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Published in:
Preventive Veterinary Medicine

Link to article, DOI:
10.1016/j.prevetmed.2017.05.009

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
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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A R T I C L E   I N F O

Article history:
Received 28 December 2016
Accepted 10 May 2017

Keywords:
Nursery pigs
Diarrhoea
ETEC
Diagnostics
qPCR
Pen floor samples

A B S T R A C T

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; Halaiehl et al., 2010; Fairbrother and Gyles, 2012). However, it has also been shown that nutritional and management...
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 ≥ 15 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.
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 Fryndahl 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 × 10^4 for F4, 1.5 × 10^3 for F18, and 2 × 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 Fisher’s 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 fimb-
bial 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 without 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 × 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 × 10^3 to 2 × 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 × 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
Quantitative PCR results for E. coli fimbrial genes F4 and F18, Lawsonia Intracellularis and Brachyspira Pilosicoli from 31 pen floor samples.

<table>
<thead>
<tr>
<th>Pen</th>
<th>Bacteria/g faeces</th>
<th>F4</th>
<th>F18</th>
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<th>Brachyspira Pilosicoli</th>
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Table 3
Relationship between presence of virulence factor genes and haemolysis in E. coli isolates.

<table>
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<tr>
<th>Virulence factors</th>
<th>Parameter</th>
<th>Estimate (95% CI)</th>
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<tr>
<td>+</td>
<td>Sensitivity</td>
<td>97.8% (92.1%–99.7%)</td>
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<tr>
<td>–</td>
<td>Specificity</td>
<td>91.8% (88.0%–94.6%)</td>
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<tr>
<td>+</td>
<td>Positive predictive value</td>
<td>78.4% (69.6%–85.6%)</td>
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<tr>
<td>–</td>
<td>Negative predictive value</td>
<td>99.3% (97.3%–99.9%)</td>
</tr>
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</table>

*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 faecal 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 fimbriated 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,
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 × 10^3 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, LT 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 floor 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.

Acknowledgements

This project was funded by The Danish Pig Levy Fund (Svineafgiftsfonden). The authors wish to thank participating herd owners and employees, and Christian Bonnerup Møller, Martin Rasmussen and Thomas Kjeldsen Kusk for their help with the data collection.

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