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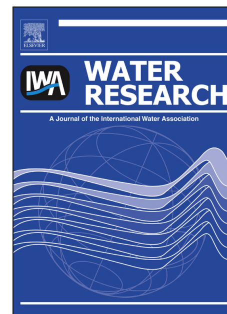

Title: Specificity and sensitivity evaluation of novel and existing Bacteroidales and Bifidobacteria specific PCR assays on feces and sewage samples and their application for fecal source tracking in Ireland

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Specificity and sensitivity evaluation of novel and existing *Bacteroidales* and *Bifidobacteria* specific PCR assays on feces and sewage samples and their application for fecal source tracking in Ireland.

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Abstract

Three novel ruminant-specific PCR assays, an existing ruminant-specific PCR assay and five existing human-specific PCR assays, which target 16S rDNA from *Bacteroidales* or *Bifidobacteria*, were evaluated. The assays were tested on DNA extracted from ruminant (n = 74), human (n = 59) and non-ruminant animal (n = 44) sewage/fecal samples collected in Ireland. The three novel PCR assays compared favourably to the existing ruminant-specific assay, exhibiting sensitivities of 91 - 100% and specificities of 95 - 100% as compared to a sensitivity of 95% and specificity of 94%, for the existing ruminant-specific assay. Of the five human-specific PCR assays, the assay targeting the *Bifidobacterium catenulatum* group was the most promising, exhibiting a sensitivity of 100% (with human sewage samples) and a specificity of 87%. When tested on rural water samples that were naturally contaminated by ruminant feces, the three novel PCR assays tested positive with a much greater percentage (52 - 87%) of samples than the existing ruminant-specific assay (17%). These novel ruminant-specific assays show promise for microbial source tracking and merit further field testing and specificity evaluation.

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47 **1. Introduction**

48

49 The need to determine the source of fecal contamination of water has led to the
50 development of various fecal source identification methods, a relatively new field
51 commonly known as microbial source tracking (MST). The methods of MST used in this
52 study are based on molecular detection of host-specific strains of bacteria from the order
53 *Bacteroidales* and the genus *Bifidobacterium*. Both these groups of bacteria are
54 abundantly present in feces and several researchers have concluded that some strains of
55 the microorganisms are confined to specific hosts (Fiksdal et al., 1985; Gavini et al.,
56 1991; Resnick and Levin, 1981). Host-specific *Bacteroidales* molecular assays have been
57 successfully used as a method of MST by a number of research groups (Gawler et al.,
58 2007; Kildare et al., 2007; Seurinck et al., 2005). Katherine Field and colleagues, in
59 particular, have performed extensive research into the use of *Bacteroidales* 16S rDNA-
60 based PCR assays for MST (Bernhard and Field, 2000a; b; Bernhard et al., 2003; Field et
61 al., 2003; Shanks et al., 2006). The use of molecular methods to detect host-specific
62 species of *Bifidobacteria* is not as well studied as detection methods for *Bacteroidales* but
63 a number of studies have been performed (Bernhard and Field, 2000a; Blanch et al.,
64 2006; Bonjoch et al., 2004; Lynch et al., 2002).

65 Fecal contamination of rural water supplies in Ireland is common (EPA, 2008)
66 and apart from human feces, the main sources of contamination are cow and sheep feces
67 (both ruminants). The principal aim of this study was the development and evaluation of
68 novel ruminant-specific PCR assays and the use of these assays for MST on contaminated
69 water samples collected from rural water supplies in Ireland. To develop the assays, novel

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70 ruminant-associated *Bacteroidales* 16S rDNA sequences were identified by terminal
71 restriction fragment length polymorphism (TRFLP) analysis of human and ruminant
72 fecal/sewage samples. These sequences were exploited for the design of ruminant-
73 specific PCR primers. A secondary aim of the study was the evaluation of a number of
74 existing putatively host-specific PCR assays on Irish fecal/sewage reference samples and
75 the application of the assays for MST on Irish naturally contaminated water samples.

76 The human-specific (HF183F & Bac708R) and ruminant-specific (CF128F &
77 Bac708R) PCR assays, designed by Bernhard and Field in 2000 for MST (2000a; b),
78 were the first set of *Bacteroidales* PCR assays chosen for evaluation in this study. The
79 second set were designed to detect *Bacteroides thetaiotaomicron* (BT 1 & 2) and
80 *Bacteroides vulgatus* (BV 1 & 2) and were developed by Wang et al. (1994; 1996) for the
81 detection of anaerobic bacteria in human and animal fecal samples. The authors found
82 that both *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* were present in large
83 numbers in adult human samples and at lower numbers or absent in animal samples
84 (Wang et al., 1996).

85 Two *Bifidobacteria*-specific PCR assays designed by Matsuki et al. (1999; 1998)
86 for microbial ecology studies of the human gastrointestinal tract were also chosen for
87 evaluation in this study. The first assay was designed to detect *B. adolescentis* (BiADO 1
88 & 2) and the second was designed to detect *B. catenulatum* and *B. pseudocatenulatum*
89 (BiCAT 1 & 2). These species were found in a high percentage of the human fecal
90 samples tested by Matsuki et al (1999).

91 All the assays developed and/or evaluated in this study are conventional PCR
92 assays as opposed to real-time PCR assays. A number of *Bacteroidales*-specific real-time

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93 PCR assays have been developed which have the potential to detect and quantify host-
94 specific targets in water (Kildare et al., 2007; Layton et al., 2006; Okabe et al., 2007;
95 Reischer et al., 2007; Reischer et al., 2006; Stricker et al., 2006). However, none of these
96 assays were available for evaluation during the timeframe of this study. The advantages
97 of real-time PCR include increased specificity, sensitivity and the ability to accurately
98 quantify the target. However, since real-time PCR technology is expensive, requires
99 additional expertise and is not generally available in Irish local authority environmental
100 monitoring laboratories, this development and evaluation of conventional PCR assays is
101 still worthwhile.

102

103 **2. Materials and methods**

104

105 **2.1 Sample collection and determination of *E. coli* densities**

106 Human sewage samples (untreated primary effluent, n = 33) were collected from two
107 different wastewater treatment works in Co. Galway, Ireland. Ruminant (n = 74) and non-
108 ruminant (n = 44) animal fecal and slurry samples were collected from various farms in
109 Co. Galway. The ruminant samples consisted of cow (n = 25), sheep (n = 39), deer (n =
110 1) and goat (n= 4) fecal samples and five cow slurry samples. The non-ruminant samples
111 consisted of horse (n = 12), donkey (n = 2), dog (n = 2), goose (n = 1), chicken (n = 2),
112 pet pig (n= 2) and farmed pig (n = 8) fecal samples and 15 pig slurry samples. Raw and
113 piped water samples were collected every two weeks from three frequently contaminated
114 rural drinking water supplies in Co. Galway over a six month period. *E. coli*
115 concentrations (most probable number [MPN] per 100-ml of water sample) were

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116 measured using Colilert-18 and Quanti-tray/2000 (Idexx, Westbrook, ME). All samples
117 were collected with sterile utensils, placed in sterile containers and transported on ice.
118 Water samples (1-L) were filtered within six hours of collection using 0.2-µm-pore-size
119 cellulose nitrate filter membranes (Sartorius AG, Goettingen, Germany). Turbid water
120 samples were pre-filtered with a 2.7-µm-pore-size glass fibre filter membrane to remove
121 debris before filtration with aforementioned 0.2-µm-pore-size filter membranes.
122 Fecal/sewage samples and filter membranes were stored at -80ºC. DNA from 26
123 individual human stool samples, which were donated by healthy human adults, was
124 kindly provided by the Microbiology Department, University College Cork, Ireland.
125

126 **2.2 DNA extraction**

127 DNA was extracted from 20 mg of sewage sediment, from fecal samples and from filter
128 membranes using the Powersoil™ DNA Isolation Kit (MoBio, Carlsbad, CA) following
129 the manufacturer's protocol, modified to include the use of four washes with solution C5
130 and a 10 min incubation at 70°C after the addition of solution C1. A mock DNA
131 extraction was included each time DNA extractions were performed to test for
132 contamination of kit components. To confirm DNA samples were free of PCR inhibitors,
133 10 ng of DNA was tested using the *Bacteroidales*-specific primer pair Bac32F & Bac
134 708R (Table 1). Amplification indicated the absence of inhibitors.
135

136 **2.3 Clone library construction**

137 The Bac32F & Bacto1080R primer pair (Table 1) was used to amplify a ~ 1060 bp
138 fragment of 16S rDNA from DNA (10 ng) extracted from six cow fecal samples, five

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139 sheep fecal samples and five human sewage samples. Each 50-μl PCR mixture contained:
140 1 × *Taq* polymerase buffer, 200μM dNTP (dATP, dCTP, dGTP, dTTP), 12.5 pmol of
141 each primer, and 1.25 U *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO). Thermal
142 cycling was performed in a Mastercycler personal PCR machine (Eppendorf, Hamburg,
143 Germany) as follows: an initial denaturation step at 94°C for 3 minutes, 30 cycles
144 consisting of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min, followed by a final 5-
145 min extension at 72°C. A positive control (fecal DNA from the target source previously
146 found positive or plasmid DNA containing the target insert) and a negative no-template
147 control were included in every experiment and all PCR assays were performed in
148 triplicate. After purification ('High Pure PCR Product Purification Kit', Roche
149 Diagnostics, Mannheim, Germany) and quantification of the PCR products (PicoGreen
150 dsDNA Quantitation Kit, Molecular Probes, Eugene, OR) three pools of PCR products,
151 each representing one of the host species, were cloned, (TOPO TA Cloning® kit,
152 Invitrogen, De Schelp, Netherlands). Plasmid DNA was extracted from 100 clones per
153 library (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and clones were resolved
154 into operational taxonomic units (OTUs) using amplified rDNA restriction analysis
155 (ARDRA) with both *Hae*III and *Alu*I (Roling and Head, 2005). A representative clone
156 from each OTU group identified was sequenced by MWG BIOTECH AG (Ebensburg,
157 Germany). The sequence data were checked for chimeric properties using Chimera Check
158 on the RDP II website and using the Mallard (Ashelford et al., 2006) and Pintail
159 (Ashelford et al., 2005) programs of the Bioinformatic Toolkit website
160 (http://www.bioinformatics-toolkit.org/index.html). Non-chimeric sequences were
161 aligned using the multiple sequence alignment program ClustalW

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162 (http://www.ebi.ac.uk/Tools/clustalw/index.html). Default parameters were used for
163 ClustalW and all other computer programs utilized. Aligned sequences showing > 97%
164 similarity were treated as a single OTU (Okabe et al., 2007) and only one of each OTU
165 was included in further analysis.

166

167 **2.4 TRFLP analysis**

168 DNA from the six cow and five sheep feces samples and six human sewage samples used
169 in clone library construction was amplified as previously described (Section 2.3) this time
170 using fluorescently labelled primers Bac32F (5'- hexachlorofluorescein [HEX] labelled)
171 and Bacto1080R (5'- [6]-carboxyfluorescein [FAM] labelled). The PCR reaction was
172 carried out in triplicate for each sample and the products were pooled and purified as
173 described in Section 2.3. PCR products were digested with *Hae*III and fragment sizes
174 were measured by polyacrylamide gel electrophoresis in an automated ABI Prism 310
175 Genetic Analyzer using the GS2500 TAMRA size marker (performed by Gene Analysis
176 Service GmbH, Berlin, Germany). Eight of the samples were analyzed in duplicate to
177 assess the reproducibility of the TRFLP profiles.

178

179 **2.5 Ruminant-specific PCR primer design**

180 Putative ruminant-specific clone sequences were aligned with all sequences from the
181 human fecal DNA clone library and putative ruminant-specific PCR primers were
182 designed adhering to general primer design guidelines (Dieffenbach et al., 1995). Primers

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183 were checked with Premier Biosoft International's free online primer analysis program
184 Netprimer (http://www.premierbiosoft.com/netprimer/index.html).
185

186 **2.6 Evaluation of host-specific PCR assays**

187 The host-specific PCR assays were tested on all the samples listed in Table 2, using the
188 PCR reaction components and conditions detailed in Section 2.3. The PCR cycling
189 conditions described by Field et al. (2003) for the HF183F & Bac708R primer pair and
190 the CF128F & Bac708R primer pair were used without modification. The optimum
191 annealing temperatures for the novel ruminant-specific PCR assays were determined
192 empirically (data not shown) as were the annealing temperatures for the other existing
193 PCR assays since these PCR assays were not originally developed for use in MST. The
194 final annealing temperatures used are listed in Table 1. The sensitivity and the specificity
195 of all the PCR assays was calculated using standard definitions (Gawler et al., 2007).
196

197 **2.7 Evaluation of the sample limit of detection (SLOD) and method detection**
198 **limit (MDL) of host-specific PCR assays**

199 The SLOD of the assays was defined as the minimum dry weight of feces per filter
200 membrane that could be detected using the assays. This was assessed by extracting DNA
201 in triplicate from decimal dilutions of 1-L water samples to which 100 mg of cow feces
202 or human sewage had been added. The MDL was defined as the minimum number of
203 copies of the 16S rRNA gene template that could be detected using the ruminant-specific
204 assays. For this method, plasmid DNA containing the target *Bacteroidales* 16S rDNA

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205 fragment was decimally diluted to give a range of DNA from 10^9 to 1 copy of plasmid
206 DNA per µl and tested with the PCR assays.
207

208 **3. Results and Discussion**

209

210 **3.1 Host-specific sequence identification**

211 **3.1.1 Clone library analysis**

212 The first step in host-specific sequence identification was the construction of clone
213 libraries using DNA extracted from cow, sheep and human feces/sewage samples. One
214 hundred clones from each library were resolved into OTUs and representative clones
215 from each OTU were sequenced. All sequences were submitted to Genbank under the
216 following accession numbers; cow clone library: EU573790 – EU573833; human clone
217 library: EU573834 – EU573866; sheep clone library: EU573867 – EU573924.
218

219 **3.1.2 TRFLP analysis**

220 The next step in host-specific sequence identification was TRFLP analysis of the same
221 DNA samples used to generate the clone libraries. Analysis of the TRFLP profiles
222 performed in duplicate confirmed the reproducibility of the method. As illustrated by the
223 representative TRFLP profiles presented in Figure 1, there were HEX-labelled ruminant-
224 associated TRFs at 190 - 191 bp and 222 - 224 bp, and sheep-associated peaks at 105 -
225 106 bp, 110 bp and 146 bp. Figure 2 illustrates the two FAM-labelled ruminant-specific
226 TRFs which were identified in the profiles, one at 69 - 70 bp and one at 81 bp.

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227 Several cloned *Bacteroidales* sequences corresponding to the ruminant-specific 228 TRFs were identified from the cow and sheep fecal DNA clone libraries. The lengths of 229 experimentally determined TRFs, as compared to sequence-determined TRFs, were 230 inaccurate by up to 4 bp (data not shown) which concurs with other studies (Bernhard and 231 Field, 2000a; Clement et al., 1998; Pandey et al., 2007). The putative ruminant-specific 232 sequences were used to design ruminant-specific primers as described in Section 2.5.

233

234 3.2 Host-specific PCR assay evaluation

235 3.2.1 Specificity and sensitivity evaluation

236 Following an initial evaluation of novel ruminant-specific PCR assays on a small number 237 of fecal/sewage samples, three assays emerged as potentially useful; Bac32F & RumD1R 238 (product 979 bp), Bac32F & RumD2R (product 997 bp) and RumB1F & BacPreR 239 (product 714 bp) (Table 1). These three PCR assays were then evaluated using a full 240 range of target and non-target fecal samples (Table 2).

241 In general, the sensitivity and specificity of the novel ruminant-specific PCR 242 assays was high, ranging from 91 – 100% sensitivity and 95 – 100% specificity (Table 2). 243 The novel ruminant-specific PCR assays compared well to the CF128F & Bac708R assay 244 (which had a sensitivity of 95% and a specificity of 94%). Although none of the novel 245 ruminant-specific PCR assays developed exhibited 100% sensitivity and 100% 246 specificity, results were consistent with other studies where ruminant-specific PCR assays 247 were developed or tested (Gawler et al., 2007; Gourmelon et al., 2007; Kildare et al., 248 2007).

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249 All three novel ruminant-specific PCR assays amplified DNA from all of the 25
250 individual cow fecal DNA samples and from all or most of the other ruminant fecal
251 samples (Table 2). Most importantly, none of the novel ruminant-specific PCR assays
252 amplified DNA from any of human sewage or fecal samples and so could be used as a
253 tool to differentiate between human and animal contamination.

254 The human-specific HF183F & Bac708R assay was 100% specific. However, the
255 assay was positive for only three of the 26 individual human fecal samples (r = 12%) and
256 only 23 of the 33 human sewage samples (r = 70%). This assay was tested on human
257 sewage samples from four European countries, including Ireland, by Gawler et al. (2007).
258 The sensitivity results obtained by these authors varied between 76% and 100%, with a
259 sensitivity of 88% for Irish samples. There appears to be a degree of variation in the
260 quantity of this target in Irish sewage samples when the results obtained in this study are
261 compared to the results obtained by Gawler et al. This would suggest a need to validate
262 this assay in the location it is to be used before its application as a MST tool.

263 The BV 1 & 2 PCR assay (Wang et al., 1994) and the BiADO 1 & 2 PCR assay
264 (Matsuki et al., 1998) had similar sensitivities for individual human fecal samples (88%
265 and 85% respectively) and both were 100% sensitive for human sewage samples (Table
266 2). These assays also had comparable specificities, amplifying DNA from many of the
267 pig samples and at least one ruminant fecal sample.

268 The BiCAT 1 & 2 PCR assay (Matsuki et al., 1998) had a sensitivity of 46% for
269 individual human fecal samples and a sensitivity of 100% for human sewage samples
270 (Table 2). The low sensitivity for individual human fecal samples is not a significant
271 drawback since from an environmental monitoring context, the ability to detect mixed

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272 sewage samples is more important than the ability to detect individual human fecal
273 samples. The assay did not test positive with any ruminant fecal samples and the only
274 non-human fecal samples which tested positive were pig slurry samples, making it
275 potentially the most useful human-specific assay tested for MST, since in many cases, pig
276 fecal contamination can be ruled out as a potential source of contamination.

277

278 **3.2.2 SLOD and MDL of host-specific PCR assays**

279 The SLOD of all PCR assays was evaluated and the MDL was evaluated for the
280 ruminant-specific assays only (because the development of ruminant-specific assays was
281 the focus of this study).

282 The ruminant-specific PCR assay with the lowest SLOD was the Bac32F &
283 RumD1R assay with a SLOD of 7.3×10^{-6} g (dry weight) of feces per filter (Table 3).
284 The assays with the next lowest SLOD were Bac32F & RumD2R, followed by RumB1F
285 & BacPreR and CF128F & Bac708R (Table 3).

286 The ruminant-specific PCR assays with the lowest MDL were RumB1F &
287 BacPreR and Bac32F & RumD2R. Both of these PCR assays tested positive with 10^2 -
288 10^3 copies of target plasmid per 50-µl PCR reaction (Table 3). The MDL for the two
289 other ruminant-specific PCR assays (Bac32F & RumD1R and CF128F & Bac708R) was
290 tenfold higher; at 10^3 - 10^4 copies of the plasmid (Table 3). The MDL of the CF128F &
291 Bac708R PCR assay was also performed in a study by Shanks et al. (2006). The authors
292 reported that the assay routinely detected 10^2 copies of target plasmid DNA, which is a 10
293 to 10^2 fold lower MDL than determined in this study. This MDL variation is possibly due
294 to the different PCR reagents, *Taq* polymerase and PCR thermal cyclers used. In a study

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295 by Gawler et al. (2007) on the CF128F & Bac708R PCR assay, a detection limit of ~ 2.5
296 $\times 10^4$ copies of target plasmid DNA was determined, similar to the results obtained in this
297 study. Inter-laboratory variation has been observed by other groups (Griffith et al., 2003)
298 which emphasizes the need to establish standard operating procedures for MST methods
299 and suggests that each new MST method should be validated in the laboratory where it is
300 to be used.

301 The human-specific PCR assays with the lowest SLODs were the BiADO 1 & 2
302 and BV 1 & 2 assays (Table 3). These assays both had SLODs of 6.6×10^{-6} g (dry
303 weight) of human sewage per filter. The BiCAT 1 & 2 assay had a SLOD of 6.6×10^{-4} g
304 and the HF183F & Bac708R assay had the highest SLOD of 6.6×10^{-2} g (dry weight) of
305 human sewage per filter. This is a relatively high SLOD which could mean the assay
306 would only be useful in cases of heavily contaminated water. However, a number of other
307 researchers have successfully used this assay in the field (Gourmelon et al., 2007;
308 Lamendella et al., 2007; Shanks et al., 2006) so this high SLOD may be due to the
309 particular sample set tested in this study.

310

311 **3.3 Application of host-specific PCR assays on naturally contaminated rural**
312 **water samples**

313 Both the novel and existing host-specific PCR assays were applied as a method of MST
314 to samples taken from contaminated rural drinking water supplies. All the rural water
315 sources were located in pasture lands for cows and sheep and so were expected to be
316 contaminated by ruminant feces. *E. coli* contamination in the rural water samples varied
317 from 0 to 2203 *E. coli* per 100 ml but only water samples with greater than 50 *E. coli* per

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318 100 ml (n = 23) were tested with the host-specific assays. The ruminant-specific PCR
319 assays with the highest detection rates were the Bac32F & RumD2R assay and the
320 RumB1F & BacPreR assay which were positive for ruminant DNA in 87% (20/23) of the
321 rural water samples tested. The Bac32F & RumD1R assay was positive for approximately
322 52% (12/23) of the rural water samples. The ruminant-specific PCR assay CF128F &
323 Bac708R assay was positive for ~ 17% (4/23) of the rural water samples. Possible
324 reasons for the superior performance of the novel ruminant-specific PCR assays include
325 the possibility that the novel assays detect microorganisms which persist for longer in the
326 environment or that the novel assays target an indigenous microorganism that is abundant
327 in local fecal pollution. The latter may indicate that methods developed in the region
328 where they are to be used may perform better than methods developed in other regions or
329 countries.

330 Two samples with relatively high levels of *E. coli* contamination (77 and 178 *E.*
331 *coli* per 100 ml) which were pre-filtered with 2.7 μm-pore-size glass fibre filter
332 membranes (Section 2.1) tested negative with all the ruminant-specific PCR assays. It is
333 possible that pre-filtration removed target species of *Bacteroidales* that may have been
334 attached to particles of manure or debris. However, these negative results could also be
335 explained by the inconsistent correlation observed in this study between the level of *E.*
336 *coli* contamination and PCR detection of *Bacteroidales* in the water (data not shown). A
337 study by Shanks et al. (2006) also found that there was poor correlation between *E. coli*
338 counts and presence of ruminant-specific *Bacteroidales.*

339 Two of the putatively human-specific assays (HF183F & Bac708R and BiCAT 1
340 & 2) did not amplify DNA from any of the rural water samples, while the other two

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341 assays (BV 1 & 2 and BiADO 1 & 2) tested positive with five and three of the rural water
342 samples, respectively. The HF183F & Bac708R and BiCAT 1 & 2 PCR assays are also
343 the only two putatively human-specific assays that did not amplify DNA from any
344 ruminant DNA samples (Table 3). It is possible that the BV 1 & 2 and BiADO 1 & 2
345 assays were detecting ruminant fecal contamination in the rural water samples rather than
346 a human fecal source of contamination. Nonetheless, the possibility of contamination of
347 the water by human waste cannot be ruled out since there may have been leaking septic
348 tanks in the area.

349 Overall, the results of the testing of the contaminated rural water samples
350 tentatively reveal, that as anticipated by land-use patterns, the main source of
351 contamination of the raw water of these three rural drinking water supplies is ruminant in
352 origin.

353

354 **4. Conclusions**

355

356 Of the five putatively human-specific published PCR assays evaluated in this
357 study, the BiCAT 1 & 2 PCR assay, which targets *Bifidobacterium catenulatum* and
358 *Bifidobacterium pseudocatenulatum*, shows most promise for use as a method of
359 detecting human fecal contamination.

360 The ruminant-specific PCR assays developed in this study show good specificity,
361 sensitivity, have low SLODs and MDLs and have been used to amplify putatively
362 ruminant-specific *Bacteroidales* strains from naturally contaminated water samples. All
363 of the assays developed in this study compared favourably to the CF128F & Bac708R

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364 PCR assay. The novel ruminant-specific PCR assays show promise for use in MST
365 studies but require more extensive evaluation both *in vitro* and in field studies before they
366 could be employed as an unambiguous method of identifying ruminant fecal pollution. As
367 mentioned in the introduction, all the PCR assays developed and/or tested in this study
368 are conventional PCR assays as opposed to real-time or quantitative PCR (qPCR) assays.
369 While qPCR offers the possibility of elucidating the quantities of different fecal inputs in
370 a contaminated water source, for true quantification of fecal sources with qPCR, not only
371 will the distribution of *Bacteroidales* in different types of feces need to be established,
372 but also the persistence of host-specific *Bacteroidales*, the stability of their relative ratios
373 in the environment and their resistance to waste treatment (Santo Domingo *et al.*, 2007).
374 Nonetheless, it would be advantageous, in the future, to convert the ruminant-specific
375 conventional PCR assays developed in this study into real-time PCR assays.
376

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511 Table 1 - Primers used in this study

512

Primers[a]	Sequence (5'-3')	Target	Annealing temp (ºC)	Reference
CF128F	CCAACYTTCCCGWTACTC	*Bacteroidales*	62ºC	(Bernhard and Field, 2000b)
HF183F	ATCATGAGTTCACATGTCCG	*Bacteroidales*	63ºC	(Bernhard and Field, 2000b)
Bac32F	AACGCTAGCTACAGGCTT	*Bacteroidales*	Variable[b]	(Bernhard and Field, 2000a)
Bac708R	CAATCGGAGTTCTTCGTG	*Bacteroidales*	Variable[b]	(Bernhard and Field, 2000a)
Bacto1080R	GCACTTAAGCCGACACCT	*Bacteroidales*	58ºC	(Dore et al., 1998)
BT 1	GGCAGCATTTCAGTTTGCTTG	*Bacteroides*	50ºC	(Wang et al., 1994)

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		thetaiotaomicron		
BT 2	GGTACATACAAAATTCCACACGT	*Bacteroides thetaiotaomicron*	50ºC	(Wang et al., 1994)
BV 1	GCATCATGAGTCCGCATGTTC	*Bacteroides vulgatus*	50ºC	(Wang et al., 1994)
BT 2	TCCATACCCGACTTTATTCCTT	*Bacteroides vulgatus*	50ºC	(Wang et al., 1994)
BiADO 1	CTCCAGTTGGATGCATGTC	*Bifidobacterium adolescentis*	55ºC	(Matsuki et al., 1998)
BiADO 2	CGAAGGCTTGCTCCCAGT	*Bifidobacterium adolescentis*	55ºC	(Matsuki et al., 1998)
BiCAT 1	CGGATGCTCCGACTCCT	*Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum*	61ºC	(Matsuki et al., 1998)
BiCAT 2	CGAAGGCTTGCTCCCGAT	*Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum*	61ºC	(Matsuki et al., 1998)
RumD1R	ATCTCTGAGCCTGTCCAG	*Bacteroidales*	60ºC	This study

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RumD2R	TGGTCCGAAGAAGGGCCC	*Bacteroidales*	63ºC	This study
RumB1F	CTCCGCATGGAGTTTCCAC	*Bacteroidales*	62ºC	This study
BacPreR	AGGTGTCGGCTTAAGTGC	*Bacteroidales*	62ºC	(Avgustin et al., 1994)

513

514 [a] The primer pairs CF128F & Bac708R, RumB1F & BacPreR, Bac32F & RumD1R and Bac32F & RumD2R are putatively ruminant-
515 feces-specific. The primer pairs HF183F & Bac708R, BT 1 & BT2, BV 1 & BV2, BiADO 1 & BiADO 2, BiCAT 1 & BiCAT 2 are
516 putatively human-feces-specific. The primer pairs Bac32F & Bacto1080R and Bac32F & Bac708R were non-host-specific.

517 [b]When Bac32F was paired with Bac708R, the annealing temperature of 53ºC was used, when paired with Bacto1080R, RumD1R or
518 RumD2R the annealing temperature listed for these reverse primers was used. When Bac708R was paired with CF128F or HF183F
519 the annealing temperature listed for these forward primers was used.

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521 Table 2 - Sensitivity and specificity of host-specific PCR assays

Sensitivity and Specificity	RumB1F & BacPreR	Bac32F & RumD1R	Bac32F & RumD2R	CF128F & Bac708R	HF183F & Bac708R	BT 1 & 2[b]	BV 1 & 2	BiADO 1 & 2	BiCAT 1 & 2
% Sensitivity (r)	97	91	100	95	12/70[c]	65/39[c]	88/100[c]	85/100[c]	46/100[c]
% Specificity (s)	97	100	95	94	100	NT	86	84	87
Sample Type	No. of positive PCR results/No. of samples tested								
Human sewage	0/33	0/33	0/33	0/33	23/33	13/33	33/33	33/33	33/33
Human feces	0/26	0/26	0/26	0/26	3/26	17/26	23/26	22/26	12/26
Cow[a]	25/25	25/25	25/25	25/25	0/25	NT	0/25	4 [w]/25	0/25
Cow[a] Slurry	5/5	4/5	5/5	1/5	0/5	NT	0/5	0/5	0/5

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Sheep[a]	37/39	34/39	39/39	39/39	0/39	NT	1/39	1[w]/39	0/39
Deer[a]	1/1	1/1	1/1	1/1	0/1	NT	0/1	0/1	0/1
Goat[a]	4/4	3/4	4/4	4/4	0/4	NT	0/4	0/4	0/4
Horse	1[w]/12	0/12	2[w]/12	0/13	0/13	NT	0/13	0/13	0/13
Donkey	0/2	0/2	0/2	0/2	0/2	NT	0/2	0/2	0/2
Dog	0/2	0/2	0/2	0/2	0/2	NT	0/2	0/2	0/2
Goose	0/1	0/1	0/1	0/1	0/1	NT	0/1	0/1	0/1
Chicken	0/2	0/2	0/2	0/2	0/2	NT	0/2	0/2	0/2
Pet Pig	2[w]/2	0/2	0/2	2/2	0/2	NT	2/2	0/2	0/2
Pig	0/8	0/8	0/8	4/8	0/8	NT	2/8	0/8	0/8
Pig Slurry	0/15	0/15	3[w]/15	0/15	0/15	NT	11/15	14/15	15/15

522 [a]Ruminant species

523 [b]These primer pairs were not tested on all the samples as they did not exhibit adequate specificity or sensitivity in initial assays.

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524 [c]Sensitivity evaluation results for human-specific PCR assays based on testing the human-specific PCR assays with individual human

525 feces (n=26)/human sewage (n=33) separately

526 [w]Indicates there was a very weak band on the gel, reflecting poor PCR product yield

527 NT – not tested

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528 Table 3 - SLOD and MDL of host-specific PCR assays

PCR Assay	SLOD[a] - g of dry feces/sewage [g of wet feces/sewage]	MDL[a] - no. of copies of plasmid per 50-µl PCR reaction
HF183F & Bac708R	6.6×10^{-2} [1×10^{-1}]	NT
BiCAT 1 & 2	6.6×10^{-4} [1×10^{-3}]	NT
BiADO 1 & 2	6.6×10^{-6} [1×10^{-5}]	NT
BV 1 & 2	6.6×10^{-6} [1×10^{-5}]	NT
CF128F & Bac708R	7.3×10^{-3} to 7.3×10^{-4} [1×10^{-2} to 1×10^{-3}]	1×10^{3} to 1×10^{4}
Bac32F & RumD1R	7.3×10^{-6} [1×10^{-5}]	1×10^{3} to 1×10^{4}
Bac32F & RumD2R	7.3×10^{-5} [1×10^{-4}]	1×10^{2} to 1×10^{3}
RumB1F & BacPreR	7.3×10^{-3} to 7.3×10^{-4} [1×10^{-2} to 1×10^{-3}]	1×10^{2} to 1×10^{3}

529 [a]10^{x} to 10^{y} indicates that all of the triplicate samples amplified at 10^{x} and some amplified

530 at 10^{y}

531 NT – not tested

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532 Fig. 1 - HEX labelled TRFLP profiles of 16S rDNA from two of each cow [(a) and (b)]
533 and sheep fecal [(c) and (d)]and human sewage DNA samples [(e) and (f)], amplified
534 with Bac32F-HEX and Bacto1080R-FAM and digested with *Hae*III.

535

536 Fig. 2 - FAM labelled TRFLP profiles of 16S rDNA fragments two of each cow [(a) and
537 (b)] and sheep fecal [(c) and (d)] and human sewage DNA samples [(e) and (f)],
538 amplified with Bac32F-HEX and Bacto1080R-FAM and digested with *Hae*III.

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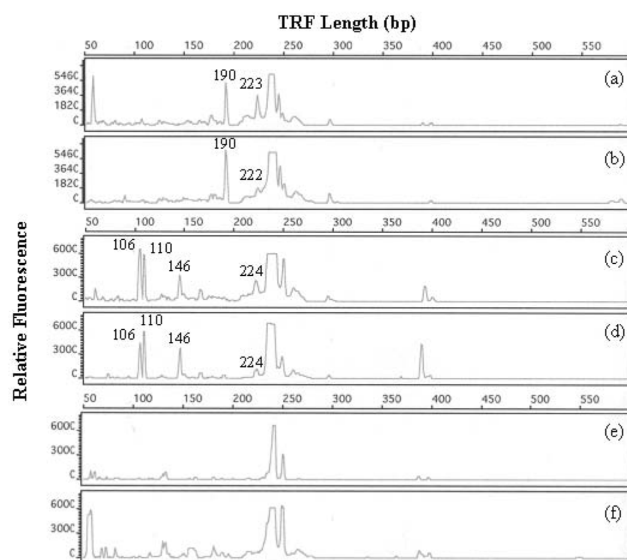

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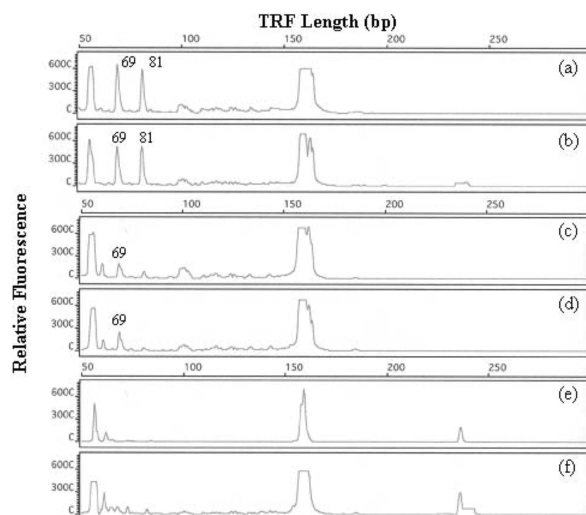

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