method was designed using a gene idiosyncratic for *Campylobacter* spp., according to the manufacturer. Further studies are needed to establish whether the overnight enrichment step used in this method facilitates the growth of all species. Nevertheless, the detection of an additional 25% of *Campylobacter*-positive results compared to routine culture points to the greater sensitivity of the EntericBio system over routine culture for *C. jejuni*, at least.

Finally, the isolation of *E. coli* O157 by routine culture without enrichment yielded eight positive results, which were augmented by an additional two positive results detected both by subculture of the EntericBio broth onto Sorbitol MacConkey agar, and by the National *E coli* Reference Laboratory using immunomagnetic concentration techniques combined with molecular detection. Nine of these isolates were VT2-positive, the tenth was non-verotoxigenic. The remaining two positive results detected by the EntericBio

system remained unconfirmed either as *E. coli* O157 or as verotoxigenic strains by the Reference Laboratory, and by the additional molecular testing which we undertook to detect VT1 and VT2, and so these results were interpreted as false positives.

An expanded gold standard was used to calculate sensitivity, specificity, positive-predictive value (PPV) and negative-predictive value (NPV) for the EntericBio method using the combination of culture and alternative molecular techniques, and showed that sensitivity was 100%, specificity 99.3%, PPV 91.5% and NPV 100%.

The detection of *E. coli* O157 is clinically more highly significant where the organism is demonstrated to be verotoxigenic (4). In this study, the two unconfirmed *E. coli* O157 isolates did not give positive signals for VT 1 or 2, thereby lessening the impact of a false positive result in these cases. It could be argued that the EntericBio system might benefit more from the facility to detect VT1 and VT2 rather then *E. coli* O157 alone, as there have been many serotypes of *E. coli* identified as being verotoxigenic (12). Future modifications of the system should address this issue.

The detection of *E. coli* O157, *Salmonella* and *Shigella* by the EntericBio system required a subsequent culture analysis of these positive samples. In each case, the identification of the pathogen to serovar or serotype level was vital for epidemiological purposes. This necessitated a delay in the generation of a report of at least 48 hours after the positive EntericBio result. However, the low rate of positives (7.6%) by the EntericBio system meant that the more than 90% of faeces samples negative for the four pathogens detected by the assay, even during a peak seasonal period as in this study (between April and June), can be reported with confidence at least one working day earlier than by using routine culture.

296 A comparison of the waste generated by culture and by the EntericBio system was also
297 made. Approximately 3,000 agar plates were generated by culture, and the total waste for
298 autoclaving amounted to approximately 306L, which contrasted sharply with that of the
299 36 litres generated by the EntericBio system. Most of the waste generated by the latter
300 was recyclable packaging.
301
302 In summary, the EntericBio system proved convenient to use, more sensitive than the
303 conventional culture used in this study, highly specific, and it generated results
304 significantly faster than routine culture for the pathogens tested. At the moment the
305 method is more expensive than conventional culture. However, this is compensated for
306 in the laboratory by not having to perform immunomagnetic separation for *E. coli* O157,
307 and by the potential savings in personnel once the method is established in the laboratory.
308 The use of an enrichment broth suitable for the enrichment of all four pathogens,
309 necessitates the use of a Category 3 containment facility, however, which may not be
310 available in all laboratories. Finally, the simultaneous detection of the four most common
311 bacterial enteric pathogens with one test and with a significantly reduced turnaround time
312 than culture is likely to be the major advantage offered by this system for clinical
313 diagnostic laboratories. The clinical impact of the decreased turnaround time means that
314 bacterial diarrhoea is more promptly outruled than with conventional culture. This
315 reduces pressure on infection control resources and, in particular, in cases of sporadic
316 diarrhoea, helps reduce the requirement for scarce isolation rooms. In addition, in
317 outbreaks which are largely community-based, the earlier results are helpful in their
318 management.

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320

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396 TABLE 1 Culture procedure for investigation of faeces samples for the presence of

397 *Campylobacter* spp., *Salmonella enterica*. *Shigella* spp. and *E. coli* O157

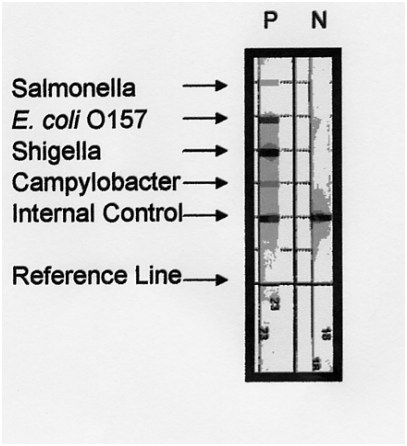
398

Pathogen	Culture method	Identification Method
Campylobacter spp.	Preston Agar (Campylobacter Agar Base, Oxoid CM689, with supplement SR204E). Microaerophilic environment at 42ºC for 48h.	Typical macroscopic and microscopic appearance; positive catalase/oxidase reactions
Salmonella enterica	DCA and XLD¶; incubation overnight at 37ºC. Enrichment overnight in Selenite broth (LP0121); subculture to Harlequin Medium (LabM Hal1),	Suspicious colonies confirmed biochemically and serologically*
Shigella spp.	DCA and XLD and incubated overnight at 37ºC.	Suspicious colonies confirmed biochemically and serologically*
E. coli O157	Cefixime Tellurite Sorbitol MacConkey Agar (LIP, W11021) (CT-SMAC); incubation overnight at 37ºC	Suspicious colonies confirmed biochemically and serologically*

399 *All suspicious isolates confirmed by reference laboratories

400 ¶ DCA=Desoxycholate Citrate Agar (Oxoid CM0227)

401 XLD=Xylose Lysine Desoxycholate Agar (LabM Lab 32)



402

403 FIGURE 1. EntericBio line-blot hybridisation results for a positive control, denoted by

404 P, and a negative control, denoted by N.

405 TABLE 2 A comparison of the results of routine bacterial culture with the EntericBio
406 system for the detection of *Salmonella enterica*, *Shigella* spp., *Campylobacter* spp., and
407 *E. coli* O157.

Organism	Routine culture	EntericBio system
	No. (%)	No. (%)
Salmonella enterica	4 (0.5)	4 (0.5)
Shigella spp.	0	1 (0.1)
Campylobacter spp.	30 (3.9)	42 (5.4)
E. coli O157*	8 (1.0)	12 (1.6)
Total positives	42 (5.4)	59 (7.6)
Negatives	731 (94.6)	714 (92.4)

408 *A total of ten positive results were generated by the National *E. coli* Reference
409 Laboratory, using PCR, and subculture from the EntericBio broth yielded an additional
410 two positive culture results over routine culture methods for the same ten samples.
411