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To cite this version:
Accepted Manuscript

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

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PII: S0378-1135(11)00151-9
DOI: doi:10.1016/j.vetmic.2011.03.013
Reference: VETMIC 5236

To appear in: VETMIC

Received date: 22-10-2010
Revised date: 22-2-2011
Accepted date: 14-3-2011

Please cite this article as: Ståhl, M., Kokotovic, B., Hjulsager, C.K., Breum, S.Ø., Angen, Ø., The use of quantitative PCR for identification and quantification of *Brachyspira pilosicoli*, *Lawsonia intracellularis* and *Escherichia coli* fimbrial types F4 and F18 in pig feces., Veterinary Microbiology (2010), doi:10.1016/j.vetmic.2011.03.013

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

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**Abstract**

Four quantitative PCR (qPCR) assays were evaluated for quantitative detection of *Brachyspira pilosicoli*, *Lawsonia intracellularis*, and *E. coli* fimbrial types F4 and F18 in pig feces. Standard curves were based on feces spiked with the respective reference strains. The detection limits from the spiking experiments were $10^2$ bacteria/g feces for Bpilo-qPCR and Laws-qPCR, $10^3$ CFU/g feces for F4-qPCR and F18-qPCR. The PCR efficiency for all four qPCR assays was between 0.91 and 1.01 with $R^2$ above 0.993. Standard curves, slopes and elevation, varied between assays and between measurements from pure DNA from reference strains and feces spiked with the respective strains. The linear ranges found for spiked faecal samples differed both from the linear ranges from pure culture of the reference strains and between the qPCR tests. The linear ranges were five log units for F4-qPCR, and Laws-qPCR, six log units for F18-qPCR and three log units for Bpilo-qPCR in spiked feces. When measured on pure DNA from the reference strains used in spiking experiments, the respective log ranges were; seven units for Bpilo-qPCR, Laws-qPCR and F18-qPCR and six log units for F4-qPCR. This shows the importance of using specific standard curves, were each pathogen is analyzed in the same matrix as sample DNA. The qPCRs were compared to traditional bacteriological diagnostic methods and found to be more sensitive than cultivation for *E.*
coli and B. pilosicoli. The qPCR assay for Lawsonia was also more sensitive than the earlier used method due to improvements in DNA extraction. In addition, as samples were not analyzed for all four pathogen agents by traditional diagnostic methods, many samples were found positive for agents that were not expected on the basis of age and case history. The use of quantitative PCR tests for diagnosis of enteric diseases provides new possibilities for veterinary diagnostics. The parallel simultaneous analysis for several bacteria in multi-qPCR and the determination of the quantities of the infectious agents increases the information obtained from the samples and the chance for obtaining a relevant diagnosis.

**Introduction**

Diarrhoea in pigs can be caused by a number of infectious agents. Diarrhoea in newborn piglets due to *Escherichia coli* is mostly associated with isolates carrying F4 fimbria, whereas diarrhoea after weaning is mostly associated with F18-positive or F4-positive *E. coli* isolates (Ojeniyi et al. 1994, Frydendahl 2002, Fairbrother and Gyles 2006). *Lawsonia intracellularis* and *Brachyspira pilosicoli* are most commonly associated with diarrhoea in grower-finishers (Hampson and Duhamel, 2006; McOrist and Gebhart, 2006). Multiple agents may occur not only in the same herd but also in the same animal (Møller et al., 1998; Jensen and Boye 2005), which often makes it difficult to unequivocally relate clinical signs and macroscopic pathology to the infectious agents present (Jensen et al., 2006). Laboratory diagnosis normally includes cultivation, histopathology and PCR. Quantification of the infectious agents involved using traditional techniques is time consuming, but due to development of quantitative PCR (qPCR) assays (Guo et al., 2008; Akase et al., 2009; Nathues et al., 2009; Song and Hampson, 2009), this has become a feasible option for diagnosis. It is likely that the amount of the infectious agents present in feces, correlated to the onset of disease, is highly relevant for correct diagnosis of disease. In the present paper, qPCR assays for four of the
most relevant infectious agents associated with pig diarrhoea are presented. The assays were evaluated on DNA from pure bacterial cultures as well as on spiked fecal samples. The results from qPCRs are compared to results from traditional diagnostic methods on 113 clinical samples from Danish pig herds.

Materials and methods

Clinical samples

Clinical samples consisted of pig fecal specimens from pigs with diarrhoea obtained through routine submissions to the National Veterinary Institute (NVI) in Copenhagen, Denmark during 2007 and 2008. A total of 113 samples from pigs in 65 different herds were analysed. Prior to analysis by qPCR all samples were stored at -20°C. The diagnostics at the time of submission were performed taking into consideration the age of the pigs and the disease history of the herd. Generally, cultivation for *E. coli* was performed for all pigs younger than 8 weeks, while investigations for *L. intracellularis* and *Brachyspira* spp. were performed on pigs older than 8 weeks. However, depending on the disease history, some older pigs were also investigated for *E. coli* and some younger pigs for *L. intracellularis*. Cultivation and serotyping of *E. coli* were performed as earlier described (Frydendahl, 2002). The *E. coli* isolates were only tested against sera to the O-types that have been associated with diarrhoea in Danish pigs, i.e. types 8, 45, 64, 138, 139, 141, 149, and 157. Strains not belonging to these serotypes were designated “O-type negative” and not expected to carry the fimbrial types F4 or F18. Subcultivation and serotyping of haemolytic *E. coli* was conducted on two colonies from all samples with haemolytic *E. coli*. Non-haemolytic *E. coli* were subcultured and serotyped only when they were the dominant part within the microflora of a
Cultivation for Brachyspira spp. was performed using trypticase soy yeast (TSYF) agar plates as earlier described (Råsbäck et al., 2005) and species identification was performed by biotyping (Fellström and Gunnarsson, 1995). Investigation for L. intracellularis was performed by real-time PCR on boiled lysates of feces suspensions (Lindecrona et al., 2002).

**Reference strains**

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

**DNA extraction**

DNA was extracted from 200 µl samples of 10% feces diluted in phosphate buffered saline (PBS) by using QIAcube™ extraction robot and QIAamp DNA Stool Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer’s instructions. The protocol was DNA-QIAamp-DNA Stool-Pathogen detection Version 1. In each DNA extraction process DNA from B. pilosicoli, E. coli F4, E. coli F18, and L. intracellularis were extracted and subsequently used as positive and negative controls in the four different qPCRs. The controls were adjusted to give Cq values around 30 in the
respective qPCR. DNA from pure cultures of the four bacterial reference strains used for spiking was extracted with Easy DNA (INVITROGEN A/S, Taastrup, Denmark) according to the manufacturer’s instructions. The DNA concentrations were measured on NanoDrop® ND-1000 v.3.1. Spectrophotometer (NanoDrop Technologies, Inc., USA) according to the manufacturer’s instructions. The concentrations of pure chromosomal DNA were used for calculations of genome equivalents (GE) used in standard curves.

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

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

qPCRs and design of standard curves for quantification

A new set of primers and probe (Table 2) for amplification and detection of a fragment of 124 bp from the 23S rDNA gene of B. pilosicoli was constructed. Specificity of the primers and probe at the species level was examined by performing BLAST searches of Gene Bank database (Zheng et
al., 2000). Primers and probe were purchased from DNA Technology A/S, Denmark. The specificity was validated by using a test panel of 44 reference strains and field isolates of the genus Brachyspira and one strain each of E. coli, Enterococcus faecalis, Campylobacter jejuni, Yersinia enterocolitica and Salmonella enterica from the strain collection at NVI (Table 1). Primers and probes for the L. intracellularis, E. coli F4, E. coli F18 assays were previously described and tested (Frydendahl et al., 2001; Lindecrona, et al. 2002). However none of these assays have previously been evaluated and used for quantification. In this study the different qPCRs were designated Bpilo-qPCR, Laws-qPCR, F4-qPCR, and F18-qPCR.

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

All amplifications were run at the same cycling conditions consisting of activation at 94 ºC for 2 min followed by 40 cycles of 94 ºC for 15 s and 60 ºC for 60 s on Rotorgene 3000 or Rotorgene 6000 (Corbett Research) in JumpStart Taq Ready Mix for Quantitative PCR (Sigma-Aldrich Danmark A/S, Brøndby, Denmark). The MgCl2 concentrations were individually optimized for each assay (Table 2). Each qPCR reaction was carried out in a total volume of 25 μl containing 3 μl of sample DNA. The linear range and efficiency of each qPCR were determined in three parallel experiments with 10-folds dilutions of pure DNA extracted from reference strains as well as one
experiment with DNA extracted from spiked feces samples. All standard curves were made in triplicate and all samples were tested in duplicate. The samples that gave negative results in all four bacterial qPCR assays were tested by conventional PCR with primers for the eubacterial 16S rDNA (Angen et al. 1998).

Results

qPCR validation

The $R^2$ values for all standard curves were above 0.993 and the PCR efficiencies for all four qPCR assays ranged from 0.91 to 1.01, both when pure bacterial DNA was used as template and when DNA was extracted from spiked feces specimens. The detection limits from the spiking experiments were $10^2$ bacteria/g feces for Bpilo-qPCR and Laws-qPCR, and $10^3$ CFU/g feces for F4-qPCR and F18-qPCR (Table 3). Detection limit was defined as the lowest concentration giving a positive quantification cycle ($C_q$) value in one or more of the triplicate samples of the standard curves. The lower limits of the linear ranges were based on the mean value of triplicates. The limits of the linear ranges define the quantification limits of each qPCR assay. The linear ranges were: five log units for F4-qPCR, and Laws-qPCR, six log units for F18-qPCR and three log units for Bpilo-qPCR in spiked feces (Figure 1 and Table 3). When measured on pure DNA from the reference strains used in spiking experiments, the respective log ranges were; seven units for Bpilo-qPCR, Laws-qPCR and F18-qPCR and six log units for F4-qPCR. Negative controls from the DNA extraction as well as no template controls (NTCs) were all negative.
The biological repeatability was determined as the average coefficient of variation in percent (CV%) of log concentrations: the average CV% was 1.3 for the 14 different double samples analysed by Laws-qPCR; the average CV% was 1.6 for the 13 double samples analysed by F4-qPCR; and the average CV% was 2.2 for the 14 double samples analysed by F18-qPCR. The technical repeatability was determined by measuring DNA from one spiked fecal sample in 15 replicates by Bpilo-qPCR, the mean log concentration was 6.81 and CV 0.8%.

Validation of the species specificity of the Bpilo-qPCR

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

Traditional bacteriological diagnosis compared to qPCR.

In 89 (79%) of 113 samples one or several of the four pathogens were detected by qPCR (Tables 4-7 supplementary material). In 50 out of the 113 (44%) samples no pathogens were diagnosed at the time of submission. Twenty (18%) of the samples were true negatives (no pathogens found in neither qPCR nor at the time of submission) whereas four (3.5%) samples were false negative in qPCR (Tables 4-7 supplementary material). All samples that were negative in the four qPCRs were
found positive when analysed with a PCR test against eubacterial 16S rDNA (data not shown), showing that amplifiable DNA was present.

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

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

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

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

Bpilo-qPCR gave positive reactions in 31 (27%) of the 113 tested samples, only 19 of these had been cultivated for Brachyspira, eight of those were positive for B. pilosicoli (Table 6
supplementary material). In four samples were *B. pilosicoli* had been identified by biotyping, Bpilo-qPCR gave negative results. The highest measured concentration was 4x10^7 *B. pilosicoli*/g feces; this sample was one of those where no *B. pilosicoli* was detected by cultivation. (Table 6 supplementary material).

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

All samples that were positive by real-time PCR on boiled lysates were positive in Laws-qPCR. Out of the 37 samples that were positive by Laws-qPCR, 23 were analysed by real-time PCR on boiled lysates, 13 gave positive real-time PCR results and 10 were negative in real-time PCR (Table 7 supplementary material). In 68 cases, samples were not analysed for *L. intracellularis* at the time of submission, 14 of those were positive for *L. intracellularis* when tested by Laws-qPCR. Up to 3x10^8 bacteria/g feces of *L. intracellularis* were detected in the clinical samples. The Laws-qPCR positive samples containing less than 1x10^4 bacteria/g feces were not found positive by PCR on boiled lysates (Table 7 supplementary material).

Quantification of mixed infections

All four pathogenic agents were found in two fecal samples. Many samples contained two or three pathogens. The amount of pathogenic *E. coli* F4 and/or *E. coli* F18 was above 1x10^7 CFU/g feces in 18 samples. When more than 10^5 *E. coli* F4 and/or *E. coli* F18 were detected, no *L. intracellularis* was found (Table 8 supplementary material). On the other hand two samples with more than 10^6 *L. intracellularis* also contained *E. coli* F4 and/or *E. coli* F18. *L. intracellularis* and *B. pilosicoli* were often found together in both high and low concentrations (Tables 9 and 10 supplementary material).
*L. intracellularis* above 1x10^6 bacteria/g feces were found in 16 samples, seven of these also contained *B. pilosicoli*.

**Discussion**

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

Determination of PCR efficiency, linear range, and detection limit are important parameters in describing a qPCR test (Bustin et al., 2009). The comparison to other published assays is made difficult by the lack of information regarding PCR efficiency in many publications. A multiplex qPCR for detection of pathogenic intestinal spirochaetes (Song et al., 2009) is published, but the authors have neither presented the dynamic ranges nor the PCR efficiencies for the different PCR reactions in the assay. Nathues et al. (2009) quantified *L. intracellularis* based on a standard curve from a plasmid containing a cloned fragment from *L. intracellularis*. The standard curve showed excellent dynamic range and PCR efficiency, however the limit of quantification was 2.8 x 10^6 GE of *L. intracellularis* per gram feces corresponding to 10 GE per µl reaction volume. In this study the limit of quantification was 3x10^3 bacteria/g feces. When testing pure bacterial DNA, the qPCR tests
had linear ranges of $10^1$-$10^8$ GE/reaction in accordance with what was found by Nathues et al. (2009). To our knowledge no other qPCR tests for *E. coli* fimbra have been published.

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

The detection limits of the tests were $10^2$ bacteria per gram feces for Laws-qPCR and Bpilo-qPCR and $10^3$ CFU/gram feces for the two *E. coli* qPCR tests. The differences between the qPCRs may partly be caused by the different methods of measuring; both *L. intracellularis* and *B. pilosicoli* were measured as total cell count while *E. coli* was measured as viable cell count. Similar detection limit for *Brachyspira* qPCR test have been reported by Song and Hampson (2009). The lower limit of linear range for Laws-qPCR is only 0.9 GE/reaction, which may seem too low to be theoretically
possible (Bustin et al., 2009). However, since *L. intracellularis* carries 6 rRNA genes (J. Craig Venter Institute, [http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntli04](http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntli04) 2010-10-13) detection of 0.9 GE/reaction corresponds to 5-6 copies/reaction. *B. pilosicoli* carries three rRNA genes (Wanchanthuek et al., 2010), which also contributes to a lower limit of linear range and detection limit of Bpilo-qPCR compared to a qPCR based on a single copy gene. The discrepancy between the standard curves of pure DNA and the standard curves based on bacteria in spiked feces could be explained by free DNA from lysed cells or by the presence of more than 1 GE/cell depending on growth phase.

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

The sensitivity of the qPCR was higher when compared to cultivation of *E. coli* F4, *E. coli* F18 and *B. pilosicoli*. In 34% of the samples that were positive in F4-qPCR and/or F18-qPCR, pathogenic *E. coli* were not detected by cultivation. This may reflect the higher sensitivity of a PCR test compared to traditional cultivation or that these fimbrial genes were carried by non haemolytic strains. When more than $10^7$ CFU/gram of *E. coli* F4 and/or *E. coli* F18 were detected this was correlated with the cultivation of a high number of potentially pathogenic *E. coli* (Table 5 supplementary material).
However, massive occurrence of *E. coli* F4 and/or F18 was detected in samples that had not been cultivated for *E. coli* at the time of submission.

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

Of the 19 Bpilo-qPCR positive samples that were analysed for *B. pilosicoli* by cultivation, 42% yielded growth of *B. pilosicoli*. The highest number of *B. pilosicoli* detected among the clinical samples was $4 \times 10^7$ cells per gram feces. From this sample no *B. pilosicoli* had been detected by cultivation. Antibiotic therapy and mixed spirochetal infections might reduce the chances of identifying *B. pilosicoli* by culture and might be an explanation for this result. For *Brachyspira* spp. similar high numbers of bacteria have only been reported in connection with clinical disease. Neef et al. (1994) report that $10^7 - 10^8$ CFU per gram of *B. hyodysenteriae* are excreted in connection with clinical disease. According to Råsbäck et al. (2006) up to $10^8 - 10^{10}$ cells per gram feces are shed in the acute phase of the disease.
The Laws-qPCR test for *L. intracellularis* detected more positive samples than the earlier used real
time PCR, reflecting the removal of inhibitors by using a better DNA extraction protocol. On the
other hand, Nathues et al. (2009) reported that 96.5% of the DNA present in a sample is lost during
the DNA extraction process. The highest concentration of *L. intracellularis* measured among the
clinical samples by Laws-qPCR was $3 \times 10^7$ bacteria per gram feces. For *L. intracellularis* a
maximum shedding of $7 \times 10^8$ bacteria per gram feces has been measured by IFT (Smith and
McOrist, 1997). A correlation between the number of *L. intracellularis* present in mucosal
scrapings and the severity of lesions was demonstrated by Guedes et al. (2003).

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

**Acknowledgements**
The project was supported by the Ministry for Food, Agriculture and Fisheries (Grant 3412-08-02226). The authors would like to thank Mahshad Rezaali, Birgitte Bjørn Møller, and Pia Thuro Hansen for skilful technical assistance.

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<table>
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<tr>
<th>Species</th>
<th>Strain (n=number of isolates)</th>
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<td>NVI&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
<td>AN 35491/03</td>
<td>SVA&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. innocens</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 29796&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
</tr>
<tr>
<td></td>
<td>E646</td>
<td>NVI</td>
</tr>
<tr>
<td></td>
<td>520</td>
<td>SVA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Isolates from pig (n=3)</td>
<td>NVI</td>
</tr>
<tr>
<td><em>B. pilosicoli</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 51139&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ATCC</td>
</tr>
<tr>
<td></td>
<td>Isolates from pig (n=8)</td>
<td>NVI</td>
</tr>
<tr>
<td>“B. suanatina”&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AN 4859/03</td>
<td>SVA&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>AN 1681:1/04</td>
<td>SVA&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>AN 2384/04</td>
<td>SVA&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>AN 3949:2/02</td>
<td>SVA&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
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<td>AN 1418:2/01</td>
<td>SVA&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Isolate from pig</td>
<td>NVI</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 29212</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Isolate from pig</td>
<td>NVI</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Isolate from pig</td>
<td>NVI</td>
</tr>
<tr>
<td><em>Salmonella enterica</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CCUG 31969</td>
<td>CCUG&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Used in validation of Bpilo-qPCR
<sup>b</sup> National Veterinary Institute, Copenhagen, Denmark
<sup>c</sup> American Type Culture Collection
<sup>d</sup> Claes Fellström, National Veterinary Institute, Uppsala Sweden
<sup>e</sup> Désirée S Jansson, National Veterinary Institute, Uppsala Sweden
<sup>f</sup> Culture Collection, University of Göteborg, Sweden
Table 2
Concentrations of primers, probes and Mg$^{2+}$ in the four qPCR´s.

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

$^a$ 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.

<table>
<thead>
<tr>
<th>qPCR assay</th>
<th>PCR efficiency</th>
<th>Linear range</th>
<th>PCR efficiency</th>
<th>Linear range</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>DNA</td>
<td>Spiked feces</td>
<td>Spiked feces</td>
<td>Spiked feces</td>
</tr>
<tr>
<td></td>
<td>(SD)</td>
<td>GE²/reaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4-qPCR</td>
<td>0.96 (0.03)</td>
<td>4.0x10⁷ - 4.0x10⁸</td>
<td>0.99</td>
<td>9.4x10⁷ - 9.4x10⁴</td>
<td>10³</td>
</tr>
<tr>
<td>F18-qPCR</td>
<td>0.96 (0.01)</td>
<td>4.5x10⁷ - 4.5x10⁸</td>
<td>1.01</td>
<td>1.9x10⁹ - 1.9x10⁴</td>
<td>10³</td>
</tr>
<tr>
<td>Laws-qPCR</td>
<td>0.97 (0.04)</td>
<td>9.0x10⁷ - 9.0x10⁸</td>
<td>0.95</td>
<td>4x10⁸ - 4x10³</td>
<td>10²</td>
</tr>
<tr>
<td>Bpilo-qPCR</td>
<td>0.95 (0.01)</td>
<td>4.2x10⁸ - 4.2x10¹</td>
<td>0.91</td>
<td>1x10⁵ - 1x10⁵</td>
<td>10²</td>
</tr>
</tbody>
</table>

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
Legend

Figure 1 Standard curves of the four qPCRs, average Cq 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).