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Comparison of antimicrobial resistance in *E. coli* isolated from rectal and floor samples in pens with diarrhoeic nursery pigs in Denmark

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ABSTRACT

Introduction: The prudent use of antibiotics in veterinary medicine necessitates the selection of antibiotic compounds with narrow-spectrums targeted against the specific pathogens involved. The same pathotype of enterotoxigenic *E. coli* (ETEC) was recently found both in diarrhoeic pigs and in samples from the pen floor where the pigs were housed. The first objective of this study was to compare resistance profiles from ETEC isolates and Non-ETEC isolates. The second objective was to evaluate the agreement between resistance profiles of ETEC isolated from pen floor samples and from individual rectal samples from pigs.

Across three Danish pig herds, faecal samples were collected from the floors of 31 pens that had a within-pen diarrhoea prevalence of > 25%, and from rectal samples of 93 diarrhoeic nursery pigs from the same pens. A total of 380 *E. coli* isolates were analysed by PCR and classified as ETEC when genes for adhesin factors and enterotoxins were detected. Minimum inhibitory concentrations of 13 antimicrobial agents were determined by the broth micro dilution method. Isolates were classified as resistant based on clinical breakpoints.

Results: Based on logistic regression models, the odds of Non-ETEC isolates (n = 291) being pan-susceptible were significantly higher compared to ETEC isolates (n = 89), (P < 0.001, OR = 20.22, CI95% = 6.35-64.35). The odds of ETEC isolates having multidrug resistance were significantly higher compared to Non-ETEC isolates (p < 0.001, OR: 7.21, CI95%: 2.87-18.10). The odds of an isolate being resistant were significantly higher in ETEC isolates compared to Non-ETEC isolates for ampicillin (p < 0.001), apramycin (p = 0.003), sulphamethoxazole (p < 0.001) and trimethoprim (p < 0.001). No overlap of resistance patterns between the three study herds was observed in the sampled ETEC isolates.

In addition, there was generally good or excellent agreement when comparing resistance profiles from isolates from the same pen (pen floor and pig samples), and perfect agreement (Kappa = 1.000, SE = 0.316) was observed for ampicillin, apramycin, gentamycin, sulphamethoxazole, tetracycline and trimethoprim.

Conclusions: We found that ETEC isolates were more resistant than Non-ETEC isolates. Furthermore, this study indicates that resistance testing of ETEC isolates from pen floor samples can be used as a convenient sampling method for resistance testing and in the selection of clinically relevant antimicrobial agents in the treatment of diarrhoeic pigs. The herd-level variation of resistance in ETEC isolates emphasises the importance of performing antimicrobial susceptibility testing at farm level when selecting antimicrobial agents for the treatment of *E. coli*-related diarrhoea.

1. Introduction

The risk of antimicrobial resistance (AMR) spreading from food-producing animals to humans is a major concern that attracts considerable political attention. The World Health Organisation (WHO) has highlighted antimicrobial resistance as a global threat for human health, and action to combat AMR must be taken to avoid a post-antibiotic era (WHO, 2014). The prudent use of antimicrobials for production animals is therefore a focus point throughout the world (European Commission, 2015; OIE, 2016). Prudent use is defined as the

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choice of antimicrobials based on combined information from clinical experience, the expected susceptibility of the target pathogen, the route of administration, expected activity at the site of infection and the epidemiological history of the production unit, in particular previous antimicrobial resistance profiles (OIE, 2016). By using antimicrobial resistance profiles, veterinarians are able to select antimicrobial compounds with the narrowest spectrum of activity sufficient to target the pathogen (European Commission, 2015).

An important element in achieving prudent use is the development of new and precise diagnostic tools in veterinary pig practice, in order to decide whether antimicrobial treatment is necessary and to achieve the most efficient treatment of diseased animals. Previous published results from our group have shown that faecal pen floor samples can be used to diagnose enteric diseases from groups of pigs (Pedersen et al., 2015; Weber et al., 2017b). Furthermore, in outbreaks of ETEC-induced diarrhoea, the same pathotype of ETEC was demonstrated in rectal faecal samples from diarrhoeic pigs and in faecal samples from the pen floor where the pigs were housed (Weber et al., 2017a). We therefore hypothesise that using ETEC isolated from pen floor samples could be a convenient and relevant method for resistance testing and selection of antimicrobial agents.

The aim of this study was to investigate resistance profiles in ETEC and Non-ETEC isolates and to evaluate whether ETEC isolates from faecal pen floor samples could be used for resistance profiling. This was achieved by comparing resistance profiles in ETEC isolates from pen floor samples to faecal samples obtained per rectum from individual pigs in the same pens. Resistance profiling of pathogenic *E. coli* is highly relevant in veterinary practice when choosing the type of antimicrobial agent for treatment.

The first objective of the study was to compare resistance profiles from ETEC isolates and Non-ETEC isolates.

The second objective was to evaluate the agreement between resistance profiles of ETEC isolated from pen floor samples and from individual rectal samples from pigs.

2. Method

2.1. Design

A cross sectional study was performed in three commercial production herds in 2014. A total of 31 pens were selected and 93 pigs from these pens were sampled 14–28 days after weaning.

2.2. Herd description

A thorough description of the herds included in the study is published in Weber et al. (2017b). The herds were previously selected for a clinical trial investigating batch medication for intestinal diseases in nursery pigs. In brief, 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 antimicrobial treatment (Pedersen et al., 2014). All herds had all-in all-out batch production in sectioned compartments, and the flooring consisted of 1/3 solid floor and 2/3 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 DanAvl crossbreds of Yorkshire/Landrace and Duroc. All herds used 3000 ppm zinc oxide in the feed during the first 14 days after weaning.

2.3. Sampling procedure

The inclusion criteria for individual pens and pigs are described in detail in Weber et al. (2017a). In brief, rectal samples from 15 randomly selected pigs were obtained by digital manipulation. A diarrhoeic pig was identified by scoring the rectal sample 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). In pens with a diarrhoea prevalence of 25% or above among the sampled pigs, rectal samples from three diarrhoeic pigs and a faecal pen floor sample were collected and stored in sealed plastic containers. The pen floor samples were collected by running a gloved hand across the full length of the slatted floor. The cooled faecal samples were transported for bacteriology to the Laboratory for Pig Diseases in Kjellerup, Denmark in a polystyrene box containing ice packs.

2.4. Laboratory analyses

2.4.1. Bacteriology

In this study, bacterial culture of faecal samples was used to identify presence of *E. coli* colonies. The pig and pen floor samples were aerobically cultured for *E. coli*. Parallel culturing was performed on Drigalski (in-house selective and indicative medium for coliforms) and blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood). Plates were incubated for 24 h at 37 °C. To identify the excepted higher diversity of *E. coli* isolates in pen floor samples, a larger number of colonies were sampled from pen floor samples than pig samples (Weber et al., 2017a). After culture, two coliform colonies with haemolytic activity (if present) and two coliform colonies with non-haemolytic activity were isolated from each pig sample. Haemolytic isolates were defined as colonies surrounded by a zone of lysis. Up to five coliform colonies with haemolytic activity and five coliform colonies with non-haemolytic activity were isolated from the pen floor samples. The selected isolates were analysed at the Danish Veterinary Institute using the 5′-nuclease assay (TaqMan PCR) previously described for the detection of virulence factor genes: F4, F5, F6, F18, F41, STa, STb, LT and VT2e (Frydendahl et al., 2001).

2.4.2. Antimicrobial susceptibility testing

Susceptibility testing was performed to determine the phenotypic susceptibilities of the sampled *E. coli* isolates to 13 antimicrobial agents. The antimicrobial concentration ranges and clinical breakpoints of the 13 antimicrobial agents included in the panel are shown in Table 1. The panel comprises clinically relevant antimicrobial agents for the treatment of porcine *E. coli* infections, in agreement with international guidelines (Burch et al., 2008; DANMAP, 2010). Minimum inhibitory concentrations (MIC) were determined by the broth micro dilution method in 96-well microtitre plates using the Sensititre system (Thermo Fisher Scientific, Waltham, Massachusetts, USA), as described in the standards manual of the Clinical and Laboratory Standards Institute (CLSI, 2015). The *E. coli* reference strain ATCC 25922 was used as a control organism. The plates were incubated for 20 h at 37 °C in an aerobic atmosphere. The Sensititre plates were manually read by trained laboratory personnel. The MIC was defined as the lowest concentration producing no visible growth. The clinical breakpoints used to interpret MIC values were a combination of CLSI breakpoints if available, and those routinely used by the Laboratory of Swine diseases, Kjellerup, Denmark and by the Danish Veterinary Institute, Frederiksberg, Denmark (CLSI, 2015; DANMAP, 2016).

2.5. Statistical analysis

The presence of resistance in ETEC and Non-ETEC isolates are presented in summary tables. Statistical analyses were performed in R version 3.1.2 with mixed models implemented using the lme4 package (R-Core-Team, 2014; Bates et al., 2015). The susceptibility to the 13 tested antimicrobials for both ETEC and Non-ETEC isolates were evaluated by determination of MIC50 and MIC90. Furthermore, to estimate the effect of the isolates' ETEC status on the occurrence of resistance, a generalised linear mixed model with logit link and binomial response (logistic regression) was used for each antimicrobial agent, with binary

Table 1
Antibiotic concentration ranges and resistance breakpoints used for susceptibility testing of E. coli (n = 380) isolated from faecal samples from weaned pigs and pen floors.

Antimicrobial class	Antimicrobial agent	Abbreviations	Concentration used (μg/ml)	Clinical breakpoint (μg/ml)*
Penicillins	Ampicillin	AMP	1 − 32	≥32[a]
B-Lactam/β-lactamase inhibitors	Amoxicillin/clavulanic acid	AUC	2/1 − 32/16	≥32/16[a]
Folate pathway inhibitors	Trimethoprim	TMP	1 − 32	≥16[b]
	Sulphamethoxazole	SMX	64 − 1024	≥512[a]
Aminoglycosides	Gentamicin	GEN	0.5 − 32	≥16[a]
	Apramycin	APR	4 − 32	≥16[b]
	Streptomycin	STR	8 − 128	≥32[b]
	Spectinomycin	SPE	16 − 256	≥128[b]
	Neomycin	NEO	2 − 32	≥16[b]
Quinolones	Ciprofloxacin	CIP	0.015 − 4	≥4[c]
Cephalosporins	Ceftiofur	XNL	0.5 − 8	≥8[d]
Tetracyclines	Tetracycline	TET	2 − 32	≥16[a]
Polymyxins	Colistin	COL	1 − 16	≥16[b]

[a] CLSI-approved breakpoints based on human data.
[b] Breakpoints routinely used by the Laboratory of Swine diseases, Kjellerup, Denmark and by the Danish Veterinary Institute, Frederiksberg, Denmark.
[c] CLSI-approved breakpoint for Enrofloxacin based on dog data used as representative for Ciprofloxacin. [d]CLSI-approved breakpoint based on cattle data.

Table 2
Distribution of sampled E. coli isolates by Herd and Batch level.

				Pig isolates	Pen isolates	
Herd	Batch	Sampled pens	Sampled pigs	Haemolytic − Non-haemolytic	Haemolytic − Non-haemolytic	Total
1	1	5	15	20 − 28	22 − 21	91
2	2	6	18	2 − 34	2 − 20	58
2	3	5	15	5 − 19	1 − 19	44
2	4	3	9	8 − 10	15 − 8	41
2	5	3	9	6 − 14	4 − 12	36
3	6	6	18	2 − 33	0 − 20	55
3	7	3	9	11 − 16	13 − 15	55
Total		31	93	54 − 154	57 − 115	380

resistance classification as the outcome and ETEC status as the sole fixed effect variable. Herd, batch and sample were used as random effects in all the statistical models to account for clustering at herd, batch and sample level. Model adequacy was assessed by visual inspection of the random effect estimates for individual herds, batches and samples in order to verify an approximately normal distribution of estimates within each random effect, and by comparison of the predicted logit probabilities between observed resistance classifications to assess the predictive ability of the model. Only a single fixed effect was considered, so no model selection procedure was performed.

To determine the odds ratio of ETEC isolates being multidrug resistant (MDR) a separate logistic regression model was used with MDR status as the binary outcome (resistance to ≥3 agents of antimicrobials/resistance to < 3 agents of antimicrobials), ETEC status as the primary explanatory variable, and herd, batch and sample as random effects. Similarly, to determine the odds ratio of ETEC isolates being sensitive to all tested antimicrobial agents (pan-susceptible) a separate logistic regression model was used with Pan-susceptible status as the binary outcome (pan-susceptible/non pan-susceptible), ETEC status as the primary explanatory variable, and herd, batch and sample as random effects.

To evaluate the agreement between resistance profiles of ETEC isolated from pen floor samples and from individual rectal samples from pigs, a total of 4 comparisons were performed in this study:

1. Comparison of resistance in ETEC isolates from the same pig sample
2. Comparison of resistance in ETEC isolates from the same pen floor sample
3. Comparison of resistance in ETEC isolates between pig samples from pen mates
4. Comparison of resistance in ETEC isolates between pig and pen floor isolates from the same pen

Agreement calculations were performed in 2 × 2 contingency tables for each tested antimicrobials. Agreement was evaluated by the calculation of observed agreement, and the statistical association was evaluated using Fisher's exact test and Cohen's kappa coefficient. The kappa values were used to interpret agreement as: < 0 = none; 0–20 = slight; 21–40 = fair; 41–60 = moderate; 61–80 = substantial; 81–100 = almost perfect, as described by Landis and Koch (1977).

3. Results

3.1. Resistance in ETEC and non-ETEC isolates

A total of 380 E. coli isolates, obtained from 93 pig samples and from 31 pen floor samples, were used for further analysis. An overview of the distribution of sampled pens, and number of *E. coli* isolates per batch are shown in Table 2. PCR testing for STa, STb, LT and VT2e toxin and F4, F5, F6, F18, F41 fimbriae genes revealed 89 isolates classified as ETEC and 291 as Non-ETEC.

Table 3 shows the proportion of resistant isolates, MIC50 and MIC90 to the 13 antimicrobial agents used in this study. Furthermore the results of generalised linear mixed models for estimating the effect of the isolates' ETEC status on the occurrence of resistance are presented. The overall proportion of resistance was above 1% for seven antimicrobial agents: sulphamethoxazole (50.3%), ampicillin (45.5%), trimethoprim (40.5%), streptomycin (38.9%), tetracycline (36.1%), spectinomycin (20.5%), apramycin (3.9%) and gentamicin (3.4%). Low resistance rates were observed in neomycin (0.5%) and amoxicillin + clavulanic acid (0.2%). Pan-sensitivity was observed for ciprofloxacin, colistin, and ceftiofur. The odds of an isolate being resistant were significantly higher in ETEC isolates compared to Non-ETEC isolates for ampicillin ($p < 0.001$), apramycin ($p = 0.003$), sulphamethoxazole ($p < 0.001$) and trimethoprim ($p < 0.001$), based on the results of the logistic regression models (Table 3). Furthermore the MIC50 and/or MIC90 were more than 4 dilution steps higher in ETEC isolates compared to Non-ETEC isolates for the above mentioned antimicrobials. Resistance to gentamycin was only observed in ETEC isolates (14.6%), but due to complete separation, the logistic model to estimate the odds of ETEC isolates being resistant to gentamycin could not be run.

Based on the logistic regression model, the odds of an isolate being sensitive to all tested antimicrobial agents were significantly higher in Non-ETEC isolates compared to ETEC isolates ($P < 0.001$, OR = 20.22, CI95% = 6.35-64.35). On average, ETEC isolates were resistant to 3.29 antimicrobial agents, whereas Non-ETEC isolates on average was resistant to 2.17 antimicrobial agents.

Table 3
Occurrence of resistance in 89 ETEC and 291 Non-ETEC isolates with results of generalised linear mixed models.

Antimicrobial agent	Isolate type	MIC 50	MIC 90	Resistant (%)	Odds ratio	CI95%	P-value
Amoxicillin + Clavulanic acid	ETEC	4	8	0.0	-[a]	–	–
	Non-ETEC	4	8	0.3			
Ampicillin	ETEC	> 32	32	60.7	7.52	2.99 – 18.93	< 0.001
	Non-ETEC	4	32	40.9			
Apramycin	ETEC	≤4	> 32	14.6	12.46	5.23–297.18	0.003
	Non-ETEC	≤4	4	0.7			
Ceftiofur	ETEC	≤0.5	≤0.5	0.0	–	–	–
	Non-ETEC	≤0.5	≤0.5	0.0			
Ciprofloxacin	ETEC	0.015	0.03	0.0	–	–	–
	Non-ETEC	0.03	0.03	0.0			
Colistin	ETEC	≤1	4	0.0	–	–	–
	Non-ETEC	≤1	≤1	0.0			
Gentamicin	ETEC	≤0.5	16	14.6	–	–	–
	Non-ETEC	≤0.5	1	0.0			
Neomycin	ETEC	≤2	≤2	0.0	–	–	–
	Non-ETEC	≤2	≤2	0.7			
Spectinomycin	ETEC	≤16	> 256	18.0	0.97	0.43 – 2.17	0.940
	Non-ETEC	≤16	> 256	21.3			
Streptomycin	ETEC	≤8	> 128	29.2	0.73	0.36 – 1.49	0.385
	Non-ETEC	≤8	> 128	41.9			
Sulphamethoxazole	ETEC	< 1024	> 1024	69.7	8.05	3.18–20.37	< 0.001
	Non-ETEC	≤64	> 1024	44.7			
Tetracycline	ETEC	≤2	> 32	47.2	1.74	0.88 – 3.46	0.111
	Non-ETEC	≤2	> 32	32.7			
Trimethoprim	ETEC	> 32	> 32	69.7	13.51	4.94 – 36.96	< 0.001
	Non-ETEC	≤1	> 32	31.6			

The effect of the isolates' ETEC status on the occurrence of resistance was estimated with a generalised linear mixed model with logit link and binomial response (logistic regression) was used for each antimicrobial agent, with binary resistance classification as the outcome and ETEC status as the sole fixed effect variable. Herd, batch and sample were used as random effects in the statistical models to account for clustering at herd, batch and sample level.

[a] Logistic regression models could not run for antimicrobials with complete separation for isolate type (zero resistance in either isolate group).

3.2. Resistance distribution at herd level

The distribution of resistant ETEC and Non-ETEC isolates at herd level is shown in Table 4. In ETEC isolates from Herd 1, resistance was observed to eight different antimicrobial agents, compared to one and four antimicrobial agents in isolates from Herds 2 and Herd 3, respectively. Resistance to ampicillin was observed in isolates from all three herds. Resistance to sulphamethoxazole, trimethoprim and streptomycin was observed in Herds 1 and 3. Resistance to apramycin, gentamicin, spectinomycin and tetracycline was only observed in isolates from Herd 1. The herd-level patterns of resistance in Non-ETEC isolates superficially appeared to be more similar than for ETEC isolates. Resistance against sulphamethoxazole, spectinomycin, streptomycin, tetracycline, and trimethoprim was present in Non-ETEC isolates from all three herds and accounted for the majority of resistance in Non-ETEC isolates.

Table 4
Proportion of resistant isolates at herd level.

Antimicrobial agent	Herd 1				Herd 2				Herd 3			
	ETEC		Non-ETEC		ETEC		Non-ETEC		ETEC		Non-ETEC	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Amoxicillin + Clavulanic acid	0	0.0	0	0.0	0	0.0	1	0.6	0	0.0	0	0.0
Ampicillin	14	33.3	7	14.3	19	86.4	2	1.3	21	84.0	0	0.0
Apramycin	13	31.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Ceftiofur	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Ciprofloxacin	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Colistin	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Gentamicin	13	31.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Neomycin	0	0.0	1	2.0	0	0.0	0	0.0	0	0.0	1	1.2
Sulphamethoxazole	41	97.6	7	14.3	0	0.0	54	34.4	21	84.0	68	80.0
Spectinomycin	16	38.1	2	4.1	0	0.0	42	26.8	0	0.0	18	21.2
Streptomycin	18	42.9	9	18.4	0	0.0	62	39.5	13	52.0	59	69.4
Tetracycline	42	100.0	18	36.7	0	0.0	50	31.8	0	0.0	27	31.8
Trimethoprim	41	97.6	6	12.2	0	0.0	34	21.7	21	84.0	52	61.2
Pan-susceptible	0	0.0	28	57.1	3	13.6	71	45.2	4	16.0	11	12.9
Total isolates	42		49		22		157		25		85	

Table 5
Profile of antimicrobial resistance in E. coli isolates in faecal samples from weaned pigs and pen floor samples.

Type	Pattern	ETEC isolates		Non-ETEC isolates	
		No.	%	No.	%
28	AMP, APR, GEN, SMX, SPE, STR, TET, TMP	13	14.6		
27	AMP, NEO, SMX, SPE, STR, TET, TMP			1	0.3
26	AMP, SMX, SPE, STR, TET, TMP			7	2.4
25	SMX, SPE, STR, TET, TMP	1	1.1		
24	AMP, SMX, STR, TET, TMP			38	13.1
23	AMP, SMX, SPE, STR, TMP			6	2.1
22	AMP, SMX, SPE, STR, TET			1	0.3
21	SMX, STR, TET, TMP	4	4.5		
20	SMX, SPE, TET, TMP	2	2.2		
19	SMX, SPE, STR, TET			14	4.8
18	AMP, SMX, TET, TMP			5	1.7
17	AMP, SMX, STR, TMP	13	14.6	16	5.5
16	AMP, SMX, SPE, STR			8	2.7
15	SPE, STR, TMP			4	1.4
14	SPE, STR, TET			9	3.1
13	SMX, TET, TMP	21	23.6	8	2.7
12	AMP, STR, TET			1	0.3
11	AMP, SPE, STR			4	1.4
10	AMP, SMX, TMP	8	9.0	3	1.0
9	AMP, SMX, STR			19	6.5
8	STR, TET			1	0.3
7	GEN, SMX			2	0.7
6	APR, STR			1	0.3
5	AMP, TET	1	1.1		
4	AMP, AUC			1	0.3
3	TET			21	7.2
2	SMX			1	0.3
1	AMP	19	21.3	10	3.4
0	Pan-susceptible	7	7.9	110	37.8
Total isolates		89		291	

Notes: For abbreviations refer to Table 1. Isolates where no resistance was observed were labelled “Pan-susceptible”.

3.3. Antimicrobial resistance profiles

Table 5 shows the 28 different antimicrobial resistance patterns observed among the pig and pen floor isolates. The ETEC isolates were clustered in fewer patterns (9) than the Non-ETEC isolates (22), and four patterns (17, 13, 10 and 1) were observed in both Non-ETEC and ETEC isolates. According to the European Centre for Disease Prevention and Control, multidrug resistance (MDR) is defined as resistance to ≥3 agents of antimicrobials (Magiorakos et al., 2012). The odds of an isolate having MDR was a significantly higher in ETEC isolates than Non-ETEC isolates based on the logistic regression model ($p < 0.001$, OR: 7.21, CI95%: 2.87-18.10).

3.4. Comparison of resistance patterns in ETEC isolates

Table 6 shows the resistance patterns of ETEC isolates from pig and pen floor samples. In 10 pens, ETEC isolates were demonstrated in both pig samples and in the pen floor samples simultaneously. Within-pen variation in resistance patterns was observed in both pig and pen floor isolates. Overall, the resistance patterns appeared to be clustered at herd level, with no overlap of resistance patterns between the three study herds. Good agreement was observed when comparing resistance patterns between pig and pen floor isolates. The same resistance patterns were observed in pig isolates and corresponding pen floor isolates in 7 of the 10 pens.

3.5. Comparison of resistance to selected antimicrobial agents

In the following sections, resistance classifications of ETEC isolates were compared for selected antimicrobial agents that had an overall resistance rate of > 1%.

3.5.1. Within-sample agreement

Table 7 shows the agreement in resistance classifications for selected antimicrobial agents between ETEC isolates obtained from the same sample. In this study, it was only possible to make 18 comparisons of resistance between two isolates from the same pig sample. Nearly perfect agreement of resistance to ampicillin and tetracycline was observed between isolates obtained from the same pig. Substantial agreement was observed in resistance to apramycin, gentamycin, spectinomycin, sulphamethoxazole and trimethoprim, and moderate agreement was observed in resistance to streptomycin.

Between 0 and 5 ETEC were isolated per pen floor sample. It was possible to make a comparison between multiple isolates from 11 pen floor samples. As with the pig samples, an overall good agreement was observed between isolates from the same pen floor sample. Nearly perfect agreement was observed in resistance to sulphamethoxalzole, tetracycline and trimethoprim, and substantial agreement was observed in resistance to ampicillin, apramycin and gentamycin. Fair agreement was observed for streptomycin and spectinomycin resistance, where only 6 of 11 and 8 of 11 pen samples showed agreement, respectively.

3.5.2. Agreement between pen mates

Within each pen, 1–3 diarrhoeic pigs were sampled. A pig was classified as resistance positive for a specific antimicrobial agent if a minimum of one ETEC isolate from the pig was found to be resistant. In 7 pens, ETEC was detected in more than one diarrhoeic pig. When comparing the resistance classification in these 7 pens, perfect agreement between pigs from the same pen was observed in apramycin, gentamicin, spectinomycin and tetracycline resistance. Substantial agreement in ampicillin resistance, moderate agreement in sulphamethoxazole and trimethoprim resistance and fair agreement in streptomycin, was observed.

3.5.3. Agreement between pig and pen floor isolates

When comparing resistance in pig isolates and in pen floor isolates from the same pen, the following definition of resistance classification was used:

Pig isolate resistance. The pigs were classified as resistance positive for a specific antimicrobial agent if one or more ETEC isolates from one or more pigs in the pen were resistant.

Pen floor resistance. A pen floor sample was classified as resistance positive for a specific antimicrobial class if one or more ETEC isolates from the sample were resistant.

It was possible to make a comparison of resistance classification between pig isolates and the corresponding pen floor samples in 10 pens. By using the previously mentioned definitions, perfect agreement was observed in ampicillin, apramycin, gentamicin, sulphamethoxazole, tetracycline and trimethoprim resistance, whereas substantial agreement in spectinomycin resistance and fair agreement in streptomycin resistance was observed.

4. Discussion

This study investigated resistance in *E. coli* isolates from pig and pen floor samples. The isolates classified as Non-ETEC can be regarded as indicator bacteria, whereas ETEC isolates are considered clinical isolates. Indicator bacteria are ubiquitous and present as commensals in both animal and human reservoirs, and can be monitored to detect the occurrence of antimicrobial resistance in different reservoirs

Table 6
Resistance patterns in ETEC isolates detected from pig and pen floor samples in the same pen

Herd Id	Pen Id	Pig isolates						Pen floor isolates				
1	1	21*	20	13	13	13	13	21	20	13	13	13
1	2	13	13					25	21	13	13	
1	3	13	13	13	13	13		21	13	13	13	13
1	4	28	28	28	28			28	28	28	28	13
1	5	28	28	5				28	28	28		
2	6	1	1					1	1	1	1	1
2	7	1	1	1	1			1	1	1		
3	8	17	17	17	10	10	0	17	17	17	10	
3	9	17	10	10	0			10	10	10		
3	10	17						17	17	17	17	17

For each pen, one to two ETEC were isolated from one to three diarrhoeic pigs. For each pen floor sample, one to five ETEC were isolated. The colour represents the resistance pattern given by the corresponding Type number in Table 5.

Table 7
Agreement of resistance in ETEC isolates from pig and pen floor isolates.

Antimicrobial agent[a]	p-value[b]	Observed agreement (samples with agreement/ total samples)	Kappa (Standard Error)
	Within-sample agreement in isolates from 18 pig samples[c]		
Ampicillin	< 0.001	0.944 (17/18)	0.889 (0.234)
Apramycin	0.020	0.944 (17/18)	0.769 (0.229)
Gentamycin	0.020	0.944 (17/18)	0.769 (0.229)
Spectinomycin	0.039	0.889 (16/18)	0.609 (0.217)
Streptomycin	0.025	0.833 (15/18)	0.571 (0.213)
Sulphamethoxazole	0.002	0.889 (16/18)	0.753 (0.228)
Tetracycline	< 0.001	1.000 (18/18)	1.000 (0.219)
Trimethoprim	0.002	0.889 (16/18)	0.753 (0.228)
	Within-sample agreement in isolates from 11 pen floor samples[d]		
Ampicillin	0.024	0.909 (10/11)	0.792 (0.295)
Apramycin	0.182	0.909 (10/11)	0.621 (0.279)
Gentamycin	0.182	0.909 (10/11)	0.621 (0.279)
Spectinomycin	0.364	0.727 (8/11)	0.298 (0.215)
Streptomycin	0.491	0.545 (6/11)	0.225 (0.191)
Sulphamethoxazole	0.006	1.000 (11/11)	1.000 (0.302)
Tetracycline	0.002	1.000 (11/11)	1.000 (0.302)
Trimethoprim	0.006	1.000 (11/11)	1.000 (0.302)
	Agreement of resistance between pen mates in 7 pens[e]		
Ampicillin	0.143	0.857 (6/7)	0.696 (0.360)
Apramycin	0.048	1.000 (7/7)	1.000 (0.378)
Gentamycin	0.048	1.000 (7/7)	1.000 (0.378)
Spectinomycin	0.048	1.000 (7/7)	1.000 (0.378)
Streptomycin	1.000	0.571 (4/7)	0.276 (0.261)
Sulphamethoxazole	0.286	0.857 (6/7)	0.588 (0.344)
Tetracycline	0.029	1.000 (7/7)	1.000 (0.378)
Trimethoprim	0.286	0.857 (6/7)	0.588 (0.344)
	Agreement between pig resistance and pen floor resistance in 10 pens[f]		
Ampicillin	0.008	1.000 (10/10)	1.000 (0.316)
Apramycin	0.022	1.000 (10/10)	1.000 (0.316)
Gentamycin	0.022	1.000 (10/10)	1.000 (0.316)
Spectinomycin	0.033	0.900 (9/10)	0.783 (0.309)
Streptomycin	0.500	0.700 (7/10)	0.348 (0.309)
Sulphamethoxazole	0.022	1.000 (10/10)	1.000 (0.316)
Tetracycline	0.008	1.000 (10/10)	1.000 (0.316)
Trimethoprim	0.022	1.000 (10/10)	1.000 (0.316)

Pig resistance: The pig isolates from one pen was classified as resistance positive for a specific antimicrobial class if a minimum of one ETEC isolate from one or more pigs in the pen was resistant. **Pen floor resistance:** Pen floor samples were classified as resistance positive if a minimum of one ETEC isolate from the sample was resistant.

[a] Selected antimicrobial agents with an overall resistance rate > 1%.
[b] Fisher's Exact test.
[c] Comparison of isolates from 18 diarrhoeic pigs where multiple ETEC were isolated.
[d] Comparison of isolates from 11 pen floor samples where multiple ETEC were isolated.
[e] A pig was classified as resistance positive for a specific antimicrobial agent if a minimum of one ETEC isolate from the pig was found to be resistant.
[f] Comparison of pig isolates and corresponding pen floor isolates in 10 pens.

throughout the food chain (DANMAP, 2016).

Overall, pan-susceptibility was observed in the two antibiotic agents ciprofloxacin and ceftiofur, which are classified by the WHO as critically important antimicrobials for human medicine (WHO, 2012). These findings correspond well with the use of flouroquinolons in pigs being strictly limited in Denmark since 2002, and the voluntary ban on the use of third- and fourth-generation cephalosporins in the Danish pig industry since 2010 (DANMAP, 2016). Furthermore, full susceptibility was observed to colistin, which has recently been classified as a critically important antimicrobial for the treatment of carbapenemase-resistant infections in human medicine (DANMAP, 2016). Resistance in Non-ETEC isolates from this study were observed for the same antimicrobial agents and with similar rates to those previously reported in indicator *E. coli* from Danish resistance surveillance (DANMAP, 2014). Furthermore, little variation in Non-ETEC resistance was observed at herd level. This indicates that the resistance found in the three farms from this study is representative of Danish pig farms in general, and that background resistance against the same antimicrobial agents is present. However, a higher between-herd variation was reported in studies of antimicrobial resistance among faecal indicator *E. coli* from North America (Bunner et al., 2007; Dunlop et al., 1998; Rosengren et al., 2008). A possible reason for the comparatively little variation observed in our study could be that factors influencing antimicrobial resistance (such as antimicrobial pressure, movement and flow of humans and animals or interaction with rodents) were similar in the three herds.

The highest overall proportion of resistance in ETEC isolates from this study was observed for ampicillin, sulphamethoxazole, tetracycline and trimethoprim. Similar findings have been reported for clinical isolates from diarrhoeic nursery pigs submitted to diagnostic laboratories in Denmark (DANMAP, 2010; Hendriksen et al., 2008).

The between-herd variation in resistance for ETEC isolates was markedly more diverse than for Non-ETEC isolates. This may be due to a more clonal distribution of virulent strains, and emphasises the importance of performing antimicrobial susceptibility testing at farm level when selecting antimicrobial agents for treatment of *E. coli*-related diarrhoea. Furthermore, susceptibility testing should always be performed on ETEC rather than Non-ETEC isolates since the resistance profiles may differ between herds.

In this study, ETEC isolates were more resistant than Non-ETEC isolates, which indicates that antimicrobial resistance may be genetically linked to virulence factor genes. This finding has previously been described by Sato et al., who observed a strong association between fimbriae and toxin genes and antimicrobial resistance in 185 *E. coli* isolates from diarrhoeic pigs in Brazil (Sato et al., 2015). The same pattern of a higher resistance rate in clinical isolates compared to indicator isolates has been observed in Denmark for many years (DANMAP, 2010). The adverse consequences of more resistance in clinical isolates underline the importance of the prudent use of

antimicrobials for treatment of ETEC-related diarrhoea in pigs. To prevent resistance from developing, the relevant susceptibility testing should be considered when selecting the antimicrobial agents to be used for treatment. Treatment of healthy pigs should be avoided to ensure the effect of antimicrobial agents on clinical isolates.

This study showed that the resistance patterns in ETEC isolates were more homogeneous than in Non-ETEC isolates. A possible explanation for this finding is that the ratio of ETEC/Non-ETEC isolates in this study was 3–1. Alternatively, it could be due to the clonal distribution of virulent strains previously described and supported by the large between-herd variation in resistance, which demonstrates that different clones of ETEC isolates predominate among different herds.

Several comparisons of resistance in ETEC isolates were performed in this study. The goal of these comparisons was to identify a convenient and representative sampling method that would provide the most precise susceptibility testing of ETEC isolates. With the exception of spectinomycin and streptomycin resistance, good agreement was observed in all the comparisons performed. The results show that no extra information on resistance is gained when multiple isolates are tested, regardless of whether the sampling is performed on isolates from diarrhoeic pigs or pen floor isolates. To our knowledge, there is no previously published report of the within-sample variation in resistance in clinical *E. coli* isolates. Publications on the variation in resistance have mainly focussed on national resistance surveillance, where the resistance of indicator *E. coli* isolated from healthy pigs has been examined (Yamamoto et al., 2014). However, low within-sample variation in resistance was reported in a Norwegian study of *E. coli* isolates from clinically healthy pigs (Brun et al., 2002).

All isolates from the current study were classified as resistant or sensitive based on MIC values above or below clinical breakpoints derived from CLSI or from Danish Veterinary diagnostic laboratories. None of the used clinical breakpoints originates from studies in swine but are based on human studies or studies in other animal species. Therefore the classification of sensitive/resistant based on MIC values in the swine *E. coli* isolates from this study has to be interpreted with caution.

Clinical breakpoints were used in this study because there are routinely used by the diagnostic veterinary laboratories in Denmark for susceptibility testing of clinical *E. coli* isolates from diarrhoeic pigs. Furthermore the same breakpoints were used for all isolates making a comparison of resistance reasonable. The greatest level of disagreement in resistance status in the within-sample comparisons and comparisons between pig and pen floor isolates was observed for spectinomycin and streptomycin. The reason for this observed disagreement could be that the MIC values for these antimicrobials were clustered around the breakpoints, making a single dilution step sufficient to change the isolate from susceptible to resistant. The results concerning resistance to spectinomycin and streptomycin must therefore be interpreted with caution due to the uncertainty of the true susceptibility status.

The comparison of resistance between pig isolates and pen floor isolates from the same pen revealed good agreement, although care should be taken with interpreting these estimates due to the small sample sizes involved as well as potential issues caused by the non-independence of observations from different animals in the same farm, section and pen. However, together with the recent finding of similar ETEC isolates with same virulence profiles in diarrhoeic pig samples and in samples from the pen, we believe that the results are sufficiently persuasive to suggest a new diagnostic approach based on pen floor samples (Weber et al., 2017a). This may be combined with susceptibility testing of the same isolates, as demonstrated in the present study.

Conclusions in this study were based on sampling ETEC isolates from diarrhoeic pigs 14–28 days post-weaning in three herds. However, the small sample size resulted in wide confidence intervals and therefore the conclusions of this study should be interpreted with care. Furthermore the high level of zinc in the starter feed used in the study farms could have had an impact on the prevalence of ETEC in the study period.

To confirm the results, this study should be further evaluated under field conditions in additional herds dealing with colibacillosis 1–2 weeks post-weaning and not using high level of zinc in the starter feed, where ETEC isolates would be considered primary pathogens.

5. Conclusion

We found that ETEC isolates were more resistant than Non-ETEC isolates. This study also indicates that resistance testing of ETEC isolates from pen floor samples can be used as a convenient sampling method for resistance testing and in the selection of clinically relevant antimicrobial agents in the treatment of diarrhoeic pigs. The herd-level variation in resistance within ETEC isolates emphasises the importance of performing antimicrobial susceptibility testing at farm level when selecting antimicrobial agents for the treatment of *E. coli*-related diarrhoea.

Conflict of interest

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

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