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# Comparison of bacterial culture and qPCR testing of rectal and pen floor samples as diagnostic approaches to detect enterotoxic *Escherichia coli* in nursery pigs

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ABSTRACT

Enterotoxigenic *E. coli* (ETEC) are a major cause of diarrhoea in weaned pigs. The objective of this study was to evaluate the agreement at pen level among three different diagnostic approaches for the detection of ETEC in groups of nursery pigs with diarrhoea. The diagnostic approaches used were: bacterial culturing of faecal samples from three pigs (per pen) with clinical diarrhoea and subsequent testing for virulence genes in *E. coli* isolates; bacterial culturing of pen floor samples and subsequent testing for virulence genes in *E. coli* isolates; qPCR testing of pen floor samples in order to determine the quantity of F18 and F4 genes. The study was carried out in three Danish pig herds and included 31 pens with a pen-level diarrhoea prevalence of > 25%, as well as samples from 93 diarrhoeic nursery pigs from these pens. All *E. coli* isolates were analysed by PCR and classified as ETEC when genes for one or more adhesin factors and one or more enterotoxins were detected. Results: A total of 208 *E. coli* colonies from pig samples and 172 *E. coli* colonies from pen floor samples were isolated. Haemolytic activity was detected on blood agar plates in 111 (29.2%) of the 380 colonies that were isolated. The only adhesin factor detected in this study was F18. When comparing bacterial culture or qPCR testing of pen floor samples with detection of ETEC-positive diarrhoeic pigs by culture, agreement was found in 26 (83.9%, Kappa = 0.665) and 23 (74.2%, Kappa = 0.488) of the pens, respectively. Agreement was observed between the detection of ETEC by bacterial culture and qPCR in the same pen floor sample in 26 (83.9%, Kappa = 0.679) pens. Conclusion: We observed an acceptable agreement for the detection of ETEC-positive diarrhoeic nursery pigs in pen samples for both bacterial culture of pen floor samples and qPCR. This study showed that both bacterial culture and qPCR testing of pen floor samples can be used as a diagnostic approach for detecting groups of ETEC-positive diarrhoeic nursery pigs.

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## 1. Introduction

Enteric diseases involving diarrhoea in nursery pigs are a major cause of antibiotic treatments in commercial pig production worldwide and have been subject to considerable research during recent years (Nagy and Fekete, 1999; Heo et al., 2013). Enteric diseases are endemic in many farms and can lead to major economic losses (Vondruskova et al., 2010; Fairbrother and Gyles, 2012). Bacterial pathogens have been identified as causative factors of enteric diseases (Frydendahl, 2002; Jacobson et al., 2003; Aarestrup et al., 2008; Halaihel et al., 2010; Fairbrother and Gyles, 2012). However, it has also been shown that nutritional and management

*Abbreviations:* ETEC, enterotoxigenic *Escherichia coli*; PWD, post-weaning diarrhoea; qPCR, quantitative polymerase chain reaction; F4, *Escherichia coli* F4; F18, *Escherichia coli* F18; LI, *Lawsonia intracellularis*; PILO, *Brachyspira pilosicoli*.

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factors also influence intestinal health in pigs (Callesen et al., 2007; Chase-Topping et al., 2007). Enterotoxigenic *Escherichia coli* (ETEC) that produce F4 or F18 adhesins are important pathogens often associated with post-weaning diarrhoea (PWD), which is characterised by diarrhoea or sudden death from 1 to 2 weeks post-weaning (Frydendahl, 2002). However, infection with ETEC can also be observed in older nursery pigs (Fairbrother and Gyles, 2012; Pedersen et al., 2014; Sato et al., 2016). There are also other bacterial causes of diarrhoea in pigs during the nursery period, such as *Lawsonia intracellularis* (LI) and *Brachyspira* spp., including *B. pilosicoli* (PILO) (Pedersen et al., 2014). For this reason, it is important that diagnostic procedures can identify the causal pathogens in such outbreaks.

ETEC is transmitted between pigs by the faecal-oral route, and it colonises the small intestine after attaching to receptors on the small intestinal epithelium using specific fimbrial adhesins. Production of enterotoxins causes a disturbance to the fluid balance in the small intestine, resulting in diarrhoea (Fairbrother et al., 2005). Proliferation of ETEC mainly takes place in the small intestine, and bacterial numbers remain constant from the ileum to the rectum, and rectal faecal samples are therefore useful for diagnosing ETEC-related diarrhoea (McAllister et al., 1979; Pedersen et al., 2010).

For many years the routine method for identifying ETEC-associated diarrhoea in weaned pigs, have been based on individual sampling of diarrheic pigs. Bacterial culture followed by either serotyping or detection of toxin (ST/LT) and fimbrial genes (F4/F18) by PCR of faecal samples collected from intestinal content or faeces is the most simple and effective way of providing a bacteriological diagnosis of the individual pig (Nagy and Fekete, 1999). Haemolytic activity of *E. coli* is a virulence marker (Frydendahl, 2002), and high concentrations of *E. coli* in a pure (or nearly pure) culture in the ileum is generally recognised as being indicative of colibacillosis (Francis, 1999). Recently, qPCR analysis of enteric pathogens in faecal samples collected by sock sampling from the floor of several pens has been described as a diagnostic tool for the simultaneous quantitative herd-level detection of different intestinal pathogens (Pedersen et al., 2015).

The aim of this study was to investigate whether bacterial culture and qPCR testing of faecal pen floor samples could be used as a diagnostic approach for identifying ETEC in groups of weaned pigs with clinical diarrhoea. The overall hypothesis was that the same *E. coli* pathotype could be detected in faecal samples from diarrhoeic nursery pigs and in samples taken from the floor of the pen where the pigs are housed.

The study was designed to evaluate the agreement at pen-level among three different diagnostic approaches for detecting ETEC in groups of diarrhoeic pigs:

D1. Bacterial culture and PCR testing for virulence genes; F4, F5, F6, F18, F41, STa, STb, LT and VT2e in selected *E. coli* colonies from three faecal samples per pen, obtained from individual pigs with clinical diarrhoea.

D2. Bacterial culture and PCR testing of virulence genes; F4, F5, F6, F18, F41, STa, STb, LT and VT2e in selected *E. coli* colonies from pen floor samples.

D3. qPCR testing for F4 and F18 genes in pen floor samples.

Furthermore, the value of using haemolytic activity as a virulence marker was evaluated.

## 2. Methods

### 2.1. Design

A cross-sectional study was performed in three commercial production herds in 2014. A total of 93 pigs in 31 different pens were sampled between 14 and 28 days post-weaning.

### 2.2. Inclusion of herds

The herds included in this study were previously selected for a clinical trial investigating batch medication for intestinal diseases in nursery pigs (Weber et al., 2017). The herds were characterised as high health herds (declared free of *Actinobacillus pleuropneumoniae* type 2, 6 and 12, porcine reproductive and respiratory syndrome virus, mange mites and lice (SPF-sus, 2015)), but with outbreaks of diarrhoea in nursery pigs requiring antibiotic treatment. The definition of an outbreak of diarrhoea requiring treatment has previously been characterised as ≥1.5 diarrhoeic pools per pen and a pen floor sample containing ≥35.000 bacteria (calculated as *E. coli* F4 + *E. coli* F18 + LI + PILO)/g faeces (Pedersen et al., 2014). All herds had all-in all-out batch production in sectioned compartments with 2300 to 3600 pen places. The flooring consisted of one-third solid floor and two-thirds slatted floor. Feed was home-mixed and formulated with wheat, barley and soybean meal as the main ingredients, and fulfilled the Danish nutrient standards (Tybirk et al., 2015). The nursery pigs were crossbred Danavl Yorkshire/Landrace and Duroc. All herds used 3000 ppm zinc oxide in the feed during the first 14 days post-weaning, which is common practice in Denmark.

### 2.3. Inclusion of pigs and pens

Eight pens were randomly selected within a batch, and 15 pigs from each of these pens were selected by systematic random sampling. If there were fewer than 15 pigs in the pen, all pigs were selected. Pigs in each herd were clinically examined on days 14, 21, and 28 post weaning. The diarrhoea status of the pigs was assessed by scoring faecal samples obtained by digital rectal manipulation. The faecal samples all were scored by one observer using a faecal consistency scale with four categories, where scores of 1 and 2 represented normal faeces and scores of 3 and 4 represented diarrhoea (Pedersen and Toft, 2011).

### 2.4. Sampling procedure

In pens with a diarrhoea prevalence of >25% among the sampled pigs, rectal samples from three diarrhoeic pigs and a pen floor sample were collected and stored in new, sealed plastic containers. The faecal pen floor sample was collected by swiping a gloved hand over the slatted floor. This sample was thoroughly stirred and the contents divided into two subsamples. The faecal samples from individual pigs and one of the pen floor samples were transported in a polystyrene box with freezing elements to the Laboratory for Pig Diseases in Kjellerup, Denmark for bacteriological examination by culture. The second pen floor sample was transported to the Danish National Veterinary Institute in Frederiksberg, Denmark for qPCR analyses.

### 2.5. Laboratory analyses

#### 2.5.1. Bacteriology

An overview of sampling procedures is shown in Fig. 1. The pig and pen floor samples were aerobically cultured for *E. coli* by parallel culturing on Drigalski (an in-house selective and indicative medium for coliforms) and blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood). The plates were incubated for 24 h at 37 °C. To identify the expected higher diversity of *E. coli* isolates in pen floor samples, a larger number of colonies were sampled from pen floor samples than pig samples. When possible, two haemolytic colonies surrounded by a clear zone of lysis and two non-haemolytic colonies were isolated from the pig samples, and where present, five haemolytic colonies and five non-haemolytic colonies were subcultured from the pen floor samples. In case of pure culture, only haemolytic or non-haemolytic were isolated.

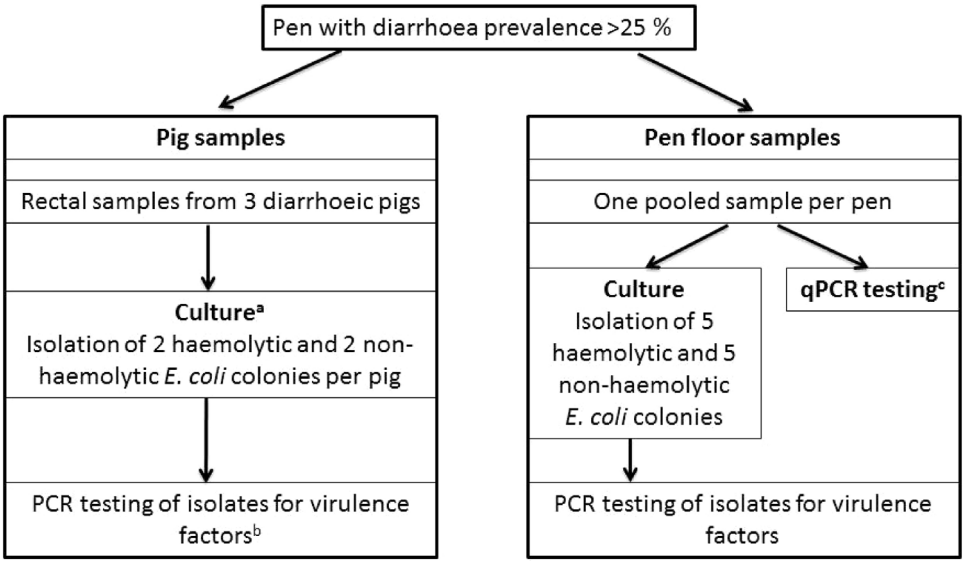


**Fig. 1.** Overview of sample procedure at pen level.
[a]Colonies surrounded by a zone of lysis after overnight growth at 37 °C on blood agar were classified as haemolytic, [b]detection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e by PCR, [c]quantitate PCR for *E. coli* fimbrial gens F4, and F18, *Lawsonia intracellularis* and *Brachyspira pilosicoli*.

The isolates were analysed at the Danish National Veterinary Institute by real-time PCR for detection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e, as previously described by Frydendahl et al. (2001), with the exception that PCR cycling was performed on a Rotor-Gene Q (QIAGEN) PCR machine.

#### 2.5.2. qPCR

Pen floor samples were thoroughly agitated and suspended in phosphate buffered saline (PBS) to obtain a 10% (w/v) faecal suspension. An aliquot of the suspension was transferred to a 2 ml microfuge tube and stored in a freezer at −20 °C until DNA extraction, as previously described by Pedersen et al. (2012). The DNA was subsequently stored in a freezer at −20 °C until the content of F4, F18, PILO and LI was quantified by qPCR, as previously described by Stahl et al. (2011), with the exception that standard curves for quantification were prepared from DNA extraction of spiked 10-fold dilution series using the same qPCR procedure as for the faecal specimens. The detection limits in bacteria or CFU per gram faeces were: $5.7 \times 10^4$ for F4, $1.5 \times 10^3$ for F18, and $2 \times 10^3$ for LI and PILO.

### 2.6. Statistics

All *E. coli* isolates were classified as ETEC when genes for one or more adhesin factors and one or more enterotoxins were detected. It was assumed that a pen contained ETEC-positive diarrhoeic pigs when the following criteria for the three diagnostic approaches were met:

D1. Minimum one pig in a pen should be ETEC-positive. An individual pig was classified as ETEC-positive when one or more ETEC isolates were demonstrated.

D2. A pen floor sample was classified as ETEC-positive when one or more ETEC isolates were demonstrated.

D3. A pen floor sample was classified as qPCR-positive when F4 and/or F18 were present in the sample.

Agreement among the three diagnostic approaches was evaluated by the calculation of observed agreement, and the statistical association was evaluated using a Fisherís exact test and Cohen's kappa coefficient.

## 3. Results

### 3.1. Data description

Pig and pen floor samples were collected from a total of 31 pens, including 5 pens in Herd 1, 17 pens in Herd 2 and 9 pens in Herd 3. The number of pigs per pen ranged from 12 to 40 with a mean of 29. Samples were taken from 10 pens at Day 14, 19 pens at Day 21 and 2 pens at Day 28 post-weaning. At the time of sampling, the mean pen-level diarrhoea prevalence of the sampled pigs was 31.6% (CL95% 27.9-35.3).

### 3.2. Bacteriology

*E. coli* colonies were isolated from 87 (93.5%) of the 93 sampled pigs, and in 27 (87.1%) of the 31 pen floor samples. An overgrowth of *proteus* was observed in four *E. coli*-negative samples which made it impossible to identify and isolate *E. coli* colonies. On six occasions, the sub-cultured isolates, selected from the dominating flora from the primary culture, was not identified as *E. coli*. Due to overgrowth of the dominating flora it was not possible to visually distinguish *E. coli* colonies from the dominating flora in these samples. A total of 208 *E. coli* isolates from pig faecal samples and 172 isolates from pen floor samples were used for further analyses. Haemolytic activity was detected on blood agar plates in 111 (29.2%) of the 380 colonies that were isolated. Pure (or nearly pure) cultures of haemolytic *E. coli* were observed in 13 (14.0%) of the 93 pig samples and in 4 (12.9%) of the 31 pen floor samples.

### 3.3. Virulence genes

The presence of virulence genes in the 380 *E. coli* isolates is shown in Table 1. The fimbrial gene F18 was detected in 95 (25.0%) isolates and was the only adhesin factor identified. Toxin genes were detected in 89 (93.7%) of the F18-positive isolates. The prevalence of toxin genes detected in F18-positive isolates were: STb (55.8%), STa (30.0%), LT (13.7%) and VT2e (2.4%). The most common virulence factor profile among the F18-positive isolates was: F18 + STb + LT, found in 45 (11.8%), F18 + STb found in 24 (6.3%), and F18 + STa + STb found in 19 (5.0%) of the isolates. Toxin genes were detected in 149 (52.3%) isolates that were negative for fim-

**Table 1**
Adhesin and toxin genes detected in 380 *E. coli* isolates from rectal samples of diarrhoeic pigs and pen floor samples 14–28 days post-weaning.

| Sample type | Adhesin factor | No. of Isolates | Toxin profile | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | LT | STa | STb | VT2e | STa,STb | STb,LT | STa,STb,VT2e | None |
| Pig | | | | | | | | | | |
| | F18+ | 46 (22.1%) | 0 | 0 | 10 | 0 | 9 | 22 | 0 | 5 |
| | None | 162 (77.8%) | 1 | 6 | 23 | 4 | 39 | 5 | 1 | 83 |
| | Total | 208 (100.0%) | 1 | 6 | 33 | 4 | 48 | 27 | 1 | 88 |
| Pen floor | | | | | | | | | | |
| | F18+ | 49 (28.5%) | 1 | 0 | 14 | 0 | 10 | 23 | 0 | 1 |
| | None | 123 (71.5%) | 0 | 11 | 18 | 3 | 37 | 0 | 1 | 53 |
| | Total | 172 (100.0%) | 1 | 11 | 32 | 3 | 47 | 23 | 1 | 54 |
| Pig + pen floor | | | | | | | | | | |
| | F18+ | 95 (25.0%) | 1 | 0 | 24 | 0 | 19 | 45 | 0 | 6 |
| | None | 285 (75.0%) | 1 | 17 | 41 | 7 | 76 | 5 | 2 | 136 |
| | Total | 380 (100.0%) | 2 | 17 | 65 | 7 | 95 | 50 | 2 | 142 |

brial genes. A total of 136 (35.8%) isolates were negative for all of the analysed virulence factors. ETEC (fimbriae- and toxin-positive) isolates were detected in 22 (23.7%) of the 93 samples from pigs housed in 12 pens, and ETEC isolates were detected in 13 (41.9%) of the 31 pen floor samples.

### 3.4. qPCR analysis of pen floor samples

Results from the qPCR tests of 31 pen floor samples are shown in Table 2. LI genes were detected in 20 (64.5%) pen floor samples, F18 genes in 16 (51.6%) pen floor samples and PILO in 4 (12.9%) pen floor samples. F4 genes were only detected in 1 (3.2%) pen floor sample. The most common pathogen profiles found from qPCR testing of pen floor samples were: LI, which was found in 10 (32.3%) pens; F18 + LI found in 8 (25.8%) pens; F18 found in 5 (16.1%) pens.

### 3.5. Relationship between virulence factors and haemolytic activity

The relationship between pathogenicity, as represented by the presence of virulence factors (adhesin and toxin genes), and haemolytic activity is presented in Table 3. Haemolytic activity was strongly associated with the presence of virulence factors defining ETEC ($p < 0.001$). A sensitivity (SE) of 97.8% (CL95% = 92.1%–99.7%) and a specificity (SP) of 91.8% (CL95% = 88.0%–94.6%) were obtained when using haemolytic activity as a marker for the presence of ETEC virulence genes. Only two of the 269 isolates with non-haemolytic activity possessed ETEC virulence genes, corresponding to a high negative predictive value (NPV) of 99.3% (CL95% = 97.3%–99.9%). However, 24 of 111 isolates with haemolytic activity did not possess either adhesin or toxin genes, resulting in a positive predictive value (PPV) of 78.4% (CL95% = 69.6%–85.6%).

### 3.6. Relationship between detection of ETEC in pen floor and pig samples

A total of five comparisons were made among the different diagnostic approaches and presented in Table 4. The association between the detection of ETEC from pen floor samples by culture (D2) or by qPCR with a lower detection limit of $1.5 \times 10^3$ CFU per gram faeces (D3) and the detection of ETEC-positive diarrhoeic pigs by culture (D1) was statistically significant for D1 vs. D2 ($p < 0.001$), and for D1 vs. D3 ($p = 0.009$). The agreement between pen floor sample testing by bacterial culture or qPCR and the detection of ETEC-positive diarrhoeic pigs by culture was observed in 26 (D1 vs. D2: 83.9%, Kappa = 0.665) and 23 (D2 vs. D3: 74.2%, Kappa = 0.488) pens, respectively.

Disagreement was observed in pens with a low concentration of F18 genes in pen floor samples analysed by qPCR. Therefore an alternative cut-off (labelled AD2) for a positive qPCR sample was introduced by increasing the detection limit from $1.5 \times 10^3$ to $2 \times 10^5$ CFU per gram faeces:

AD3. A pen floor sample was classified as qPCR-positive when the concentration of F18 genes was above $2 \times 10^5$ CFU per gram faeces in the sample.

When applying this new alternative cut-off for qPCR-positive samples, agreement between qPCR testing of pen floor samples and the detection of ETEC-positive diarrhoeic pigs in the same pen increased to 27 (D1 vs. AD3: 87%, Kappa = 0.728) pens.

The relationship between the detection of ETEC by bacterial culture (D2) and qPCR in the same pen floor sample (D3) is shown in Table 4. The association between ETEC detection by bacterial culture and qPCR was statistically significant (D2 vs. D3: $p < 0.001$). Agreement between bacterial culture and qPCR of pen floor samples was observed in 26 (D2 vs. D3: 83.9%, Kappa = 0.679) pens. Applying the alternative cut-off value for the qPCR-positive samples (AD3) resulted in agreement in 28 (D2 vs. AD3: 90.3%, Kappa = 0.799) pens.

## 4. Discussion

In this study, faecal samples were collected from diarrhoeic pigs and pen floors 14, 21 and 28 days post-weaning in three Danish commercial pig farms. F18 and LI were the most frequently detected pathogens in the 31 pen floor samples tested by qPCR and the mean diarrhoea prevalence at pen level of the sampled pigs was 32.6%. Similar findings concerning pathogen profiles and diarrhoea prevalence have previously been reported from outbreaks of diarrhoea in flocks of Danish nursery pigs (Pedersen et al., 2015). Pure (or nearly pure) cultures of haemolytic *E. coli* were observed in 14% of the diarrhoeic pigs, indicating that, in a large proportion of the pigs, *E. coli* was not the primary pathogen. Furthermore, qPCR results from the pen floor samples showed that *L. intracellularis* were frequently present and could therefore be the causative agent of some of the clinical diarrhoea observed in this study.

Haemolytic activity was found to be a useful marker of ETEC, as demonstrated by virulence genes with a sensitivity of 97.8% and a specificity of 91.8%, which is similar to previous published results from PWD pigs (Frydendahl, 2002; Do et al., 2005; Chapman et al., 2006; Zhang et al., 2007). The dominant fimbrial gene that was found was F18, which was detected in 25% of the isolates. In previous studies of isolates from PWD cases, F4 and F18 were the most frequently detected fimbrial genes, with a reported prevalence ranging from 22.9% to 64.6% for F4 and 12.9% to 46.9% for F18 (Frydendahl, 2002; Chapman et al., 2006; Zhang et al., 2007; Zajacova et al., 2012; Sato et al., 2016). The lack of F4 fimbrial genes

**Table 2**
Quantitate PCR results for *E. coli* fimbrial genes F4 and F18, *Lawsonia Intracellularis* and *Brachyspira Pilosicoli* from 31 pen floor samples.

| Pen | Bacteria/g faeces | | | | |
|---|---|---|---|---|---|
| | F4 | F18 | *Lawsonia Intracellularis* | *Brachyspira Pilosicoli* | Sum |
| 1 | 0 | 564,000 | 1,370,000 | 0 | 1,426,400 |
| 2 | 0 | 239,000 | 3,030,000 | 0 | 3,269,000 |
| 3 | 0 | 924,000 | 1250 | 0 | 925,250 |
| 4 | 0 | 5,430,000 | 0 | 0 | 5,430,000 |
| 5 | 0 | 666,000 | 0 | 0 | 666,000 |
| 6 | 0 | 498,000 | 0 | 0 | 498,000 |
| 7 | 0 | 0 | 59,700 | 0 | 59,700 |
| 8 | 0 | 0 | 338,000 | 0 | 338,000 |
| 9 | 0 | 0 | 7730 | 0 | 7730 |
| 10 | 0 | 138,000 | 1640 | 0 | 139,640 |
| 11 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | 27,200 | 10,300 | 0 | 37,500 |
| 13 | 0 | 0 | 8230 | 0 | 8230 |
| 14 | 0 | 21,300 | 0 | 6070 | 27,370 |
| 15 | 0 | 0 | 33,300,000 | 0 | 33,300,000 |
| 16 | 0 | 0 | 1,110,000 | 0 | 1,110,000 |
| 17 | 0 | 536,000 | 0 | 0 | 536,000 |
| 18 | 0 | 427,000 | 0 | 0 | 427,000 |
| 19 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 2550 | 0 | 2550 |
| 21 | 0 | 56,400 | 1,300,000 | 0 | 1,356,400 |
| 22 | 0 | 922,000 | 2400 | 0 | 924,400 |
| 23 | 0 | 0 | 0 | 8320 | 8320 |
| 24 | 0 | 0 | 0 | 0 | 0 |
| 25 | 0 | 0 | 9,740,000 | 0 | 9,740,000 |
| 26 | 0 | 0 | 3,410,000 | 0 | 3,410,000 |
| 27 | 0 | 0 | 43,000 | 10,800 | 53,800 |
| 28 | 0 | 0 | 18,400 | 0 | 18,400 |
| 29 | 0 | 1,300,000 | 1260 | 968 | 1,302,228 |
| 30 | 0 | 669,000 | 992 | 0 | 669,992 |
| 31 | 260,000 | 14,100,000 | 1,370,000 | 0 | 14,360,000 |
| Total positive pens | 1 | 16 | 20 | 4 | |

**Table 3**
Relationship between presence of virulence factor genes and haemolysis in *E. coli* isolates.

| | | Virulence factors [a] | | | Parameter | Estimate (95% CI) |
|---|---|---|---|---|---|---|
| | | + | – | Total | Sensitivity | 97.8% (92.1%–99.7%) |
| Haemolytic activity[b] | + | 87 | 24 | 111 | Specificity | 91.8% (88.0%–94.6%) |
| | – | 2 | 267 | 269 | Positive predictive value | 78.4% (69.6%–85.6%) |
| | Total | 89 | 291 | 380 | Negative predictive value | 99.3% (97.3%–99.9%) |

[a] Isolates harbouring both adhesin and toxin genes were assigned '+', all other were assigned '–'.
[b] Colonies surrounded by a zone of lysis after overnight growth at 37 °C on blood agar were assigned '+'.

in the current study could be coincidental because samples were acquired from only three herds, or because the sampled diarrhoeic pigs in the current study were older than pigs in the previous studies. In the study farms, high levels of zinc oxide was added in the feed during the first two weeks post weaning which could have resulted in delayed outbreaks of diarrhoea and could thereby favour infections with F18 rather than F4 ETEC. Another possible explanation is that all pigs from the study farms were obtained from the Danavl breeding company. In 2002, resistance genes against F4 receptors were identified in pigs (Jorgensen et al., 2003) and Danavl have implemented selective breeding for F4 resistance since 2003.

The association between clinical disease and fimbriae detection is not evident. In a study from Australia, F18 fimbriae genes were only detected in diarrhoeic pigs and not in healthy pigs (Chapman et al., 2006). In contrast, it has also been demonstrated that F18-positive ETEC can be detected in healthy pigs with no signs of diarrhoea (Schierack et al., 2006; Weber et al., 2015).

Of the F18-positive isolates, 93.7% were toxin-positive, indicating that detection of fimbrial genes is a good predictor of virulence. In contrast, detection of toxins as a sole predictor of pathogenicity proved to be problematic. Approximately half of all analysed isolates possessed toxin genes but were negative for fimbrial genes and therefore not considered ETEC. In this study, only five of the most common adhesin factors (F4, F5, F6, F18 and F41) were included in the analysis. Other adhesin factors, known to contribute to the virulence of ETEC in nursery pigs (such as intimin and AIDA) were not included in the analysis and could potentially have influenced the conclusions drawn from the study (Frydendahl, 2002; Fairbrother and Gyles, 2012). Furthermore other still unknown adhesin factors could also be involved in the pathogenesis of the diseased pigs.

The sampling procedure in this study was based on isolation of *E. coli* colonies with or without haemolytic activity after culture of faecal samples. Depending on presence after culture, we examined a maximum of two isolates with haemolytic activity and two non-haemolytic isolates from three diarrhoeic pigs per pen, in order to establish the ETEC status of all the pigs within the same pen. Furthermore, we examined a maximum of five haemolytic,

**Table 4**
Comparison of diagnostic approaches for detecting ETEC in pen floor and pig faecal samples by bacterial culture and PCR or quantitative PCR.

| Comparison of diagnostic approaches | p-value[e] | Observed agreement (Pens with agreement/total pens) | Kappa[f] (Standard Error) |
|---|---|---|---|
| D1[a] vs. D2[b] | <0.001 | 0.839 (26/31) | 0.665 (0.179) |
| D1 vs. D3[c] | 0.009 | 0.742 (23/31) | 0.488 (0.174) |
| D1 vs. AD3 [d] | <0.001 | 0.871 (27/31) | 0.728 (0.180) |
| D2 vs. D3 | <0.001 | 0.839 (26/31) | 0.679 (0.176) |
| D2 vs. AD3 | <0.001 | 0.903 (28/31) | 0.799 (0.179) |

[a] D1: pig samples analysed by culture and real-time PCR were classified as ETEC-positive when one or more of the four potential isolates from each pig in a pen were harbouring both adhesin and toxin genes.
[b] D2: Pen floor samples analysed by culture and real-time PCR were classified as ETEC-positive when one or more of the ten potential isolates from each pen were harbouring both adhesin and toxin genes.
[c] D3: pen floor samples analysed by qPCR were considered positive for fibrial F18 genes when the amount of bacteria per gram faeces was above the detection limits ($1.5 \times 10^3$ CFU per gram faeces).
[d] AD3: pen floor samples analysed by qPCR were considered positive for fibrial F18 genes when the detection of bacteria per gram faeces was above $2 \times 10^5$ CFU per gram faeces.
[e] Fisher's exact test.
[f] Cohen's kappa coefficient significance value <0.05.

and five non-haemolytic isolates per pen floor sample to establish the ETEC status of the sample. If the sample size (i.e. pigs per pen and examined colonies per sample) had been increased, it is likely that the sensitivity of these diagnostic approaches to detect ETEC-positive pigs would also increase. The selected sample size was chosen partly for convenience and economic factors related to laboratory analysis, as well as to simulate the commonly used diagnostic approach of Danish veterinary practitioners.

This study demonstrates that it is possible to detect the same ETEC pathotypes from individual pig samples and pen floor samples. The results show that pen floor samples might be a useful diagnostic approach for detecting ETEC-positive pigs that can be applied in veterinary practice. Using direct qPCR testing of faecal samples for diagnosing ETEC have the advantages of easy handling of samples and a short response time for test results, whereas bacterial culture is a cheap diagnostic method which can be performed without advanced laboratory equipment. There was a good agreement between the detection of virulent *E. coli* isolates from both diarrhoeic pigs and from pen floor samples when using bacterial culture and subsequent demonstration of virulence genes in selected *E. coli* colonies. It is likely that the lower agreement observed when using qPCR detection of fimbrial genes in pen floor samples is due to the qPCR analysis being more sensitive than bacterial culture from three diarrhoeic pigs. This is further supported by the higher agreement observed when the lower detection limit of the qPCR analysis was raised to $2 \times 10^5$ CFU per gram faeces.

The study also revealed a good agreement when comparing qPCR detection of fimbrial genes to demonstration of virulence genes following bacterial culture of selected *E. coli* colonies in pen floor samples. This finding indicates that the qPCR analysis used in this study is appropriate for detecting F18 fimbrial genes from pen floor samples where virulent F18-positive *E. coli* bacteria are present.

The prudent use of antimicrobials in production animals is a focus point throughout the world (European-Commission, 2015; OIE, 2016). In veterinary practice, this involves making the right decision regarding the initiation of antimicrobial treatment with the aim of achieving the most efficient treatment of diseased animals while maintaining the lowest possible antibiotic consumption.

This study suggests a new diagnostic approach for detecting ETEC-positive nursery pigs by examining the pen floor rather than faecal samples from diarrhoeic pigs. Recently, sock samples have been described as an effective way of obtaining samples from pen floors (Pedersen et al., 2015). Applying qPCR testing to faecal samples has the advantage of including F4/F18 ETEC, LI and PILO simultaneously. These new diagnostic approaches can improve the diagnosis of enteric diseases in nursery pigs and thereby help to achieve the goal of a more prudent use of antibiotics in pig production. Direct testing of pen floor samples for multiple enteric pathogens can give a fast and precise diagnosis, supporting the decision for initiation of treatment and antimicrobial compound selection. The diagnostic approaches suggested in this study should be further evaluated under field conditions to confirm the results. Ideally this study should be repeated in herds dealing with PWD 1–2 weeks post weaning where F4 and F18 ETEC would be considered as primary pathogens. Future research on the value of resistance profiling of ETEC isolated from pen floor samples is recommended in order to assist in the selection of narrow-spectrum antibiotics for treatment of diarrhoea caused by ETEC.

## 5. Conclusions

This study showed that both bacterial culture and qPCR testing of pen floor samples can be used as a diagnostic approach for detecting groups of ETEC-positive diarrhoeic nursery pigs. An acceptable agreement for the detection of ETEC-positive diarrhoeic nursery pigs in pen samples was observed for both the bacterial culture of pen floor samples and qPCR. Furthermore, haemolytic activity was a useful marker for isolates containing both adhesins and virulence genes.

## Competing interests

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

## Authors' contributions

KP, SH and NW designed the sampling protocol and selected methods; SH conducted the bacteriology; CH conducted the qPCR analyses; NW performed the data sampling; NW performed the statistical analysis; JPN, CFH, KP, SH, SEJ, CH and NW devised the study and drafted the manuscript. All authors contributed to finalising the manuscript. All authors read and approved the final manuscript.

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