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1 The use of quantitative PCR for identification and quantification of *Brachyspira pilosicoli*,
2 *Lawsonia intracellularis* and *Escherichia coli* fimbrial types F4 and F18 in pig feces.

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8

9 **Abstract**

10 Four quantitative PCR (qPCR) assays were evaluated for quantitative detection of *Brachyspira*
11 *pilosicoli*, *Lawsonia intracellularis*, and *E. coli* fimbrial types F4 and F18 in pig feces. Standard
12 curves were based on feces spiked with the respective reference strains. The detection limits from
13 the spiking experiments were 10^2 bacteria/g feces for Bpilo-qPCR and Laws-qPCR, 10^3 CFU/g
14 feces for F4-qPCR and F18-qPCR. The PCR efficiency for all four qPCR assays was between 0.91
15 and 1.01 with R^2 above 0.993. Standard curves, slopes and elevation, varied between assays and
16 between measurements from pure DNA from reference strains and feces spiked with the respective
17 strains. The linear ranges found for spiked faecal samples differed both from the linear ranges from
18 pure culture of the reference strains and between the qPCR tests. The linear ranges were five log
19 units for F4-qPCR, and Laws-qPCR, six log units for F18-qPCR and three log units for Bpilo-qPCR
20 in spiked feces. When measured on pure DNA from the reference strains used in spiking
21 experiments, the respective log ranges were; seven units for Bpilo-qPCR, Laws-qPCR and F18-
22 qPCR and six log units for F4-qPCR. This shows the importance of using specific standard curves,
23 where each pathogen is analyzed in the same matrix as sample DNA. The qPCRs were compared to
24 traditional bacteriological diagnostic methods and found to be more sensitive than cultivation for *E.*

25 *coli* and *B. pilosicoli*. The qPCR assay for *Lawsonia* was also more sensitive than the earlier used
26 method due to improvements in DNA extraction. In addition, as samples were not analyzed for all
27 four pathogen agents by traditional diagnostic methods, many samples were found positive for
28 agents that were not expected on the basis of age and case history. The use of quantitative PCR tests
29 for diagnosis of enteric diseases provides new possibilities for veterinary diagnostics. The parallel
30 simultaneous analysis for several bacteria in multi-qPCR and the determination of the quantities of
31 the infectious agents increases the information obtained from the samples and the chance for
32 obtaining a relevant diagnosis.

33

34 **Introduction**

35 Diarrhoea in pigs can be caused by a number of infectious agents. Diarrhoea in newborn piglets due
36 to *Escherichia coli* is mostly associated with isolates carrying F4 fimbria, whereas diarrhoea after
37 weaning is mostly associated with F18-positive or F4- positive *E. coli* isolates (Ojeniyi et al. 1994,
38 Frydendahl 2002, Fairbrother and Gyles 2006). *Lawsonia intracellularis* and *Brachyspira pilosicoli*
39 are most commonly associated with diarrhoea in grower-finishers (Hampson and Duhamel, 2006;
40 McOrist and Gebhart, 2006). Multiple agents may occur not only in the same herd but also in the
41 same animal (Møller et al., 1998; Jensen and Boye 2005), which often makes it difficult to
42 unequivocally relate clinical signs and macroscopic pathology to the infectious agents present
43 (Jensen et al., 2006). Laboratory diagnosis normally includes cultivation, histopathology and PCR.
44 Quantification of the infectious agents involved using traditional techniques is time consuming, but
45 due to development of quantitative PCR (qPCR) assays (Guo et al., 2008; Akase et al., 2009;
46 Nathues et al., 2009; Song and Hampson, 2009), this has become a feasible option for diagnosis. It
47 is likely that the amount of the infectious agents present in feces, correlated to the onset of disease,
48 is highly relevant for correct diagnosis of disease. In the present paper, qPCR assays for four of the

49 most relevant infectious agents associated with pig diarrhoea are presented. The assays were
50 evaluated on DNA from pure bacterial cultures as well as on spiked fecal samples. The results from
51 qPCRs are compared to results from traditional diagnostic methods on 113 clinical samples from
52 Danish pig herds.

53

54 **Materials and methods**

55

56 *Clinical samples*

57

58 Clinical samples consisted of pig fecal specimens from pigs with diarrhoea obtained through routine
59 submissions to the National Veterinary Institute (NVI) in Copenhagen, Denmark during 2007 and
60 2008. A total of 113 samples from pigs in 65 different herds were analysed. Prior to analysis by
61 qPCR all samples were stored at -20°C. The diagnostics at the time of submission were performed
62 taking into consideration the age of the pigs and the disease history of the herd. Generally,
63 cultivation for *E. coli* was performed for all pigs younger than 8 weeks, while investigations for *L.*
64 *intracellularis* and *Brachyspira* spp. were performed on pigs older than 8 weeks. However,
65 depending on the disease history, some older pigs were also investigated for *E. coli* and some
66 younger pigs for *L. intracellularis*. Cultivation and serotyping of *E. coli* were performed as earlier
67 described (Frydendahl, 2002). The *E. coli* isolates were only tested against sera to the O-types that
68 have been associated with diarrhoea in Danish pigs, i.e. types 8, 45, 64, 138, 139, 141, 149, and
69 157. Strains not belonging to these serotypes were designated “O-type negative” and not expected
70 to carry the fimbrial types F4 or F18. Subcultivation and serotyping of haemolytic *E. coli* was
71 conducted on two colonies from all samples with haemolytic *E. coli*. Non-haemolytic *E. coli* were
72 subcultured and serotyped only when they were the dominant part within the microflora of a

73 sample. Cultivation for *Brachyspira* spp. was performed using trypticase soy yeast (TSYF) agar
74 plates as earlier described (Råsbäck et al., 2005) and species identification was performed by
75 biotyping (Fellström and Gunnarsson, 1995). Investigation for *L. intracellularis* was performed by
76 real-time PCR on boiled lysates of feces suspensions (Lindecrona et al., 2002).

77

78 *Reference strains*

79

80 Reference strains used for spiking of feces were *B. pilosicoli* (ATCC 51139^T), *L. intracellularis*
81 (ID # 15540), *E. coli* F4 (A1) and *E. coli* F18 (94/1) (Table 1). *L. intracellularis* was grown and
82 counted as described by Boesen et al. (2004). *B. pilosicoli* was grown in Tryptic Soy Broth (TSB;
83 Becton Dickinson, Franklin Lakes, NJ USA) with 0.1% cysteine, 0.2% glycerol, 0.0001%
84 resazurine and 5% foetal calf serum. The concentration of *B. pilosicoli* was determined by
85 fluorescent *in situ* hybridisation (FISH) (Boye et al. 1998). *E. coli* strains were grown in Veal
86 Infusion Broth (Difco, Lawrence, KS USA) and colony forming units (CFU) were determined on
87 Columbia agar (Difco) with 5% bovine blood.

88

89 *DNA extraction*

90

91 DNA was extracted from 200 µl samples of 10% feces diluted in phosphate buffered saline (PBS)
92 by using QIAcubeTM extraction robot and QIAamp DNA Stool Mini Kit (Qiagen, GmbH, Germany)
93 according to the manufacturer's instructions. The protocol was DNA-QIAamp-DNA Stool-
94 Pathogen detection Version 1. In each DNA extraction process DNA from *B. pilosicoli*, *E. coli* F4,
95 *E. coli* F18, and *L. intracellularis* were extracted and subsequently used as positive and negative
96 controls in the four different qPCRs. The controls were adjusted to give C_q values around 30 in the

97 respective qPCR. DNA from pure cultures of the four bacterial reference strains used for spiking
98 was extracted with Easy DNA (INVITROGEN A/S, Taastrup, Denmark) according to the
99 manufacturer's instructions. The DNA concentrations were measured on NanoDrop® ND-1000
100 v.3.1. Spectrophotometer (NanoDrop Technologies, Inc., USA) according to the manufacturer's
101 instructions. The concentrations of pure chromosomal DNA were used for calculations of genome
102 equivalents (GE) used in standard curves.

103

104 To validate the species specificity of the qPCR for detection of *B. pilosicoli* boiled lysates from
105 colonies of isolates and reference strains were tested. One loop-full of bacteria (1 µl) was suspended
106 in 200 µl PBS and boiled for 10 minutes. The lysate was diluted 100 times in PBS and subsequently
107 used as template in qPCR.

108

109 Biological repeatability was determined by taking double samples of feces from 14 *Lawsonia*
110 positive samples, 13 *E. coli* F4 positive samples and 14 *E. coli* F18 positive samples respectively.
111 The fecal samples were diluted to 10% in PBS, DNA was extracted by QIAcube and subsequently
112 analysed by qPCR as parallel samples. Technical repeatability was determined by measuring the
113 concentration of *B. pilosicoli* in one DNA extract from spiked feces as 15 parallel samples in Bpilo-
114 qPCR.

115

116 *qPCRs and design of standard curves for quantification*

117

118 A new set of primers and probe (Table 2) for amplification and detection of a fragment of 124 bp
119 from the 23S rDNA gene of *B. pilosicoli* was constructed. Specificity of the primers and probe at
120 the species level was examined by performing BLAST searches of Gene Bank database (Zheng *et*

121 *al.*, 2000). Primers and probe were purchased from DNA Technology A/S, Denmark. The
122 specificity was validated by using a test panel of 44 reference strains and field isolates of the genus
123 *Brachyspira* and one strain each of *E. coli*, *Enterococcus faecalis*, *Campylobacter jejuni*, *Yersinia*
124 *enterocolitica* and *Salmonella enterica* from the strain collection at NVI (Table 1). Primers and
125 probes for the *L. intracellularis*, *E. coli* F4, *E. coli* F18 assays were previously described and tested
126 (Frydendahl et al., 2001; Lindecrona, et al. 2002). However none of these assays have previously
127 been evaluated and used for quantification. In this study the different qPCRs were designated
128 Bpilo-qPCR, Laws-qPCR, F4-qPCR, and F18-qPCR.

129

130 Standard curves were made by spiking of 0.9 ml 10% feces with 0.1 ml suspensions of the different
131 reference bacteria in 10-fold dilutions, prior to DNA extraction. Three μ l extracted DNA was used
132 as template in the qPCR assays. Each standard curve was made with triplicate samples and included
133 one reference point of extracted DNA (in triplicate) from pure culture from the strain used for
134 spiking. Each subsequent qPCR experiment included the same reference concentrations of pure
135 DNA in triplicate and facilitated adjustment of the standard curves to each new qPCR run (Rotor-
136 Gene 6000 Operator Manual, Corbett Research, Mortlake, NSW, Australia).

137

138 All amplifications were run at the same cycling conditions consisting of activation at 94 °C for 2
139 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 60 s on Rotorgene 3000 or Rotorgene
140 6000 (Corbett Research) in JumpStart Taq Ready Mix for Quantitative PCR (Sigma-Aldrich
141 Danmark A/S, Brøndby, Denmark). The $MgCl_2$ concentrations were individually optimized for each
142 assay (Table 2). Each qPCR reaction was carried out in a total volume of 25 μ l containing 3 μ l of
143 sample DNA. The linear range and efficiency of each qPCR were determined in three parallel
144 experiments with 10-folds dilutions of pure DNA extracted from reference strains as well as one

145 experiment with DNA extracted from spiked feces samples. All standard curves were made in
146 triplicate and all samples were tested in duplicate. The samples that gave negative results in all four
147 bacterial qPCR assays were tested by conventional PCR with primers for the eubacterial 16S rDNA
148 (Angen et al. 1998).

149

150

151 **Results**

152

153

154 *qPCR validation*

155

156 The R^2 values for all standard curves were above 0.993 and the PCR efficiencies for all four qPCR
157 assays ranged from 0.91 to 1.01, both when pure bacterial DNA was used as template and when
158 DNA was extracted from spiked feces specimens. The detection limits from the spiking experiments
159 were 10^2 bacteria/g feces for Bpilo-qPCR and Laws-qPCR, and 10^3 CFU/g feces for F4-qPCR and
160 F18-qPCR (Table 3). Detection limit was defined as the lowest concentration giving a positive
161 quantification cycle (C_q) value in one or more of the triplicate samples of the standard curves. The
162 lower limits of the linear ranges were based on the mean value of triplicates. The limits of the linear
163 ranges define the quantification limits of each qPCR assay. The linear ranges were: five log units
164 for F4-qPCR, and Laws-qPCR, six log units for F18-qPCR and three log units for Bpilo-qPCR in
165 spiked feces (Figure 1 and Table 3). When measured on pure DNA from the reference strains used
166 in spiking experiments, the respective log ranges were; seven units for Bpilo-qPCR, Laws-qPCR
167 and F18-qPCR and six log units for F4-qPCR. Negative controls from the DNA extraction as well
168 as no template controls (NTCs) were all negative.

169

170 The biological repeatability was determined as the average coefficient of variation in percent
171 (CV%) of log concentrations: the average CV% was 1.3 for the 14 different double samples
172 analysed by Laws-qPCR; the average CV% was 1.6 for the 13 double samples analysed by F4-
173 qPCR; and the average CV% was 2.2 for the 14 double samples analysed by F18-qPCR. The
174 technical repeatability was determined by measuring DNA from one spiked fecal sample in 15
175 replicates by Bpilo-qPCR, the mean log concentration was 6.81 and CV 0.8%.

176

177 *Validation of the species specificity of the Bpilo-qPCR*

178

179 All *B. pilosicoli* isolates tested were positive in the Bpilo-qPCR. The Cq for boiled and diluted
180 lysates of *B. pilosicoli* ATCC 51139^T as well as for seven of the eight *B. pilosicoli* isolates from pig
181 feces were between 16 and 20. One isolate from pig feces isolated at the NVI tested positive with a
182 Cq of 33, that isolate was biotyped as *B. innocens*. The “isolate” was shown to contain a mixture of
183 *Brachyspira* species, including approximately 0.1% *B. pilosicoli* by FISH (data not shown). All
184 other isolates and reference strains used (Table 1) were negative in the Bpilo-qPCR.

185

186 *Traditional bacteriological diagnosis compared to qPCR.*

187

188 In 89 (79%) of 113 samples one or several of the four pathogens were detected by qPCR (Tables 4-
189 7 supplementary material). In 50 out of the 113 (44%) samples no pathogens were diagnosed at the
190 time of submission. Twenty (18%) of the samples were true negatives (no pathogens found in
191 neither qPCR nor at the time of submission) whereas four (3.5%) samples were false negative in
192 qPCR (Tables 4-7 supplementary material). All samples that were negative in the four qPCRs were

193 found positive when analysed with a PCR test against eubacterial 16S rDNA (data not shown),
194 showing that amplifiable DNA was present.

195

196 *Comparison of F4-qPCR and F18-qPCR results with results of cultivation and serotyping of E. coli*

197

198 Out of the 113 feces samples analysed, 49 were positive in one or both of F4-qPCR and F18-qPCR
199 (Table 4 and 5 supplementary material). Cultivation and serotyping of *E. coli* identified 19 samples
200 with O-typable *E. coli* strains expected to carry the fimbrial types F4 and F18, all of those were
201 positive by F4-qPCR and/or F18-qPCR (Table 4 supplementary material). Among the 46 samples
202 which were not cultivated for detection of *E. coli* but only tested in qPCR, a total of 14 samples
203 were positive in either one or both of F4-qPCR and F18-qPCR.

204

205 The measured concentration of *E. coli* in feces samples ranged between 10^4 to 10^{10} CFU/g feces. In
206 one sample where no growth of pathogenic *E. coli* was detected at the time of submission, 9.4×10^8
207 CFU/g feces were detected by F4-qPCR (Table 5 supplementary material). Between 1.0×10^5 –
208 1.0×10^7 CFU/g feces of *E. coli* F4 and/or F18 were detected in four samples where no pathogenic
209 *E. coli* were cultivable. That high occurrence, above 1.0×10^5 CFU/g feces, of *E. coli* F4 and/or F18
210 were otherwise only detected in samples also being positive by culture or in samples not cultivated
211 for *E. coli*.

212

213 *Comparison of detection by Bpilo-qPCR and cultivation and biotyping of B. pilosicoli*

214

215 Bpilo-qPCR gave positive reactions in 31 (27%) of the 113 tested samples, only 19 of these had
216 been cultivated for *Brachyspira*, eight of those were positive for *B. pilosicoli* (Table 6

217 supplementary material). In four samples were *B. pilosicoli* had been identified by biotyping, Bpilo-
218 qPCR gave negative results. The highest measured concentration was 4×10^7 *B. pilosicoli*/g feces;
219 this sample was one of those where no *B. pilosicoli* was detected by cultivation. (Table 6
220 supplementary material).

221

222 *Comparison of detection of L. intracellularis by real-time PCR on boiled lysates of feces and*
223 *detection by Laws-qPCR*

224

225 All samples that were positive by real-time PCR on boiled lysates were positive in Laws-qPCR. Out
226 of the 37 samples that were positive by Laws-qPCR, 23 were analysed by real-time PCR on boiled
227 lysates, 13 gave positive real-time PCR results and 10 were negative in real-time PCR (Table 7
228 supplementary material). In 68 cases, samples were not analysed for *L. intracellularis* at the time of
229 submission, 14 of those were positive for *L. intracellularis* when tested by Laws-qPCR. Up to
230 3×10^8 bacteria/g feces of *L. intracellularis* were detected in the clinical samples. The Laws-qPCR
231 positive samples containing less than 1×10^4 bacteria/g feces were not found positive by PCR on
232 boiled lysates (Table 7 supplementary material).

233

234 *Quantification of mixed infections*

235

236 All four pathogenic agents were found in two fecal samples. Many samples contained two or three
237 pathogens. The amount of pathogenic *E. coli* F4 and/or *E. coli* F18 was above 1×10^7 CFU/g feces in
238 18 samples. When more than 10^5 *E. coli* F4 and/or *E. coli* F18 were detected, no *L. intracellularis*
239 was found (Table 8 supplementary material). On the other hand two samples with more than 10^6 *L.*
240 *intracellularis* also contained *E. coli* F4 and/or *E. coli* F18. *L. intracellularis* and *B. pilosicoli* were
241 often found together in both high and low concentrations (Tables 9 and 10 supplementary material).

242 *L. intracellularis* above 1×10^6 bacteria/g feces were found in 16 samples, seven of these also
243 contained *B. pilosicoli*.

244

245 **Discussion**

246 The use of quantitative PCR tests for diagnosis of enteric diseases provides new possibilities for
247 veterinary diagnostics. The advantage of offering a diagnostic package of four of the most relevant
248 pathogens causing diarrhoea in pigs is of course to lessen the risk of not detecting the relevant
249 pathogens in each specific case. Also, the determination of the quantities of the infectious agents
250 increases the information obtained from the samples. The analyses will also be less time consuming
251 and less expensive than traditional diagnostics. Since the four qPCR tests have the same
252 thermocycling profile it is possible to analyse parallel samples in all assays, in the same
253 thermocycler, at the same time.

254

255 Determination of PCR efficiency, linear range, and detection limit are important parameters in
256 describing a qPCR test (Bustin et al., 2009). The comparison to other published assays is made
257 difficult by the lack of information regarding PCR efficiency in many publications. A multiplex
258 qPCR for detection of pathogenic intestinal spirochaetes (Song et al., 2009) is published, but the
259 authors have neither presented the dynamic ranges nor the PCR efficiencies for the different PCR
260 reactions in the assay. Nathues et al. (2009) quantified *L. intracellularis* based on a standard curve
261 from a plasmid containing a cloned fragment from *L. intracellularis*. The standard curve showed
262 excellent dynamic range and PCR efficiency, however the limit of quantification was 2.8×10^6 GE
263 of *L. intracellularis* per gram feces corresponding to 10 GE per μl reaction volume. In this study the
264 limit of quantification was 3×10^3 bacteria/g feces. When testing pure bacterial DNA, the qPCR tests

265 had linear ranges of 10^1 - 10^8 GE/reaction in accordance with what was found by Nathues et al.
266 (2009). To our knowledge no other qPCR tests for *E. coli* fimbria have been published.

267

268 The PCR efficiencies of the bacterial qPCR tests when testing pure bacterial DNA were all within
269 the range 0.95-0.97. The PCR efficiencies when testing feces samples spiked with bacteria were
270 between 0.95 and 1.01 for all tests except Bpilo-qPCR where a value of 0.91 was found. The reason
271 for the relatively lower efficiency of the Bpilo-qPCR on fecal samples is not clear; however the
272 sensitivity of the PCR reaction to inhibiting substances might be dependent on the probe and
273 primers. Recently it has been shown that different PCR reactions may show different susceptibility
274 to inhibitors (Huggett et al., 2008). This is in accordance with the findings in this study that the
275 standard curves of the four qPCRs are affected to different degrees by the feces extracts. It is
276 important to note that all four standard curves show some degree of difference in dynamic range,
277 slope or elevation when comparing qPCR on pure bacterial DNA and qPCR on DNA extracted
278 from spiked feces (Figure 1). This shows the importance of specific standard curves were each
279 pathogen is analyzed in the same matrix as the samples. However, this is not yet common practise,
280 as most publications report the use of standard curves prepared from dilutions of pure bacterial
281 DNA (Guo et al., 2008; Akase et al., 2009; Furet et al., 2009; Nathues et al., 2009).

282

283 The detection limits of the tests were 10^2 bacteria per gram feces for Laws-qPCR and Bpilo-qPCR
284 and 10^3 CFU/gram feces for the two *E. coli* qPCR tests. The differences between the qPCRs may
285 partly be caused by the different methods of measuring; both *L. intracellularis* and *B. pilosicoli*
286 were measured as total cell count while *E. coli* was measured as viable cell count. Similar detection
287 limit for *Brachyspira* qPCR test have been reported by Song and Hampson (2009). The lower limit
288 of linear range for Laws-qPCR is only 0.9 GE/reaction, which may seem too low to be theoretically

289 possible (Bustin et al., 2009). However, since *L. intracellularis* carries 6 rRNA genes (J. Craig
290 Venter Institute, <http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntli04> 2010-10-13)
291 detection of 0.9 GE/reaction corresponds to 5-6 copies/reaction. *B. pilosicoli* carries three rRNA
292 genes (Wanchanthuek et al., 2010), which also contributes to a lower limit of linear range and
293 detection limit of Bpilo-qPCR compared to a qPCR based on a single copy gene. The discrepancy
294 between the standard curves of pure DNA and the standard curves based on bacteria in spiked feces
295 could be explained by free DNA from lysed cells or by the presence of more than 1 GE/cell
296 depending on growth phase.

297
298 The performance of the qPCR tests was further assessed by analysis of 113 clinical samples
299 originating from pigs with diarrhoea submitted to NVI for routine diagnostics. At the time of
300 submission, samples were only analysed for those bacteria prescribed according to the age of the
301 pigs and the disease history of the herd. The distribution of the bacterial agents in relation to age
302 (Figure 2 supplementary material) was generally as expected according to literature (Frydendahl,
303 2002; Fairbrother and Gyles, 2006; Hampson and Duhamel, 2006; McOrist and Gebhart, 2006).
304 However, for all bacteria quantified in the present investigation, samples were found positive for
305 agents that were not expected on the basis of age and case history.

306
307 The sensitivity of the qPCR was higher when compared to cultivation of *E. coli* F4, *E. coli* F18 and
308 *B. pilosicoli*. In 34% of the samples that were positive in F4-qPCR and/or F18-qPCR, pathogenic *E.*
309 *coli* were not detected by cultivation. This may reflect the higher sensitivity of a PCR test compared
310 to traditional cultivation or that these fimbrial genes were carried by non haemolytic strains. When
311 more than 10^7 CFU/gram of *E. coli* F4 and/or *E. coli* F18 were detected this was correlated with the
312 cultivation of a high number of potentially pathogenic *E. coli* (Table 5 supplementary material).

313 However, massive occurrence of *E. coli* F4 and/or F18 was detected in samples that had not been
314 cultivated for *E. coli* at the time of submission.

315

316 A higher number of samples were found positive for *B. pilosicoli* by qPCR than by cultivation
317 (Table 6 supplementary material). Komarek et al. (2009) also found more samples positive by using
318 PCR tests against the different *Brachyspira* spp. than by cultivation. In the present investigation,
319 four out of the 12 samples from which *B. pilosicoli* had been cultivated were found negative by
320 Bpilo-qPCR. Similarly, Komarek et al. (2009) reported that several samples were PCR negative
321 although they were found cultivation positive. Råsbäck et al. (2006) however, reported that
322 compared to cultivation PCR lowered the sensitivity by a factor $10^3 - 10^4$ for detection of *B.*
323 *hyodysenteriae* and *B. pilosicoli* in feces. This may reflect the different performance of different
324 PCR tests in different matrices. Clearly the Bpilo-qPCR was the one most adversely affected by the
325 feces extracts in this study.

326

327 Of the 19 Bpilo-qPCR positive samples that were analysed for *B. pilosicoli* by cultivation, 42%
328 yielded growth of *B. pilosicoli*. The highest number of *B. pilosicoli* detected among the clinical
329 samples was 4×10^7 cells per gram feces. From this sample no *B. pilosicoli* had been detected by
330 cultivation. Antibiotic therapy and mixed spirochetal infections might reduce the chances of
331 identifying *B. pilosicoli* by culture and might be an explanation for this result. For *Brachyspira* spp.
332 similar high numbers of bacteria have only been reported in connection with clinical disease. Neef
333 et al. (1994) report that $10^7 - 10^8$ CFU per gram of *B. hyodysenteriae* are excreted in connection
334 with clinical disease. According to Råsbäck et al. (2006) up to $10^8 - 10^{10}$ cells per gram feces are
335 shed in the acute phase of the disease.

336

337 The Laws-qPCR test for *L. intracellularis* detected more positive samples than the earlier used real
338 time PCR, reflecting the removal of inhibitors by using a better DNA extraction protocol. On the
339 other hand, Nathues et al. (2009) reported that 96.5% of the DNA present in a sample is lost during
340 the DNA extraction process. The highest concentration of *L. intracellularis* measured among the
341 clinical samples by Laws-qPCR was 3×10^7 bacteria per gram feces. For *L. intracellularis* a
342 maximum shedding of 7×10^8 bacteria per gram feces has been measured by IFT (Smith and
343 McOrist, 1997). A correlation between the number of *L. intracellularis* present in mucosal
344 scrapings and the severity of lesions was demonstrated by Guedes et al. (2003).

345

346 It can be concluded that the qPCR analyses of several important pathogens from one sample in the
347 veterinary laboratory gives a much more complete picture of the microbiological status to the
348 consulting veterinarian, both regarding which pathogens that are present as well as their possible
349 relevance. An important question is how to interpret the quantitative PCR data in terms of clinically
350 relevant diagnostics. The quantity of a microorganism present in the pig intestine is dependent on a
351 number of factors including the age of the animal, stage in the disease process, virulence of the
352 actual microorganism, and host immunity. A number of publications report on a link between the
353 amounts of pathogens present in feces and clinical symptoms. However, only a few publications
354 have used qPCR methods and no publications are available that can be used as a basis for giving
355 clear indications for how to interpret these quantitative data and the presence of several pathogenic
356 agents simultaneously. Studies in progress on Danish pig herds will hopefully make it feasible to
357 evaluate the diagnostic potential of the qPCR tests presented in the present paper.

358

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363

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Table 1
Reference strains and strains used for evaluation of sensitivity and specificity.

Species	Strain (n=number of isolates)	Source
<i>L. intracellularis</i>	ID # 15540	Boehringer Ingelheim Vetmedico
<i>E. coli</i>	A1, F4 O149: K91 94/1, F18 O139: K12: H1	Ørskov et al. (1969) Aarestrup et al. (1997)
<i>E. coli</i> ^a	Isolate from pig	NVI ^b
<i>B. hyodysenteriae</i> ^a	ATCC 27164 ^T AN 1050 AN 1425 AN 849	ATCC ^c SVA ^d SVA ^d SVA ^d
<i>B. intermedia</i> ^a	Isolates from pig (n=3) ATCC 51140 ^T 8109	NVI ATCC NVI
<i>B. murdochii</i> ^a	Isolates from pig (n=8) ATCC 51284 ^T C301 C378 AN 181/1/04 AN 4737/03 AN 35491/03	NVI ATCC SVA ^e SVA ^e SVA ^e SVA ^e SVA ^e
<i>B. innocens</i> ^a	ATCC 29796 ^T E646 520	ATCC NVI SVA ^d
<i>B. pilosicoli</i> ^a	Isolates from pig (n=3) ATCC 51139 ^T	NVI ATCC
“ <i>B. suanatina</i> ” ^a	Isolates from pig (n=8) AN 4859/03 AN 1681:1/04 AN 2384/04 AN 3949:2/02 AN 1418:2/01	NVI SVA ^e SVA ^e SVA ^e SVA ^e SVA ^e
<i>Enterococcus faecalis</i> ^a	Isolate from pig ATCC 29212	NVI ATCC
<i>Campylobacter jejuni</i> ^a	Isolate from pig	NVI
<i>Yersinia enterocolitica</i> ^a	Isolate from pig	NVI
<i>Salmonella enterica</i> ^a	CCUG 31969	CCUG ^f

^a Used in validation of Bpilo-qPCR

^b National Veterinary Institute, Copenhagen, Denmark

^c American Type Culture Collection

^d Claes Fellström, National Veterinary Institute, Uppsala Sweden

^e Désirée S Jansson, National Veterinary Institute, Uppsala Sweden

^f Culture Collection, University of Göteborg, Sweden

Table 2
Concentrations of primers, probes and Mg²⁺ in the four qPCR's.

Assay	Primer and probe sequences 5'-3' direction	Concentrations (nM)	Mg ²⁺ (mM)	Sequence accession number
Bpilo-qPCR	GTA GTC GAT GGG AAA CAG GT	600	5.0	U72703.1
	TTA CTC ACC ACA AGT CTC GG	300		
	FAM ^a -TAT TCG ACG AGG ATA ACC ATC ACC T-3 BHQ-1 ^b	150		
Laws-qPCR	GCG CGC GTA GGT GGT TAT AT	900	5.0	L15739
	GCC ACC CTC TCC GAT ACT CA	900		
	FAM-CAC CGC TTA ACG GTG GAA CAG CCT T-TAMRA ^c	200		
F4-qPCR	CAC TGG CAA TTG CTG CAT CT	600	3.5	M29374
	ACC ACC GAT ATC GAC CGA AC	600		
	FAM-TCA CCA GTC ATC CAG GCA TGT GCC-TAMRA	200		
F18-qPCR	GGC GGT TGT GCT TCC TTG T	600	5.0	M61713
	CCG TTC ACG GTT TTC AGA GC	600		
	FAM-TAA CTG CCC GCT CCA AGT TAT ATC AGC TGT T-TAMRA	200		

^a 6-carboxy-fluorescein (FAM)

^b black hole quencher-1 (BHQ-1)

^c 6-carboxy-tetramethyl-rhodamine (TAMRA)

Table 3

Linear range and detection limits of the qPCR assays when tested with pure DNA from the reference strains and DNA extracted from 10% feces spiked with the same reference strains.

qPCR assay	PCR efficiency DNA	Linear range ^a DNA	PCR efficiency Spiked feces	Linear range Spiked feces	Detection limit ^b Spiked feces
	(SD)	GE ^c /reaction		Bacteria ^d /g feces	Bacteria ^d /g feces
F4-qPCR	0.96 (0.03)	4.0x10 ⁷ - 4.0x10 ¹	0.99	9.4x10 ⁹ - 9.4x10 ⁴	10 ³
F18-qPCR	0.96 (0.01)	4.5x10 ⁸ – 4.5x10 ¹	1.01	1.9x10 ¹⁰ -1.9x10 ⁴	10 ³
Laws-qPCR	0.97 (0.04)	9.0x10 ⁷ - 9.0x10 ⁰	0.95	4x10 ⁸ - 4x10 ³	10 ²
Bpilo-qPCR	0.95 (0.01)	4.2x10 ⁸ - 4.2x10 ¹	0.91	1x10 ⁸ -1x10 ⁵	10 ²

^a Triplicate samples positive in the lowest concentration

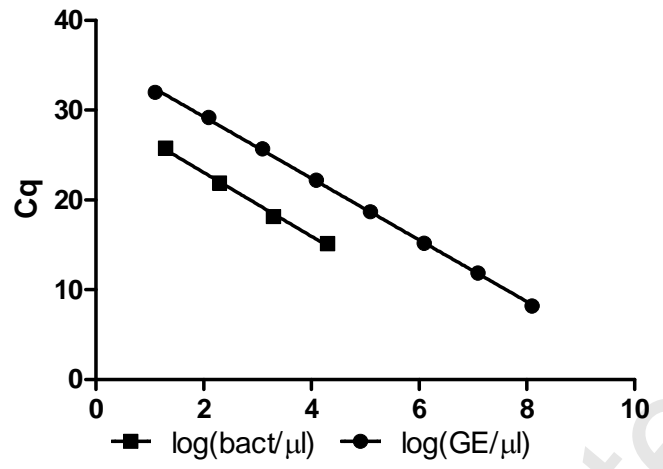
^b Detection limit is defined as the lowest concentration giving a positive Cq value in one or more of the triplicate samples of the standard curves

^c genome equivalents

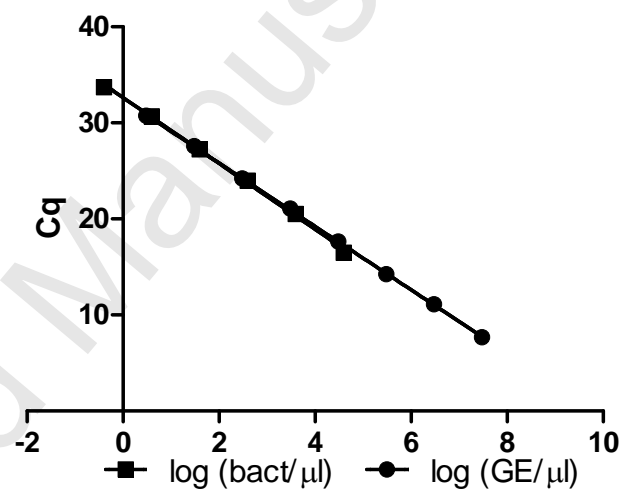
^d For F4-qPCR and F18-qPCR colony forming units (CFU); for Bpilo-qPCR and Laws-qPCR cell count

Figure 1

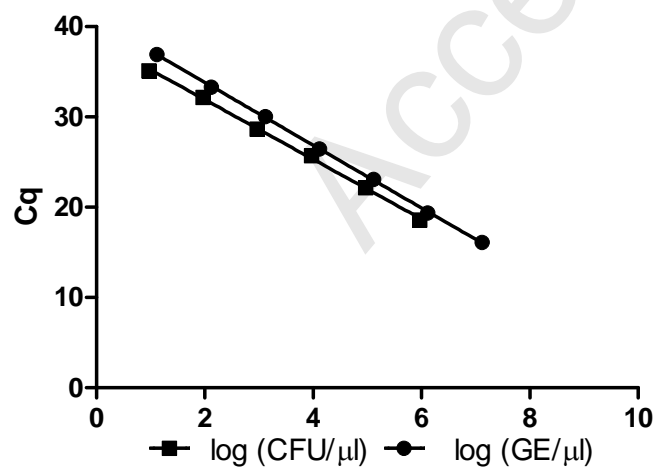
Bpilo-qPCR



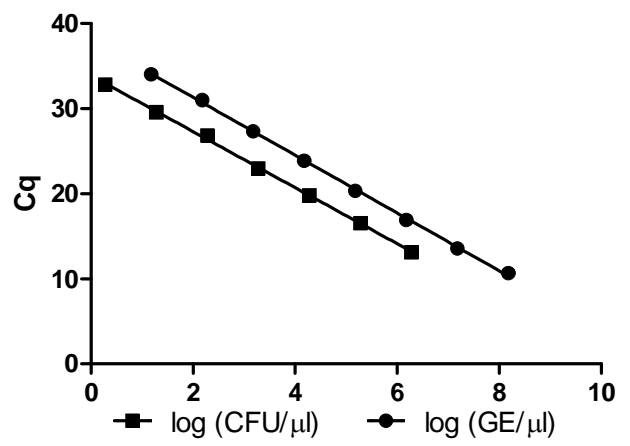
Laws-qPCR



F4-qPCR



F18-qPCR



Legend

Figure 1 Standard curves of the four qPCRs, average C_q values and log concentration. The concentration for *B. pilosicoli* and *L. intracellularis* are measured as log (bact/μl) in spiked feces, each compared to respective standard curve from pure bacterial DNA measured as log (genome equivalents (GE)/μl). The concentrations of *E. coli* fimbrial type F4 and *E. coli* fimbrial type F18 are measured as log (colony forming units (CFU)/μl) in spiked feces, each compared to respective standard curve from pure bacterial DNA measured as log (GE/μl).

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