

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

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19 Running title: EntericBio® multiplex PCR system for enteric pathogens

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21 Abstract

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

42

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

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

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

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96 Following overnight incubation the enriched broths were mixed thoroughly by inversion
97 after removal from the incubator. The broth was allowed to settle for 5min at room
98 temperature to allow large particles to settle. To perform DNA extraction a volume of
99 200µL of the supernatant from the enriched faecal samples was transferred to an
100 appropriately-labelled EntericBio extract tube (containing a two-phase separation
101 system). Each extract tube was mixed by inversion approximately 20 times, and
102 sonicated for 30min using the Transsonic Digital Sonicator (S – TP 680DH), according to
103 the manufacturer's instructions. The remainder of the enriched EntericBio broths was
104 placed in a freezer at -80°C for storage. These broths were thawed, mixed and
105 subcultured in cases where conventional culture did not yield a matching positive result
106 to that of EntericBio PCR.

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

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

117 The PCR tubes were capped firmly and a touchdown PCR programme was run. A pre-
118 PCR incubation at 21°C for 10min was followed by a two-cycle step of 95°C for 30s,
119 65°C for 30s and 72°C for 30s. This was followed by single cycles using the same step
120 times but using incrementally decreasing annealing temperatures of 64, 63, 62 and 61°C
121 respectively. The programme continued with a further 29 cycles at an annealing
122 temperature of 60°C and was followed by a final extension step of 72°C for 10min. After
123 amplification the PCR tubes were removed from the thermocycler and the samples were
124 denatured by adding 25 μ L of denaturation solution to each tube, which was then
125 incubated at room temperature for 10min.

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

129

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

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

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147 **Molecular confirmation of results:**

148 In cases where the molecular results were not confirmed by conventional culture (this
149 also included in every case subculture from the incubated EntericBio broth), alternative
150 molecular confirmation methods were used.
151 For *Shigella* spp., the PCR method of Aranda *et al.* (1) was used to detect the *ipaH* gene.
152 However, in the single case where culture for *Shigella* spp. remained negative, a
153 subculture of the EntericBio broth was made on MacConkey agar (MAST DM140D), and
154 was incubated overnight at 37°C. Fermentative and non-fermentative colonies were
155 cultured for purity onto further MacConkey agar plates. Following overnight incubation,
156 each colony type had its DNA extracted by a standard boiling method, and this DNA was
157 tested for the presence of the *ipaH* gene. The colony on which the *ipaH* gene was located

158 was subcultured for purity and re-tested for the presence of the gene, and a species
159 identification was conducted using API 20E (BioMérieux, France).
160 For *Campylobacter* spp. the PCR method of Linton *et al.*, (8) was used. This detects the
161 hippuricase gene of *C. jejuni*, and the method of Maher *et al.*, (9) was used to detect a
162 genus-specific product for the samples where positive results were not obtained using the
163 Linton method.
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165 For *E. coli* O157, the method of Müller *et al.*, (10) was used to investigate the presence of
166 verotoxin 1 and/or 2 genes among the positive specimens detected by the EntericBio
167 system. Furthermore, each of the faeces samples from which positive results were derived
168 was sent for confirmatory testing to the National *E coli* Reference Laboratory (Dublin,
169 Ireland).
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181 Results

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

183 All conventional positive culture results were matched by positive results generated by
184 the EntericBio system. However, seventeen additional positive results were generated by
185 the EntericBio system. A single *Shigella ipaH* gene target was detected by the
186 EntericBio system but *Shigella* spp., was not detected by routine culture, or by
187 retrospective culture of the EntericBio broth sample for that patient. The *ipaH* gene was,
188 however, also detected by the method of Aranda *et al.*(1), and this target was located to
189 *Klebsiella pneumoniae*, isolated from the patient's sample. Sequence analysis showed
190 >98% sequence similarity with published sequences of *ipaH* gene amplicons from
191 *Shigella* spp. for short segments flanking the central section of the amplicon which
192 corresponded to sequences of *K. pneumoniae* (data not shown). Ten of the twelve
193 additional positive campylobacter results generated by the EntericBio system gave
194 positive results by hippuricase gene PCR, indicating a species identification of *C. jejuni*
195 in each case. The remaining two positive results remained unconfirmed either by the
196 methods of Linton *et al.* (8) or Maher *et al.* (9) The twelve *E. coli* O157 positive results
197 obtained by EntericBio system, when tested by the National *E. coli* Reference Laboratory
198 generated ten positive results for *E. coli* O157, of which nine were identified as VT2
199 positive. These remaining two EntericBio results were not confirmed either by the
200 method of Müller *et al.*(10), or by the National *E coli* Reference Laboratory.

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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

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

237 For *Campylobacter* spp., a total of 30 culture isolations were made from the 773 samples,
238 all of which were detected by the EntericBio system. However, the EntericBio system
239 detected an additional 12 positive results for *Campylobacter*, of which ten were
240 confirmed as *C. jejuni* by the PCR method of Linton *et al.* (8). The PCR method of
241 Maher *et al.* (9) was applied to the remaining two unconfirmed EntericBio positive
242 *Campylobacter* results, but these remained unconfirmed by this method also. These
243 results were thus considered to be false positives. It should be noted that these additional
244 12 positive results were also subcultured from the EntericBio broths onto Preston agar
245 (incubated at 37 and 42°C) but did not result in any positive cultures. The finding that
246 currently-used culture-based methods may miss a substantial proportion of
247 *Campylobacter* infections has been noted previously (6). It probably reflects the
248 detection of *Campylobacter* cells in metabolic states that are less amenable to culture on
249 selective media, whether damaged, viable but non-culturable, or dead (7). The clinical

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

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

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

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

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

287 The detection of *E. coli* O157, *Salmonella* and *Shigella* by the EntericBio system required
288 a subsequent culture analysis of these positive samples. In each case, the identification of
289 the pathogen to serovar or serotype level was vital for epidemiological purposes. This
290 necessitated a delay in the generation of a report of at least 48 hours after the positive
291 EntericBio result. However, the low rate of positives (7.6%) by the EntericBio system
292 meant that the more than 90% of faeces samples negative for the four pathogens detected
293 by the assay, even during a peak seasonal period as in this study (between April and
294 June), can be reported with confidence at least one working day earlier than by using
295 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.

319 Acknowledgements

320

321 The authors gratefully acknowledge Mr Tim Buckley, Laboratory Manager at the
322 Microbiology Department of Cork University Hospital for facilitating this work, and the
323 Health Service Executive for bearing the cost of the validation of the EntericBio system
324 prior to its incorporation for routine use in the department.

325 We thank M. Pierre Douarre at the Department of Biological Sciences in Cork Institute of
326 Technology for his confirmatory work on a subset of the *Campylobacter* results, and the
327 National *E. coli* Reference Laboratory for their assistance in the investigation of putative
328 EntericBio-positive *E. coli* O157 results. Our gratitude also to our colleagues for their
329 support of this project.

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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

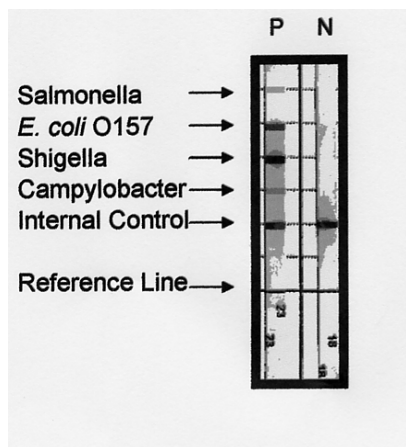
398

Pathogen	Culture method	Identification Method
<i>Campylobacter</i> spp.	Preston Agar (Campylobacter Agar Base, Oxoid CM689, with supplement SR204E). Microaerophilic environment at 42°C for 48h.	Typical macroscopic and microscopic appearance; positive catalase/oxidase reactions
<i>Salmonella enterica</i>	DCA and XLD¶; incubation overnight at 37°C. Enrichment overnight in Selenite broth (LP0121); subculture to Harlequin Medium (LabM Hal1),	Suspicious colonies confirmed biochemically and serologically*
<i>Shigella</i> spp.	DCA and XLD and incubated overnight at 37°C.	Suspicious colonies confirmed biochemically and serologically*
<i>E. coli</i> O157	Cefixime Tellurite Sorbitol MacConkey Agar (LIP, W11021) (CT-SMAC); incubation overnight at 37°C	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 cultureEntericBio system	
	No. (%)	No. (%)
<i>Salmonella enterica</i>	4 (0.5)	4 (0.5)
<i>Shigella</i> spp.	0	1 (0.1)
<i>Campylobacter</i> spp.	30 (3.9)	42 (5.4)
<i>E. coli</i> 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