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# Pooling of porcine fecal samples for quantification of *Lawsonia intracellularis* by real-time polymerase chain reaction

Ken Steen Pedersen,<sup>1</sup> Markku Johansen, Sven Erik Jorsal, Jens Peter Nielsen, Poul Bækbo, Øystein Angen

**Abstract.** Procedures in which biological specimens are mixed and tested as 1 sample (pooling) have been applied for various biological specimens and laboratory examinations. The objective of the current study was to investigate agreement between laboratory testing of fecal pools and theoretical values obtained by averaging test results from individual fecal samples in relation to a quantitative polymerase chain reaction (qPCR) test for *Lawsonia intracellularis*. Ten diarrheic and 10 normal fecal samples were submitted from each of 43 Danish swine herds ( $n = 860$  fecal samples). Pools ( $n = 43$ ), each containing 20 individual fecal samples from the same herd, were prepared in the laboratory by pooling 10% fecal phosphate buffered saline solutions. All pools and individual fecal samples were subjected to qPCR testing for *L. intracellularis*. The theoretical number of *L. intracellularis* in the pools was calculated as the mean number of bacteria from the 20 individual fecal samples contributing to each pool. Agreement between the laboratory testing of pools and theoretical calculations based on individual sample results was evaluated. Pooling resulted in fewer *L. intracellularis*-positive herds (41.9%) compared with testing 20 fecal samples (53.5%). Agreement between the laboratory and the theoretical pools for dichotomized test results was 100% (95% confidence interval: 91.8–100%). For the quantitative test results, Lin concordance correlation coefficient was 0.997. The mean difference between the laboratory testing and the theoretical values was not different from zero (mean difference =  $0.039 \log_{10}$  bacteria/g feces;  $P = 0.26$ ).

**Key words:** Feces; *Lawsonia intracellularis*; pooling; quantitative polymerase chain reaction.

## Introduction

Pooling is the procedure in which biological specimens are mixed and tested as 1 sample. The advantages of pooling biological specimens include reduced laboratory costs. Furthermore, increasing the number of biological specimens in a pool can potentially result in higher diagnostic sensitivity at group level.<sup>7,14</sup> In contrast, pooling can also result in lowered diagnostic sensitivity of a test caused by dilution under some circumstances.<sup>7</sup> The majority of the reported pooling procedures have involved dichotomized test results demonstrating the presence or absence of a biological substance or organism. Quantitative investigations of pooled specimens have been evaluated for herd-level diagnosis of postweaning multisystemic wasting syndrome in pigs,<sup>2</sup> although pooling has primarily been used in relation to microarray experiments. Quantitative investigations using microarray technology have biological averaging as a basic assumption in relation to pooling.<sup>15</sup> This means that the quantitative test result of a pool equals the mean of the test results obtained from the individual samples that contributed to the pool. This assumption has been the subject of discussion in relation to microarray experiments.<sup>5</sup>

*Lawsonia intracellularis* causes proliferative enteropathy and is an important cause of diarrhea and production loss in

pigs.<sup>6</sup> The development of quantitative polymerase chain reaction (qPCR) tests has made it possible to determine the number of *L. intracellularis* bacteria in feces on a routine basis.<sup>8</sup> Excretion levels for *L. intracellularis* indicative of proliferative enteropathy have been reported, and qPCR could potentially be applied for the assessment of severity of infection with *L. intracellularis* in practice.<sup>9,11</sup> Pooling of fecal samples could reduce laboratory costs associated with qPCR testing for *L. intracellularis*. However, for *L. intracellularis* PCR testing of fecal samples, inhibition has been a matter of concern.<sup>4</sup> Inhibition of the PCR reaction could result in a lower than expected number of bacteria cells in a pool of fecal samples, violating the assumption of biological

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averaging. Therefore, the objective of the current study was to investigate agreement between laboratory testing of fecal pools and theoretical values obtained by averaging test results from individual fecal samples in relation to quantitative qPCR investigation for *L. intracellularis*.

## Materials and methods

### Design

Fecal samples were randomly obtained from individual Danish growing pigs and pooled in the laboratory. The number of *L. intracellularis* bacteria in the individual fecal samples and laboratory pools was determined by qPCR. Theoretical values for the pools were obtained by calculation of the mean number of *L. intracellularis* based on the individual test results of the fecal samples contributing to each pool. Agreement between the laboratory testing of pools (laboratory pools) and the theoretical values (theoretical pools) was evaluated.

### Sample size

No prior information on agreement was available for sample size calculations. The difference in the number of *L. intracellularis* bacteria between the laboratory and theoretical pools was assumed to have a mean of  $0 \log_{10}$  bacteria/g feces and standard deviation (SD) of  $1.0 \log_{10}$  bacteria/g feces based on expert judgment. Investigating 43 paired pools would provide a statistical power of 0.90 ( $\alpha = 0.05$ ) for detection of a mean difference between the laboratory and theoretical pools larger than  $0.5 \log_{10}$  bacteria/g feces. This was considered acceptable, and a minimum of 43 pools ( $20 \times 43 = 860$  individual fecal samples) would be included in the study.

### Selection of fecal samples

A total of 28 Danish swine veterinarians were selected, stratified by geography and specialized swine practices in Denmark. From each veterinarian, 2 herds representing typical Danish production herds were randomly selected for the study. The inclusion criteria were as follows: metaphylactic treatment of diarrhea in nursery pigs (10–70 days postweaning) using in-water and/or feed antibiotic batch medication, a 1,000-head (or more) nursery, and the application of batch production managed all-in/all-out (by room/farm). Metaphylactic treatment was defined as previously reported.<sup>1</sup> The exclusion criteria were as follows: herds with a known history of *Brachyspira hyodysenteriae* and nucleus or multiplier herds.

From each of the selected herds, the herd veterinarian collected 20 fecal samples randomly from nonmedicated growing pigs during outbreaks of diarrhea. The herd veterinarians collected approximately 2 g of fecal material from freshly deposited feces and were asked to include approximately 10

fecal samples representing normal feces and 10 fecal samples representing diarrheic feces. Previously used pictures<sup>12</sup> of normal and diarrheic feces were provided to the herd veterinarians along with equipment for collecting the feces. During collection, the herd veterinarians recorded the fecal score (normal or diarrheic) for each fecal sample. On the day of collection, all fecal samples were collected in plastic containers, packed in a polystyrene box containing freezer packs, and sent to the National Veterinary Laboratory (Copenhagen, Denmark), where they arrived the following day.

### Microbiological examination of fecal samples

In the laboratory, the individual fecal samples were mixed with a spoon and diluted to 10% (g/g) fecal solutions (0.1 g feces and 0.9 g phosphate buffered saline [PBS]). From each herd, 1 laboratory pool was prepared from the 20 fecal solutions (pooling of 10% [g/g] fecal solutions). The 20 fecal solutions (10% g/g) were equally pooled by weight (0.1 g from each sample) to one 2-ml sample and homogenized for 1 min using a vortex mixer. The individual fecal samples and the laboratory pools were subjected to DNA extraction and qPCR testing for *L. intracellularis* as previously described.<sup>10,13</sup>

### Statistical analysis

The theoretical number of *L. intracellularis* in each pool was calculated as the mean number of *L. intracellularis* from the 20 individual fecal samples contributing to each pool. The qPCR results were also used in the calculations for those individual fecal samples containing a number of *L. intracellularis* between 3.3 and  $4.3 \log_{10}$  bacteria/g feces, which is between the qPCR limit of detection ( $3.3 \log_{10}$  bacteria/g feces) and linear range ( $4.3$ – $8.3 \log_{10}$  bacteria/g feces).<sup>13</sup>

$$\text{Theoretical pool} = \frac{\text{Sum of } L. \textit{intracellularis} \text{ in the individual samples}}{20}$$

The measured numbers of *L. intracellularis* for each laboratory pool and the calculated numbers in the theoretical pool were  $\log_{10}$  transformed ( $\log_{10}$  bacteria/g feces). Theoretical pools calculated to contain less than  $3.3 \log_{10}$  bacteria/g feces (qPCR limit of detection)<sup>13</sup> were classified as test negatives. All laboratory and theoretical pools were classified as *L. intracellularis* positives or negatives (dichotomized qPCR test results). Agreement for dichotomized test results was calculated. The quantitative test results were statistically compared using Lin concordance correlation coefficient and performing a Bland–Altman plot as previously described for evaluation of agreement for tests with continuous outcomes.<sup>3</sup> A *t*-test was performed to test whether the mean difference between the laboratory and theoretical pools was significantly different from zero. All statistical analyses were performed using commercial software.<sup>a</sup>

## Results

A total of 26 veterinarians submitted fecal samples from 43 different herds. A total of 860 individual fecal samples were submitted, with 49.9% ( $n = 429$ ) of the samples representing normal feces and 50.1% ( $n = 431$ ) representing diarrheic feces. *Lawsonia intracellularis* was demonstrated by qPCR in 27.2% ( $n = 117$ ) of the diarrheic fecal samples and 23.1% ( $n = 99$ ) of the normal fecal samples. In the qPCR-positive fecal samples, the median number of *L. intracellularis* was 5.31  $\log_{10}$  bacteria/g feces and 4.77  $\log_{10}$  bacteria/g feces in samples from diarrheic and normal feces, respectively. At the herd level, 53.5% ( $n = 23$ ) of the herds tested *L. intracellularis* positive in at least 1 of the individual fecal samples. In the *L. intracellularis*-positive herds, the number of *L. intracellularis*-positive samples ranged between 1 and 20 (median = 7).

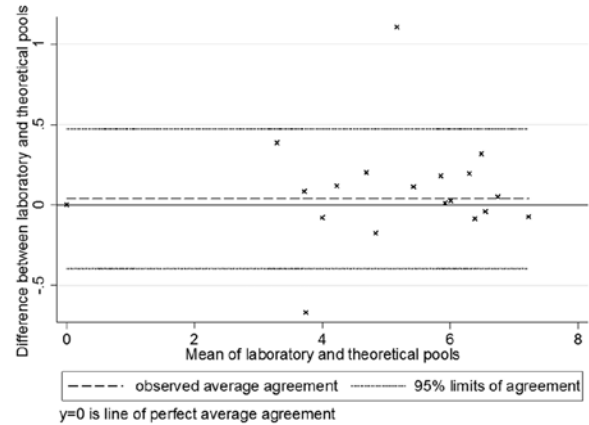
A total of 43 laboratory pools were prepared and subjected to qPCR testing, and 41.9% ( $n = 18$ ) of these were *L. intracellularis* positive. In the qPCR-positive laboratory pools, the median number of *L. intracellularis* was 5.57  $\log_{10}$  bacteria/g feces. Based on the qPCR results from the 860 individual fecal samples, the quantity of *L. intracellularis* was calculated for 43 theoretical pools. A total of 41.9% ( $n = 18$ ) of the theoretical pools were classified as *L. intracellularis* positives containing a median number of *L. intracellularis* equal to 5.83  $\log_{10}$  bacteria/g feces.

Agreement between the laboratory pool and the theoretical pool for dichotomized test results was 100% (95% confidence interval [CI]: 91.8–100%;  $n = 43$ ). For the quantitative test results, Lin concordance correlation coefficient was 0.997 (Pearson correlation coefficient = 0.997; bias correction factor = 1.0). The mean difference between the laboratory and theoretical pools was not significantly different from zero (mean difference = 0.039  $\log_{10}$  bacteria/g feces; SD = 0.22  $\log_{10}$  bacteria/g feces; 95% CI: -0.11 to 0.29  $\log_{10}$  bacteria/g feces;  $P = 0.26$ ). The observed differences are displayed in a Bland–Altman plot (Fig. 1). Two extreme differences of -1.11  $\log_{10}$  bacteria/g feces and 0.67  $\log_{10}$  bacteria/g feces, respectively, were demonstrated. The 95% CI for the individual pairwise differences between laboratory and theoretical pools was -0.40 to 0.47  $\log_{10}$  bacteria/g feces.

## Discussion

The fecal samples used in the current study were obtained from 43 herds representing different types of production systems including different types of diets. The prevalence of *L. intracellularis* in both normal and diarrheic feces and the quantitative load of *L. intracellularis* in the positive samples were similar to those previously reported in Danish growing pigs.<sup>10,11</sup>

The performance of quantitative diagnostic tests is evaluated in terms of accuracy and precision. Lin concordance correlation coefficient evaluates both aspects and is computed based on 3 different parameters. The product of 2 of the parameters constitutes the bias correction factor, which is



**Figure 1.** Bland–Altman plot showing the difference between 43 laboratory and theoretical pools in relation to the number of  $\log_{10}$  *Lawsonia intracellularis* bacteria/g feces.

a measure of accuracy. The last parameter is the normal Pearson correlation coefficient, which measures precision. In the case of perfect agreement between 2 tests, Lin concordance correlation coefficient has a value of 1.<sup>3</sup> Both the bias correction factor and the Pearson correlation coefficient had a high value for agreement between the laboratory and theoretical pools, demonstrating perfect accuracy and near perfect precision. This was also reflected in the agreement for the dichotomized test results and in the mean quantitative difference between the laboratory and theoretical pools, which was close to zero. The 95% CI for the individual differences implies that the difference for 95% of future laboratory and theoretical pools can be expected to lie between -0.40 and 0.47  $\log_{10}$  bacteria/g feces. However, the observation of 2 extreme differences demonstrates that a large difference between laboratory and theoretical pools can be expected in a small number of cases.

The assumption or concept of biological averaging is fulfilled in relation to *L. intracellularis* qPCR testing of pooled fecal samples. Therefore, pooling can be simulated by simple calculation of the mean of the test results for the individual fecal samples that contribute to the pooled sample. This can be of interest in research studies in which data from individual fecal samples is available. It is also useful for interpreting qPCR results from pooled samples in the field, because a pooled sample containing all pigs within a pen will in fact provide the mean excretion level from the pigs within the pen. This can potentially be useful in practice because a relationship between the mean excretion of *L. intracellularis* and the average daily gain has been reported.<sup>9</sup>

The demonstrated agreement was observed for pools containing 20 individual fecal samples (half diarrheic and half normal feces) pooled as 10% (g/g) fecal PBS solutions in the laboratory. The results of the study potentially only apply to samples obtained during outbreaks of diarrhea. The effect of fecal consistency could not be evaluated in the current study design. Whether inhibition is a smaller problem in diarrheic

fecal samples because of dilution by increased water content remains to be investigated. If this is the case, a lower agreement between laboratory and theoretical pools could be expected when normal fecal samples contribute exclusively to a pool (i.e., during subclinical infection with *L. intracellularis*). Pooling was performed using 10% (g/g) fecal PBS solutions in the laboratory. In practice, pooling may be performed by pooling feces from individual fecal samples in the laboratory or simply by pooling fecal material on the pen floor during collection in the field.

At the herd level, pooling resulted in fewer herds being classified as *L. intracellularis* positive compared to the test results from the 20 individual fecal samples (41.9% vs. 53.5%). This illustrates that pooling can result in reduced diagnostic sensitivity compared with testing of individual samples in relation to detection of infection at herd level. This is probably caused by dilution, where single fecal samples containing a low level of *L. intracellularis* will result in the pool being below the limit of detection for the qPCR test. Hence, if the diagnostic purpose is a simple detection of infection at herd level, testing of 20 individual fecal samples would be preferable to testing pooled samples. In conclusion, perfect agreement between laboratory testing of fecal pools and theoretical values obtained by averaging test results from individual fecal samples was demonstrated in relation to qPCR testing for *L. intracellularis*.

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### Sources and manufacturers

a. Stata/IC, version 12, StataCorp LP, College Station, TX.

### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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