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Development of a multiplex qPCR for detection and quantitation of pathogenic intestinal spirochaetes in the faeces of pigs and chickens

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Running title: qPCR for *Brachyspira* spp.

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Abstract

Anaerobic intestinal spirochaetes of the genus *Brachyspira* include several important pathogenic species, particularly those infecting pigs and chickens. In this study a multiplex-quantitative polymerase chain reaction (M-qPCR) assay was developed based on amplification of a 198 base pair portion of the NADH oxidase gene, using TaqMan probes for detecting and quantifying the three main pathogenic species, *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia*. The specificity of the assay was validated using 130 spirochaete strains belonging to members of the seven officially named and two provisionally named *Brachyspira* species. The detection limit for all three targeted species was 1-10 viable cells and 10 fg DNA per reaction. Further detection limit testing was conducted on porcine and chicken faecal specimens that were spiked with spirochaete cells before DNA extraction. The assay could detect 10^2 to 10^3 cells per 0.2 g of sample, giving an improved detection threshold compared to standard PCRs. The M-qPCR was further developed by incorporating a novel internal control (IC) that employed host cells as template DNA. This adaptation allowed monitoring of the quality of the extracted DNA and ensured that there was no inhibition of the PCR reaction. Use of the IC further improved the detection limits of the assay and increased confidence in being able to detect low numbers of pathogens in faecal samples. Taken together, the results indicate that the new M-qPCR assay is a valuable tool for detecting and quantifying low numbers of pathogenic intestinal spirochaetes in the faeces of pigs and chickens, and potentially other species.

1. Introduction

The genus *Brachyspira* currently comprises seven species of anaerobic intestinal spirochaetes (Stanton, 2006). In pigs the two most common diseases associated with these spirochaetes are swine dysentery caused by *Brachyspira hyodysenteriae* (Hampson et al., 2006) and intestinal spirochaetosis caused by *B. pilosicoli* (Hampson and Duhamel, 2006), with *B. intermedia* being a possible cause of colitis (Hampson et al., 2006). In poultry, avian intestinal spirochaetosis is caused by one or more species (Hampson and Swayne, 2008), but mainly *B. intermedia* and *B. pilosicoli* (Stephens and Hampson, 1999; Bano et al., 2008). Less commonly *B. alvinipulli* or *B. hyodysenteriae* may be involved (Feberwee et al., 2008).

Laboratory diagnosis of *Brachyspira* infections traditionally has relied on selective anaerobic culture, with biochemical testing of isolates. More recently polymerase chain reaction (PCR) methodology has been developed, including the use of multiplex formats which, for example, allow identification of *B. hyodysenteriae* and *B. pilosicoli* in porcine faeces (La et al., 2003), *B. intermedia* and *B. pilosicoli* in chicken faeces (Phillips et al., 2006), and *Brachyspira* species together with other pathogens such as *Salmonella* spp. or *Lawsonia intracellularis* (Elder et al., 1997; Suh and Song, 2005; La et al., 2006; Nathues et al., 2007).

Quantitative real time PCR (qPCR) assays based on either SYBR green for quantification of amplified DNA or TaqMan probe technology are now widely used for diagnosis. A SYBR green-based qPCR has been described for quantitation of *B. hyodysenteriae* in experimentally infected mice (Davis et al., 2005), but there have been no reports on the use of qPCR systems for routine diagnosis of *Brachyspira*

spp.. The present study describes the development of a multiplex assay for detecting *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia* in samples from pigs and chickens.

2. Materials and methods

2.1. Spirochaete strains, culture conditions and DNA preparation

A total of 130 well-characterized *Brachyspira* spp. strains were obtained from the collection held at the Australian Reference Centre for Intestine Spirochaetes at Murdoch University. These strains were *B. hyodysenteriae* (n=43), *B. intermedia* (n=24), *B. pilosicoli* (n=23), *B. innocens* (n=17), *B. murdochii* (n=7), "*B. pulli*" (n=5), "*B. canis*" (n=5), *B. alvinipulli* (n=4), and *B. aalborgi* (n=2). The spirochaetes were originally isolated from pigs, chickens, dogs and human beings, and came from diverse geographical origins including Australia, North America and Europe. The strains were routinely grown at 37°C in Kunkle's pre-reduced anaerobic broth containing 2% (v/v) foetal bovine serum and 1% (v/v) ethanolic cholesterol solution (Kunkle et al., 1986). Spirochaete DNA was purified from pelleted cells using the DNeasy Tissue Kit (Qiagen Pty Ltd, Doncaster, Australia), and DNA concentrations were determined using a spectrophotometer. Cells of *B. hyodysenteriae* strain $B78^T$, *B. pilosicoli* strain 95/1000 and *B. intermedia* strain PWS/A^T in broth cultures were enumerated with a counting chamber and subjected to tenfold serial dilutions from 10^6 to 10^0 cells. The spirochaete DNA was extracted from each dilution using the boiling method (Fellström et al., 2001), and this was used to evaluate the performance and set up standard curves for the qPCR. The diluted cells also were

used to spike porcine and chicken faeces to determine the limits of qPCR detection following DNA extraction from the faeces.

2.2. Faecal samples from pigs

A total of 212 faecal samples were collected from grower pigs on six farms. Two of the farms (100 samples) were of high health status, and the other four had reported recent outbreaks of swine dysentery. Faecal samples from another 39 pigs that had been experimentally challenged with cultures containing 10^{10} cells of *B. hyodysenteriae* over three successive days also were examined. The samples were collected 15 days post-infection, when they were not showing clinical signs.

2.3. Faecal samples from chickens

A total of 100 faecal samples from adult laying chickens on six farms with unknown *Brachyspira* spp. status were collected for testing.

2.4. Culture and culture-PCR

The porcine faecal samples were streaked onto selective Trypticase Soy agar (BBL, Becton and Dickinson Microbiology Systems, Cockeysville, Md.) containing 5% (v/v) defibrinated sheep blood, 400 µg/ml spectinomycin and 25 µg/ml each of colistin and vancomycin (Sigma-Aldrich, Caste Hill, Australia) (Jenkinson and Wingar, 1981). The plates were incubated for 5-7 days at 37°C in a jar with an anaerobic environment generated using a GasPak Plus™ disposable hydrogen plus

carbon dioxide generator envelope with a palladium catalyst (BBL). The presence of low flat spreading growth of spirochaetes on the plates was recorded. Suspected areas of spirochaete growth were resuspended in phosphate buffered saline and examined under a phase contrast microscope at 400 × magnification. The DNA was extracted from spirochaete cells picked from the plates, as described above for cell pellets, and then subjected to normal PCRs (nPCR) and the new M-qPCR for *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia*. The three species-specific nPCRs were performed separately, targeting a 354 base pair (bp) region of the *nox* gene for *B. hyodysenteriae*, an 823 bp region of 16S rRNA gene of *B. pilosicoli* and a 567 bp region of the *nox* gene for *B. intermedia*. The PCR primers and conditions have been described previously (La et al., 2003; Phillips et al., 2005), and the method was designated culture-PCR. Culture-PCR was not applied to the chicken faeces.

2.5. DNA extraction from faecal samples and spiking protocol

DNA was extracted from the faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, 1 g faeces was suspended in 10 ml buffer ASL and vortexed until thoroughly homogenized, and 2ml of lysate was used for DNA extraction, as previously described (La et al., 2005). In the spiking experiment, 2 ml of lysate from pig and chicken faecal samples that were free of spirochaetes was mixed with serial 10-fold dilutions of *B. hyodysenteriae* B78^T, *B. pilosicoli* 95/100 and/or *B. intermedia* PWS/A^T individually or together prior to further processing.

2.6. Primers and probes

142

143 A total of 38 *nox* gene accessions for *Brachyspira* species strains were
144 analysed. Multiple alignments were performed with the sequences retrieved from
145 GenBank (http://www.ncbi.nlm.nih.gov) using ClustalW (European Bioinformatics
146 Institute). The analysed sequences included those from 8 *B. hyodysenteriae* strains
147 (U19610, AF060800, AF060801, AF060802, DQ487115, DQ487116, DQ487117,
148 DQ487118), 5 *B. pilosicoli* strains (EF517547, AF060806, AF060807, AF060808,
149 AF060809), 7 *B. intermedia* strains (EF517542, EF517543, DQ458796, AH015259,
150 AF060810, AF060811, AF060812), 4 *B. innocens* strains (EF517544, EF517546,
151 AF060804, AF060805), 6 *B. murdochii* strains (EF517548, EF517545, AF060803,
152 AF060813, AH015257, AH015260), 1 *B. aalborgi* strain (AF060816), 1 *B.*
153 *alvinipulli* strain (AF060814) and 6 "*B. suanatina*" strains (DQ487119, DQ487120,
154 DQ487121, DQ487122, DQ487123 and DQ487124). A 198 bp region of the gene
155 then was used to design primer sets from sequences that were present and conserved
156 in the three target species, and probes with little or no sequence variation amongst
157 strains of the target species, but with a high divergence from the sequences of the
158 other species.

159

160 The internal control (IC) was designed on the housekeeping β-actin gene. The
161 β-actin genes from pigs, chickens, human beings, dogs, horses and cattle were
162 downloaded from GenBank (U07786, L08165, NM_001101, AF021873 U39357 and
163 AF035774 respectively), aligned, and conserved sequences were selected for
164 designing primers and probes. A final 98 bp amplification product was used.

165

166 The oligonucleotide qPCR primers and TaqMan probes were designed using
167 Primer Express version 2.0 (Applied Biosystems) and were examined for specificity
168 using Blastn homology searches. The hybridization probes were 5' labelled with
169 FAM for *B. hyodysenteriae*, JOE for *B. pilosicoli*, ROX for *B. intermedia*, and CY5
170 for IC. All probes were labelled with a non-fluorescent Black Hole Quencher (BHQ)
171 dye: BHQ-1 for FAM and JOE, and BHQ-2 for ROX and CY5. The primers and
172 FAM probe were ordered from Geneworks (Adelaide, Australia) while the rest of the
173 probes were purchased from Sigma-Proligo (Sigma-Aldrich). The sequences of the
174 primers and probes are listed in Table 1.

175 **[Table 1 about here]**

176 *2.7. qPCR optimization and M-qPCR protocol*

177

178 Initially, the qPCR was optimised on annealing temperature as a monoplex
179 format for each species. The M-qPCR then was established on the lowest optimal
180 temperature, and further optimized on each reagent by comparative analysis of
181 detection limits using serial dilutions of spirochaete DNA.

182

183 Cycling condition consists of an initial 15 min at 95°C, followed by 40-45
184 amplification cycles of a denaturing step at 95°C for 30 s, annealing at 55°C for 30 s
185 and extension at 70°C for 20 s. The final PCR protocol involved a 20 µl reaction
186 containing 1 × PCR buffer, 4.5 mM $MgCl_2$, 0.5 mM of each DNTP, 2.5 pmol of each
187 *Brachyspira* primer, 0.62 pmol of the IC primers, 0.19 pmol of each probe, 0.1U
188 HotStar Taq (Qiagen) and 1-5 µl template DNA. Where the qPCR was applied to the
189 culture format, the IC plasmid template β-actin DNA was included in the reaction to
190 a concentration of 0.1 pg plasmid DNA. The DNA was prepared from the swine

faecal samples by PCR amplification using the IC primers of the faecal-qPCR (Table 1) and then cloned into a pGEM®-T-Easy Vector (Promega, Annandale, Australia). The forward primer was replaced with the oligonucleotide complementary to the pGEM plasmid sequence (labelled "forward for culture-qPCR" in Table 1) to ensure amplification only of the genetically modified IC template.

2.8. Interpretation of the qPCR output

Reactions were carried out with a negative control containing sterile water and the 10-fold serial dilutions of known amounts of positive control DNA from 10^6 to 10^0 cells per reaction for generating standard curves. Due to the presence of a single nucleotide polymorphism (SNP) at the probe-binding site in *B. pilosicoli* P43/6/78^T, detection limit tests also were undertaken using the same amount of DNA from this strain. The assays were performed using a Rotor-gene 3000 (Corbett Life Science, Mortlake, NSW, Australia). By monitoring the increase in fluorescence, the threshold cycle (Ct) values representing the cycle number were obtained directly via Rotor-gene 3000 software when the measured fluorescence increased above the calculated background fluorescence in the positive samples. The positive signal of the target DNA was automatically assigned to a cell copy number with reference to the set standard curves.

In the faecal-qPCR, the sample was designated negative if no signal was produced in the three detection channels (FAM, JOE and ROX), and a stable amplification curve was obtained in the IC channel (CY5), except for the negative control. If all four channels showed negative results, the result was considered to be a

false-negative reaction. Validation of the culture-qPCR was different from the faecal qPCR in that even the negative control was required to give a satisfactory signal, as IC template DNA (plasmid) was included in the reaction. For assays that appeared to be inhibited, the samples were re-diluted (1:3 serial dilution) and/or bovine serum albumin was added to a final concentration of 0.1 µg/µl to remove the PCR inhibitors. If inhibition was still present the DNA was re-extracted.

2.9. Cloning and sequencing

DNA extracted from seven chicken faecal samples gave amplicons in the M-qPCR assay but not in the nPCRs. Due to their low concentration, the products were cloned into the pGEM®-T-Easy Vector to enrich the sequences of interest. Plasmids harbouring inserts of PCR products were purified using a Qiagen MiniPrep Kit. Cycle sequencing of the plasmids was carried out using the Dye Terminator Cycle Sequencing Reaction Mix (PE Applied Biosystems, Foster City, CA, USA), using the vector specific primers (Forward: 5'- TACGACTCACTATAGGGC-3'. Reverse: 5'-TTGGGAGCTCTCCCATATG-3) with the dideoxy method employing an ABI 3730 capillary machine (PE Applied Biosystems). The sequences obtained were compared with data from the GeneBank database under accession numbers U19610 for *B. hyodysenteriae* B204; AF060806 for *B. pilosicoli* HRM7; and AF060812 for *B. intermedia* 4482.

2.10. Statistical analysis

The correlation coefficients (R^2) of the standard curves were produced automatically by the Rotor-gene 3000 software. The coefficient of variation (CV) was calculated using SPSS software using the equation: standard deviation/mean x 100.

3. Results

3.1. In silico analysis of target oligonucleotides

Alignment of the 38 *Brachyspira nox* genes over the selected 198 bp region revealed conservation of the selected primer regions and some divergence of the probe regions for *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia*. SNPs were present at the probe binding regions for *B. pilosicoli* P/43/6/78^T (T at the 7th nucleotide) and *B. intermedia* AN519/97 (A at the 16th nucleotide).

3.2. Detection limits of the M-qPCR

The optimal annealing temperatures of the qPCR as single assays with 10-fold dilution series of *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia* cells are shown in Table 1. Thereafter, a multiplex qPCR was established on these optimal conditions with comparable results to the single formats, having detection limits of 1-10 viable cells from pure culture. In a comparison of the M-qPCR and the nPCRs, the former detected 10 fg of DNA for all three species while the nPCRs detected only 10 pg, 100 pg and 1 pg for *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia*,

respectively. The amplification curves showed no systemic deviation when DNA from *B. pilosicoli* P43/6/78^T (with a SNP) was used.

For the three *Brachyspira* species seeded into 0.2 g of pig or chicken faeces, 100-1000 copies could be identified in the assays with cycle threshold (Ct) values of 35-40 cycles.

3.3. Specificity of the M-qPCR

Even with large numbers (10^6) of *Brachyspira* cells in the samples, no cross-species reactivity was observed in the different channels. Similarly when using the 130 *Brachyspira* strains, only the target species strain was detected in the respective channel, whilst no amplification of the other *Brachyspira* species was detected.

3.4. Compatibility and reproducibility of the M-qPCR and establishment of standard curves

Using mixed DNA standards containing equal concentrations of serial dilutions of the three *Brachyspira* spp., the M-qPRC was able to identify the presence of the species correctly with an equivalent test detection level on each sample. Therefore, serial ten-fold dilution samples was mixed equally to serve as positive controls in the reaction, and to create standard curves. The R^2 values for the standard curves were more than 0.99 from the three detection channels. Amplification interference was only observed in dilution 10^0 to 10^1 cells from a species when this concentration was mixed with 10^6 cells from the other two species.

When the assay was performed three times with serial ten-fold DNA dilutions (10^6 – 10^0 cells) the intra-run CV ranged from 1.6% to 4.6%.

3.5. Comparison of M-qPCR with nPCR

When the nPCRs and qPCR were compared after primary plate culture of the pig samples, the assays had perfect agreement, with an 18.7% positive detection rate for *B. hyodysenteriae*, 3.2% for *B. pilosicoli* and 3.2% for *B. intermedia* (Table 2). The detection limits of the assays based on culture and PCR were better than with direct DNA extraction from faeces, except for the identification of *B. intermedia* in faecal-qPCR (5.6%). However, the identity of the extra positive *B. intermedia* samples in the faecal-qPCR could not be confirmed as these samples also were positive for *B. hyodysenteriae* and/or *B. pilosicoli*, making the qPCR universal primers unsuitable for sequence analysis. In comparison, when both assays were applied to DNA extracted from the porcine faeces, the qPCR identified 10 more *B. hyodysenteriae* positive samples and eight more *B. intermedia* samples than the nPCR for the pig faeces (Table 2). The additional *B. hyodysenteriae* samples that were positive in the M-qPCR also were positive by culture-PCR.

[Table 2 about here]

In the faecal-PCR from the chicken samples, three more *B. pilosicoli* and four more *B. intermedia* were identified by qPCR than by nPCR, and the identities of the additional positives were confirmed by sequencing the products.

3.6. Application of the internal control

The concentrations of the IC primers and probe were minimised to produce a clear signal without interfering with amplification in the other channels. In faecal-PCR, the Ct values obtained for porcine samples ranged from 26.45 to 36.50, with a CV of 6.8%, while in chicken samples the values ranged from 29.83 to 36.27 with a CV of 5.2%. DNA isolated from 18 of the 200 (9%) negative porcine samples and one of 40 (2.5%) negative chicken samples was considered either of poor quality or had PCR inhibitors present, as the IC signal also was not produced. Re-evaluation of these samples after dilution, addition of BSA or re-extraction identified three more positive porcine samples (2 *B. hyodysenteriae* and 1 *B. pilosicoli*) in both the nPCR and qPCR. These three new reactivities came from DNA samples that had been diluted, however the Ct values of the IC in some of the diluted samples remained identical. In culture-qPCR, all samples produced uniform signals in the IC channel at approximately 27 cycles with a CV of 3.1%.

The potential of IC normalization was assessed in five porcine samples positive for *B. hyodysenteriae,* from which DNA was extracted at three different times. The copy number of target cells in each sample calculated by the Rotor-gene software was multiplied by a correction factor that was calculated as the mean copy number for β-actin genes in all the samples divided by the copy number of the β-actin gene in the individual sample. After this normalization the mean CV decreased from 27.49% to 20.32% (Table 3).

[Table 3 about here]

3.7. Quantitation in experimentally infected pigs

The apparent number of *Brachyspira* species cells detected by M-qPCR in DNA extracted from the faeces of the 39 pigs that had been experimentally inoculated with *B. hyodysenteriae,* but were not showing clinical signs after 15 days, are shown in Table 4. Predicted numbers of *B. hyodysenteriae* cells in the individual samples ranged from 0 to 5800. One sample contained 8027 cells of *B. intermedia.* Samples from seven pigs had no *B. hyodysenteriae* detected, samples from five pigs had no *Brachyspira* spp. detected, and samples from 14 pigs had more than one *Brachyspira* spp. detected. None of the pigs went on to develop SD.

[Table 4 about here]

4. Discussion

Certain *Brachyspira* species are important pathogens of pigs and poultry, and improved methods for their rapid detection and/or identification are needed. The M-qPCR developed in this study was suitable for use with samples from both pigs and poultry, as it covered the three most common pathogenic species – *B. hyodysenteriae* and *B. pilosicoli* for pigs, and *B. pilosicoli* and *B. intermedia* for chickens. *B. intermedia* is considered to be a possible pathogen of pigs, and *B. hyodysenteriae* is an occasional pathogen of chickens, hence these two assays also had utility for both species. The Rotor-gene 3000 that was used allowed the detecting of four targets in a single reaction, ie the three pathogenic spirochaete species and a novel internal control. In the future the use of apparatus with more detection channels would allow the simultaneous detection of other species; for example, for some studies it could be informative also to include probes for detection of potentially pathogenic *B. alvinipulli* and/or "*B. suanatina*" (Råsbäck et al., 2007).

364 The target used in the study was the *nox* gene, as previously it has been
365 applied to the development of PCR-based assays for detecting and/or identifying
366 various *Brachyspira* spp. (Atyeo et al., 1999). Although SNPs were found in the
367 probe region used for one strain of *B. pilosicoli* and one of *B. intermedia*, the one
368 base pair mismatch was considered unlikely to cause systemic deviation in the
369 detection signal (Yao et al., 2006; Wacharapluesadee et al., 2008). This was
370 confirmed when the assay was used with the *B. pilosicoli* strain with the SNP,
371 although the *B. intermedia* strain with the SNP was not available for testing.
372 Confirmation of the test sensitivity and specificity of the M-qPCR was obtained by
373 testing 90 strains of the target species and 40 strains of other *Brachyspira* spp..
374
375 The detection limit of the M-qPCR for the three species was analysed in a
376 number of ways. First, using DNA extracted from serial dilution of pure cultures, it
377 was shown to be able to detect 1-10 cell copies of each species with clear signals.
378 Second, in testing with DNA, the detection limit was 10 fg for each species, while
379 the conventional PCR methods had detection limits of 1-100 pg, depending on the
380 species. The latter result was in agreement with previously reported limits of
381 conventional PCR where the products are visualized in gels (Suh and Song, 2005).
382 Third, on artificially seeded faeces, 100 to 1000 cells could be detected in 0.2 g
383 samples. Similar studies of *Brachyspira* spp. with conventional PCRs have achieved
384 detection thresholds ranging from 10^2 to 10^{10} cells (Atyeo et al., 1998; La et al., 2003;
385 Råsbäck et al., 2006). Therefore, the preliminary data demonstrated that the qPCR
386 had lower detection limits than the nPCR.
387

388 Further study was conducted using DNA extracted from porcine and chicken
389 faecal samples. As expected, the qPCR could identify more positive samples than
390 nPCR from both types of specimen. When both assays were applied to cultures from
391 porcine faeces, they had an equal detection limit that was also higher than when they
392 were used with the faecal-qPCR. Previously, the use of culture and biochemical
393 testing has been found to have detection limits as low as 140 spirochaete cells per
394 gram of faeces (Fellström et al., 2001), and an equivalent level of detection was
395 achieved in culture-PCR (Råsbäck et al., 2006). Nevertheless, it is generally
396 preferred to perform diagnostic tests directly on the template from the primary source
397 (Persson and Olsen, 2005; Råsbäck et al., 2006), as this greatly reduces the analysis
398 time for slow-growing bacteria. Furthermore, the application of M-qPCR directly to
399 DNA extracted from faeces provides an easy and reliable means of directly
400 measuring relative bacterial load. An example of this is shown in Table 4, where
401 relatively low numbers of *B. hyodysenteriae* cells were detected in the faeces of 32
402 of 39 experimentally inoculated pigs. These pigs were effectively sub-clinical
403 carriers, as they did not go on to develop SD. Furthermore, 14 of these experimental
404 pigs were unexpectedly shown to be carrying *B. intermedia,* and two were carrying *B.*
405 *pilosicoli.* Isolates of *B. intermedia* subsequently were recovered from some of these
406 pigs, confirming that these spirochaetes were present, and were probably being
407 carrying at low levels by one or more pigs when they were purchased from a
408 commercial source for the experiment.
409
410 One of the problems with the use of PCR directly with clinical samples is the
411 uncertainty of the DNA extraction efficiency, and the potential presence of PCR
412 inhibitors that may lead to false negative results. To help overcome these difficulties

internal controls (IC) can be used. ICs can be classified as exogenous and endogenous (Hoorfar et al., 2004). Exogenous controls are spiked into the samples at a defined copy number of cells before nucleic acid extraction, while endogenous controls are included in the PCR reaction at a known quantity. An exogenous control has the advantage over and endogenous control in that it monitors the whole process of effectiveness from DNA extraction to PCR test, and helps to standardize for difference in efficiency of sample extraction (Pal et al., 2008). In a new development, the current study utilized background non-specific host cell DNA as an IC template for monitoring DNA extraction and PCR efficiency in faecal PCR. The use of a conserved region of the β-actin gene meant that the IC could be used on samples from a range of host species. Using this strategy, 9.0% and 2.5% of the negative samples from pigs and chickens respectively were identified as having either poor DNA quality or having PCR inhibitors present. Additionally the coefficient of variation of the IC in the porcine samples was higher than for the chicken samples. These results demonstrated that inhibition was particularly associated with porcine samples, and indicates that there is a need to develop improved methods for DNA extraction from porcine faeces. Sample dilutions, addition of BSA or DNA re-extracted did result in the identification of three more positive samples from pigs; however, in some samples the dilutions seemed only to reduce the inhibitory effect, as the Ct values of the IC remained the same.

Another consideration of designing an IC is based on controlling for intrinsic differences arising from variation of DNA preparation, in order to make a precise quantitative analysis. During multiplexing, the high concentration of target DNA (equivalent to 10^6 cells) had no significant effect on IC amplification, which

provided the opportunity of standardizing the cell copy numbers into the IC within the same reaction. DNA extraction performed three times on five porcine samples positive for *B. hyodysenteriae* demonstrated that less variation was observed after implementation of IC normalization, as the CV decreased. Although the corrected CV was still high, the result helped to validate the general approach taken.

In conclusion, the M-qPCR that was developed should prove to be a useful adjunct to the rapid, sensitive and specific detection of pathogenic intestinal spirochaete species colonizing pigs and chickens. This new capacity to both identify and quantitated different species will be particularly useful for investigating field cases where multiple *Brachyspira* species may be present and may be interacting in the disease process, in experimental pathogenicity studies, and in investigations of the use of vaccines or antimicrobials for control of the infections.

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Table 1. Primers and probes for M-qPCR with optimized annealing temperature on a single PCR amplification

Name	Sequence	Optimized Tm
Brachyspira spp.		
Forward	5'-TTGCTACTGGTTCTTGGCCTG-3'	55°C
Reverse	5'- GAATGCTTCTATAAGTTCAACACCTAT-3'	
B. hyodysenteriae-probe	FAM-5'-CGAAGGCTTAAAACAAGAAGGA-3'-BHQ1	60°C
B. pilosicoli-probe	JOE-5'-TGAGGGCTTAAAACAAGAAGGT-3'-BHQ1	55°C
B. intermedia-probe	ROX-5'-AGAAGGATTAAAACAGGAAGGA-3'-BHQ2	57°C
Internal control		
Forward for faecal-qPCR	5'- TCCCTGTACGCCTCTGG - 3'	55°C
Forward for culture-qPCR	5'- TACGACTCACTATAGGGC-3'	
Reverse (for faecal and culture q-PCR)	5'- TGGTGGTGAAGCTGTAG - 3'	
Internal control-probe	Cy5-5'-AGGATCTTCATGAGGTAGTC-3'-BHQ2	60°C

Table 2. Results of a comparison of qPCR and nPCR applied to DNA extracts from faecal samples and/or primary plate cultures from pigs and chickens

Animal species	Health status	No. of samples	Species tested for	Culture-nPCR	Culture-qPCR	Faecal-nPCR	Faecal-qPCR
Pigs	Farms negative for swine dysentery	100	*B. hyodysenteriae*	0	0	0	0
			B. pilosicoli	0	0	0	0
			B. intermedia	0	0	0	0
Pigs	Farms with swine dysentery diagnosed	112	*B. hyodysenteriae*	12	12	7	9
			B. pilosicoli	8	8	5	5
			B. intermedia	0	0	0	0
Pigs	Experimentally infected with *B. hyodysenteriae*	39	*B. hyodysenteriae*	35	35	24	32
			B. pilosicoli	0	0	2	2
			B. intermedia	8	8	6	14

Laying chickens	Unknown	100	*B. hyodysenteriae*	NT	NT	0	0
			B. pilosicoli	NT	NT	43	46
			B. intermedia	NT	NT	30	34
% positive pigs		251	*B. hyodysenteriae*	18.7%	18.7%	12.4%	16.3%
			B. pilosicoli	3.2%	3.2%	2.0%	2.0%
			B. intermedia	3.2%	3.2%	2.4%	5.6%
% positive		100	*B. hyodysenteriae*	NT	NT	0%	0%
chickens			*B. pilosicoli*	NT	NT	43.0%	46.0%
			B. intermedia	NT	NT	30.0%	34.0%

NT, not tested.

Table 3. Comparison of deduced number of *B. hyodysenteriae* cells in five porcine samples using faecal q-PCR with and without implementation of internal control normalization

	Samples	Experiment 1	Experiment 2	Experiment 3	Mean (Ct)	SD[a]	CV[b]
Original	1	273.82	183.68	208.59	222.03	46.55	20.96%
	2	13.44	33.83	27.02	24.76	10.38	41.93%
	3	39.61	20.64	49.63	36.62	14.72	40.19%
	4	98.29	69.00	78.82	82.04	14.90	18.17%
	5	77.23	83.55	104.56	88.44	14.31	16.18%
	Mean						27.49%
Normalized	1	190.57	229.23	260.83	226.88	35.19	15.51%
	2	22.42	30.18	22.06	24.89	4.59	18.44%
	3	38.93	31.06	50.67	40.22	9.87	24.53%
	4	113.28	75.33	91.85	93.49	19.03	20.35%
	5	45.18	53.16	70.23	56.19	12.80	22.78%
	Mean						20.32%

[a]Standard deviation. [b]Coefficient of variation.

Table 4. Predicted number of *Brachyspira* spp. cells in DNA extracted from 0.2 g of faeces from pigs experimentally inoculated with *B. hyodysenteriae*, using M-qPCR. Five other pigs had no *Brachyspira* spp. detected.

	Cell numbers of the three species detected by M-qPCR		
Pig number	*B. hyodysenteriae*	*B. intermedia*	*B. pilosicoli*
1	398	0	0
2	4	0	742
3	1720	0	0
4	540	0	0
5	0	866	0
6	28	124	0
7	788	0	0
8	30	0	0
9	126	814	0
10	4686	0	0
11	2	0	0
12	74	0	0
13	734	0	232
14	46	454	0
15	1294	241	0
16	32	0	0
17	22	0	0
18	178	396	0
19	26	0	0

20	172	247	0
21	224	438	0
22	0	347	0
23	18	0	0
24	1482	0	0
25	2	232	0
26	160	0	0
27	2	0	0
28	5678	0	0
29	310	143	0
30	674	0	0
31	586	00	0
32	5800	8027	0
33	616	691	0
34	2666	2851	0