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Extended-spectrum beta-lactamases (ESBL)/AmpC beta-lactamases-producing *Escherichia coli* in German fattening pig farms: a longitudinal study

Extended-Spektrum-Beta-Laktamase (ESBL)/AmpC Beta-Laktamase-produzierende Escherichia coli in deutschen Schweinemastbetrieben: eine Langzeitstudie

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Summary

The presence of ESBL/AmpC-producing Enterobacteriaceae in healthy livestock, such as pigs, was frequently reported worldwide in the last years. The development and potential spread of these resistant microorganisms in farm animals is discussed critically. Therefore, the main objectives of this longitudinal study were to determine potential sources and prevalence dynamics of ESBL/AmpC-producing *Escherichia coli* in seven German ESBL/AmpC-positive conventional fattening pig farms in the course of the fattening period. Samples tested were taken at three different times within one finishing fattening period and included 20 individual faeces samples as well as various samples of the animals' housing environment such as pooled faeces, boot swabs, dust and environmental swabs. In individual faeces average carriage levels of 45%, 29% and 36% at the three sampling times were accompanied by decreasing faecal counts from 2.97×10^4 cfu/g at the first to 2.17×10^3 cfu/g at the third visit. In the animals' housing environment 47.6% of pooled faeces and boot swab samples respectively and 5.9% of environmental swabs but none of the dust samples were tested positive. Altogether 228 *E. coli* isolates were analysed by combination disc tests, PCR and partly via sequencing. Thereby, a novel gene $bla_{\text{TEM-206}}$ was detected. This study shows different detection levels of ESBL/AmpC-producing *E. coli* amongst the different farms and in the course of the fattening period. Pooled faeces and boot swab samples but not dust samples seem to be appropriate to assess the herd status of pigs with respect to ESBL/AmpC-producing *Escherichia coli*.

Keywords: pig production, animal farming, antimicrobial resistance, CTX-M, *E. coli*

Zusammenfassung

In den letzten Jahren wurde weltweit vielfach über das Vorkommen von ESBL/AmpC-produzierenden Enterobacteriaceae in gesunden Nutztieren, wie beispielsweise Schweinen, berichtet. Die Entwicklung und potenzielle Verbreitung dieser resistenten Mikroorganismen bei den Nutztieren wird kritisch diskutiert. Daher waren Hauptziele dieser Langzeitstudie die Untersuchung potenzieller Quellen sowie der Prävalenzdynamik von ESBL/AmpC-bildenden *Escherichia coli* in sieben deutschen ESBL/AmpC-positiven konventionellen Schweinemastbetrieben im zeitlichen Verlauf der Mastperiode. Probenahmen erfolgten dabei zu drei verschiedenen Zeitpunkten innerhalb einer Endmastperiode und umfassten 20 Einzeltierkotproben sowie verschiedene Proben der Tierumgebung wie Sammelkot, Sockentupfer, Staub und Umgebungstupfer. Die durchschnittlichen Nachweishäufigkeiten von ESBL/AmpC-*E. coli* in den Einzeltierkotproben lagen zu den drei Probenahmezeitpunkten bei 45 %, 29 % und 36 %, wobei eine Abnahme der Konzentration von $2{,}97 \times 10^4$ KbE/g zum ersten Zeitpunkt zu $2{,}17 \times 10^3$ KbE/g

zum dritten Zeitpunkt zu verzeichnen war. In der Tierumgebung wurden jeweils 47,6 % der Sammelkot- und Sockentupferproben und 5,9 % der Umgebungstupfer, jedoch keine der Staubproben positiv auf ESBL/AmpC-produzierende *E. coli* getestet. Insgesamt wurden 228 *E. coli*-Isolate mittels Blättchendiffusionstest zur antimikrobiellen Empfindlichkeitstestung, PCR und teilweise durch Sequenzierung analysiert. Dabei wurde ein neues Gen $bla_{TEM-206}$ beschrieben. Diese Studie zeigt unterschiedliche Nachweishäufigkeiten von ESBL/AmpC-bildenden *E. coli* in den untersuchten Schweinemastbetrieben und im Verlauf der Mast. Sammelkot- und Sockentupferproben scheinen im Gegensatz zu Staubproben geeignet zu sein, um den ESBL/AmpC-Status von Schweinebeständen zu bestimmen.

Schlüsselwörter: Schweineproduktion, landwirtschaftliche Tierhaltung, antimikrobielle Resistenz, CTX-M, *E. coli*

Introduction

When antibiotics were discovered they were seen as "miracle drugs". Indeed, these powerful therapeutic tools, able to fight bacterial diseases, revolutionized medical care in the 20th century. However, in the last decades antimicrobial resistance became an increasing issue in human as well as veterinary medicine. Two of the main mechanisms of resistance which are currently in the focus of public attention are the production of extended-spectrum beta-lactamases (ESBL) and plasmid-mediated AmpC beta-lactamases among Enterobacteriaceae. These enzymes have the ability to hydrolyse and thus inactivate many beta-lactam antibiotics including the extended-spectrum cephalosporins of the 3rd and 4th generation recognized as "critically important antimicrobials" by the WHO. Furthermore, these resistances are often linked with resistance to other classes of antibiotics such as fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazol (Ramos et al., 2013). As a consequence these enzymes are limiting dramatically the therapeutic options.

The first ESBL was described in a human *Klebsiella pneumoniae* isolate in 1983 in Germany (Knothe et al., 1983). However, it was as recently as 1999–2000 that the first extended-spectrum cephalosporin-resistance in livestock was reported in an AmpC-producing *Salmonella* spp. isolate in the United States (Fey et al., 2000; Winokur et al., 2000). Similar reports from *E. coli* isolates in animals soon followed from all over the world: ESBL/AmpC-producing *E. coli* were isolated among others from poultry (Costa et al., 2009; Horton et al., 2011), cattle (Geser et al., 2011; Horton et al., 2011), horses, dogs and cats (Dierikx et al., 2012). Also in pigs these resistant microorganisms seem to be widely spread: There are reports from the USA (Lutz et al., 2011), Asia (Tamang et al., 2013) and many European countries such as Spain (Escudero et al., 2010), Portugal (Goncalves et al., 2010; Ramos et al., 2013), Switzerland (Geser et al., 2011), the UK (Horton et al., 2011) and Denmark (Hansen et al., 2013). Even pork samples were tested positive for ESBL-producing Enterobacteriaceae, with *E. coli* being the predominant bacteria (Ojer-Usoz et al., 2013). This may pose a human health hazard due to a possible transfer of resistances from animals to humans via the food chain.

However, there is only little information available on the occurrence of ESBL/AmpC-producing *E. coli* in pig farms in Germany (Friese et al., 2013). Moreover, there is little known on the detection level of these bacteria in pigs in the course of one fattening period (Hansen et al., 2013). Therefore, the purpose of this study was to determine the amount and dynamics of ESBL/AmpC-producing *E. coli* in ESBL/AmpC-positive fattening pig farms. Within the national research consortium RESET a long-term investigation of seven conventional fattening pig farms in North-East and East Germany was carried out to answer this question.

Material and Methods

Pig farms

The fattening pig farms were preselected by a positive ESBL/AmpC-status of pooled faeces samples in a prior screening (Friese et al., 2013): In this screening pooled faeces samples of seven out of 16 (43.8%) fattening pig farms were tested positive for ESBL/AmpC-producing *E. coli* and these seven farms were therefore, with the consent of the farmers, chosen to be part in this study. On each farm, located in North-East and East Germany and housing between 1800 and 10 800 animals per farm, one barn with between 80 and 760 animals was selected for the samplings (Tab. 1). This barn was investigated three times within one finishing fattening period (starting after the pre-fattening period): At the beginning (one to seven days after housing), in the middle (41 to 62 days after housing) and at the end (81 to 118 days after housing) of the finishing fattening period. Only the 1st sampling of farm 7 took place in the pre-fattening pig barn one day before housing for the finishing pig barn on the same farm. The age of the pigs varied between 70 and 100 days of life for the first sampling date. Sampling was carried out from January 2011 to October 2012. A questionnaire was used to register antibiotic use and housing conditions of each farm. All farms applied the all-in/all-out system for the investigated barns. However, on farm 5 and 7 a few animals were already gone for slaughtering by the third visit. Farm 3, 4, 5 and 7 raised the animals by themselves (closed system). Farm 1 bought the pigs from one source, farm 2 from two sources and farm 6 from more than two sources. Antibiotic applications within the finishing fattening period and the pre-fattening period are shown in Table 1.

Samplings at pig farms

At each of the three samplings on the seven farms (n = 21) between 5 g (young animals) and 25 g (adult animals) of rectal faeces were taken from 20 randomly selected, healthy pigs from different areas within the

barn (n = 420). In parallel, one pooled faeces sample (n = 21) of approximately 200 g and one pooled dust sample (n = 21) of approximately 2 g were collected at each sampling. Both pooled samples were taken from at least five different spots within the barn. The pooled dust sample was taken from locations without contact to the animals such as handrails, exposed brick work and excluding window sills. Moreover, a boot swab sample (n = 21) (Finnimport, Hamburg, DE) was taken at each sampling by walking the whole length of the stable alley. In addition, four environmental swabs (n = 80) (Sarstedt AG & Co. KG, Nümbrecht, DE) were taken at each sampling date if possible from watering places, feeding troughs, walls and toys. Ten different places were sampled by swabbing a surface of approximately 15 cm^2. Therefore, dry cotton swabs (watering places and feeding troughs) or cotton swabs humidified with phosphate buffered saline (PBS) (Oxoid, Wesel, DE) (walls and toys) were used for sampling.

Laboratory analyses of animal and environmental samples

All samples were stored at 4°C and laboratory investigations were performed within 24 h. Each sample was investigated directly without a preenrichment and, in parallel, with a non-selective preenrichment procedure using Luria-Bertani (LB) broth (Merck kGaA, Darmstadt, DE). MacConkey agar plates (Oxoid, Wesel, DE), supplemented with 1 µg/ml Cefotaxime Sodium Salt (AppliChem GmbH, Darmstadt, DE) (MC+), were used for the detection of ESBL/AmpC-producing *E. coli.*

Animal samples

5/25 g faeces were inoculated in 45/225 ml LB broth and homogenised using a stomacher (260 rpm, 2 min). For quantitative analysis 100 µl of the first (faeces homogenised in LB broth), second and third 10-fold dilution of the faeces were directly transferred onto three MC+ agar plates. The agar plates and the preenrichments were aerobically incubated at 37°C for 24 h. Thereafter, *E. coli* colonies were counted and simultaneously a loop-full of the preenrichment broth was streaked on MC+ agar. After another 24 h of incubation at 37°C colonies were qualitatively analysed.

Environmental samples

Pooled faeces and dust samples were quantitatively analysed. For this, 25 g of the pooled faeces were treated in the same manner as the individual faeces described above. To analyse the dust samples 0.1 g dust was dissolved in 10 ml PBS + 0.01% TWEEN 20 and analysed quantitatively in triplicate as described above. In addition, 1 ml of dust solution was placed into 9 ml LB broth for preenrichment. After 24 h of incubation at 37°C one loop of the LB broth was streaked onto MC+ agar and incubated again under the same conditions. The boot swab samples and environmental swabs were investigated only qualitatively. The boot swab samples were inoculated in 225 ml LB broth, homogenised using the stomacher (260 rpm, 2 min) and 100 µl was streaked directly onto MC+ agar. The environmental swabs were analysed individually by streaking them directly onto MC+ agar. The swabs were then placed into 9 ml LB broth for preenrichment. The agar plates and the preenrichments were aerobically cultured at 37°C for 24 h. The preenrichments of all environmental samples were further processed as described above.

Confirmation and characterisation of ESBL/AmpC beta-lactamases in E. coli

For each sample one suspected colony with typical *E. coli* morphology was randomly selected and subcultured on sheep blood agar (Oxoid, Wesel, DE). After incubation at 37°C for 24 h the species was confirmed using MALDI-TOF identification (MALDI Microflex® LT and Biotyper® database, Bruker Daltonics, Bremen, DE). All *E. coli* isolates were tested by the CLSI disc diffusion method (CLSI M02–A11; M100–S23) for their antimicrobial susceptibility against cefotaxime (30 µg), cefoxitin (30 µg), cefepime (30 µg), ceftazidime (30 µg) and amoxicillin/clavulanic acid (30 µg) (Oxoid, Wesel, DE and Mast Diagnostica GmbH, Reinfeld, DE). ESBL production was phenotypically confirmed by the combination disc method (M100–S23) testing also cefotaxime/clavulanic acid (30 µg and 10 µg). Resistance to cefoxitin, clavulanic acid and lack of synergy with clavulanic acid was used as indicator for AmpC production. In addition, the DNA of each isolate was prepared with the Nexttec genomic DNA Isolation Kit (Biozym Scientific GmbH, Hess. Oldendorf, DE) and polymerase chain reaction (PCR) amplification was carried out to detect the beta-lactamase genes $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV} and the plasmid-mediated AmpC beta-lactamase gene bla_{CMY} as described previously (Guerra et al., 2001; Zhao et al., 2001; Weill et al., 2004; Batchelor et al., 2005) or by using protocols kindly provided by the ESBL SafefoodEra Consortium (EU-SAFEFOODERA project 08176). Sequence analysis was performed for all isolates with the genes bla_{TEM} and bla_{SHV} by using primers as described by Olesen et al. (2004) and Weill et al. (2004).

TABLE 1: *Detection frequencies of ESBL/AmpC-positive Escherichia coli (after preenrichment) in individual faeces samples of seven fattening pig farms at three stages within the fattening period*

	(%) ESBL/AmpC-positive samples per farm*							
Animals/barn	**All Farms**	**1 298**	**2 460**	**3 286**	**4 760**	**5 80**	**6 497**	**7 200**
AB PFP		**COL**	**COL/AMX**	**COL**	**COL**	**COL/AMX/CTC**	**TIA**	**COL**
1st sampling	**45 (140)**	100 (20) AMX	70 (20) AMX	30 (20)	55 (20)	0 (20)	60 (20) DOX/TIA	0 (20)
2nd sampling	**29 (140)**	10 (20)	65 (20) TLS	0 (20)	75 (20)	0 (20)	20 (20)	35 (20)
3rd sampling	**36 (140)**	25 (20)	65 (20)	0 (20)	85 (20)	10 (20)	70 (20)	0 (20)
All samplings	**37 (420)**	**45 (60)**	**67 (60)**	**10 (60)**	**72 (60)**	**3 (60)**	**50 (60)**	**12 (60)**

* Numbers in parentheses are numbers of individual faeces samples; 1st sampling = one to seven days after housing; 2nd sampling = 41 to 62 days after housing; 3rd sampling = 81 to 118 days after housing; AB PFP = Antibiotic treatment in pre-fattening period before the finishing fattening period; antibiotic treatment between samplings are listed after the numbers of samples; COL = Colistin; AMX = Amoxicillin; CTC = Chlortetracycline; TLS = Tylosin; DOX = Doxycycline; TIA = Tiamulin

The PCR products were sequenced by LGC Genomics (Berlin, DE) and the obtained sequences were analysed using the Lasergene 10 Genomic Suite (DNASTAR, Inc. Madison, WI 53705, US). All sequence data were compared to already known sequences deposited in the Lahey database (http://www.lahey.org/Studies/) and also investigated with the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). *E. coli* isolates, which were phenotypically positive for ESBL/AmpC in the combination disc test and in which ESBL/AmpC-genes were detected by PCR/sequencing, were defined as ESBL/AmpC-producers.

Statistical analyses

Statistical analyses were performed as a matter of data description only. Statistical tests used were therefore performed not simultaneously without any adjustment of multiple factors or testing. Differences were interpreted as significant if p-value < 0.05 using the Chi-square test for proportions and the Wilcoxon test for bacterial counts. The software R [version 3.0.0 (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, AT. URL http://www.R-project.org/.)] and the software SPSS Version 20 (SPSS, Inc., Chicago, IL, US) was used respectively.

Results

Animal and environmental samples

In four out of seven investigated farms ESBL/AmpC-positive *E. coli* were detected at all times of investigation in at least one sample matrix. In farm 3 the resistant microorganisms were only found at the first sampling date, in farm 5 only at the first and third sampling date and in farm 7 only at the second and third sampling date.

Of the 420 individual faeces samples taken throughout 21 visits 37% were tested positive for ESBL/AmpC-producing *E. coli* after the preenrichment step. Using this method the detection levels of ESBL/AmpC-producing *E. coli* in individual faeces samples were 45% (n = 140) for the first visit, 29% (n = 140) for the second visit and 36% (n = 140) for the third visit (Tab. 1). The detection frequency in individual faeces samples decreased significantly from the first to the second sampling (p = 0.009). The detection frequencies of ESBL/AmpC-positive *E. coli* in the individual faeces samples differed amongst the different pig farms (Tab. 1): Farm 4 showed the highest prevalence of resistant *E. coli* strains with altogether 72% positive individual faeces samples for all three samplings and also farm 2 was high-prevalent with 65–70% positive individual faeces samples during the fattening period. In contrast, farm 5, 3 and 7 showed the lowest detection rates with 10%, 30% and 35% positive individual faeces samples respectively at only one sampling date. The other two farms (1, 6) showed detection frequencies in between (Tab. 1). The geometric mean bacterial count of suspected ESBL/AmpC-producing *E. coli* was 1.42 x 10^4 cfu/g for all individual faeces samples (n = 420) (Tab. 2). The bacterial count of these pathogens decreased significantly from the 1st to the 2nd sampling and the 1st to the 3rd sampling (Wilcoxon test: p = 0.001, p = 0.000 respectively).

Also various environmental samples were tested positive for ESBL/AmpC-producing *E. coli.* Including the preenrichment step the detection frequencies were 47.6% for pooled faeces samples and boot swab samples respectively and 5.9% for the environmental swabs. None of the pooled dust samples were tested positive for ESBL/AmpC-producing *E. coli* (Tab. 3). The detection frequencies of ESBL/AmpC-positive *E. coli* in environmental samples varied similarly amongst the different pig farms as in the individual faeces samples: Considering all environmental samples together, ESBL/AmpC-producing *E. coli* were most frequently detected in the environmental samples of farm 4, and least frequently in farm 3 and 5 (Tab. 3). The geometric mean bacterial count of suspected ESBL/AmpC-producing *E. coli* was 6.46 x 10^3 cfu/g for pooled faeces samples (n = 21) (Tab. 2). The differences in bacterial counts of resistant *E. coli* in the pooled faeces samples at the three times of investigation proved to be statistically not significant (Wilcoxon test: p = 0.456).

Confirmation and characterisation of ESBL/AmpC beta-lactamases in *E. coli*

For each sample one suspected *E. coli* colony was investigated by phenotypical and genotypical methods. In total, 228 *E. coli* were analysed by these methods. Out of them, 210 (92.11%) *E. coli* showed an ESBL or AmpC phenotype (defined by the susceptibility patterns and synergy results: 186 ESBL and 24 AmpC producers). The 228 *E. coli* isolates were further examined by PCR to detect the $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and bla_{CMY} resistance genes. 179 (78.51%) isolates were phenotypically and genotypically confirmed as ESBL/AmpC-producers (Tab. 4). In the majority of isolates with an ESBL phenotype single $bla_{\text{CTX-M}}$ (110/186) or the gene combination $bla_{\text{CTX-M}}$ & $bla_{\text{TEM-1}}$ (65/186) was detected. In 1/24

TABLE 2: *Bacterial counts (cfu/g) of suspected ESBL/AmpC-producing Escherichia coli in individual faeces and pooled faeces of seven fattening pig farms at three stages within the fattening period*

	No. of suspected ESBL/AmpC-producing *E. coli* (cfu/g)								Pooled faeces (n = 7)
	All farms (n = 140)	1 (n = 20)	2 (n = 20)	3 (n = 20)	4 (n = 20)	5 (n = 20)	6 (n = 20)	7 (n = 20)	
1st sampling	2.97 x 10^4	2.49 x 10^5	7.75 x 10^4	3.65 x 10^2	3.43 x 10^2	–	6.25 x 10^2	–	7.52 x 10^3
2nd sampling	1.48 x 10^4	–	3.33 x 10^3	–	4.00 x 10^2	–	–	4.30 x 10^5	2.26 x 10^4
3rd sampling	2.17 x 10^3	4.00 x 10^3	2.90 x 10^3	–	2.48 x 10^3	–	2.67 x 10^2	1.19 x 10^3	1.51 x 10^3
All samplings	(n = 420) 0 **1.42 x 104** 2.90 x 10^6	(n = 60) 0 **2.05 x 105** 2.90 x 10^6	(n = 60) 0 **1.04 x 104** 2.90 x 10^6	(n = 60) 0 **3.65 x 102** 1.07 x 10^3	(n = 60) 0 **8.81 x 102** 7.07 x 10^4	(n = 60) – – –	(n = 60) 0 **4.71 x 102** 1.47 x 10^3	(n = 60) 0 **1.16 x 105** 1.27 x 10^6	(n = 21) 0 **6.46 x 103** 3.00 x 10^5

Bacterial counts in bold typeface = geometric mean; Last line reading top-down: minimum, geometric mean, maximum; – = negative without preenrichment; n = number of faeces samples; 1st sampling = one to seven days after housing; 2nd sampling = 41 to 62 days after housing; 3rd sampling = 81 to 118 days after housing

isolates with an AmpC phenotype the gene combination *bla*$_{CMY}$ & *bla*$_{TEM-1}$ was detected (Tab. 4).

Moreover, in two individual faeces samples of farm 2 a new *bla*$_{TEM}$ gene was detected during this study and registered in the Lahey-database as *bla*$_{TEM-206}$. Compared to the DNA sequence of *bla*$_{TEM-1}$ (genbank accession number J01749; Sutcliffe, 1978) *bla*$_{TEM-206}$ showed four mutations at the following positions: 18 (C to T), 25 (G to A), 396 (G to T) and 773 (G to T). The appropriate sequence information is stored in the NCBI database, genbank accession number KC783461. Translation of the DNA sequence using the Lasergene software package and the comparison with different *bla*$_{TEM-1}$ sequences indicated that three out of the four mutations were silent ones. Only the mutation at position 25 leads to an amino acid change within the signal peptide of the *bla*$_{TEM-206}$ protein exchange from A (alanine) to T (threonine) at position 11 (numbering according to Ambler et al., 1991). Additionally performed disc diffusion analyses confirmed that this mutation did not alter the function of the mature protein.

Discussion

The results of this study show different detection levels of ESBL/AmpC-producing *E. coli* in animal as well as housing environmental samples of the investigated ESBL/AmpC-positive fattening pig farms in Germany. ESBL/AmpC-producing *E. coli* were detected throughout the fattening period, a finding which had not been shown in other pig studies in Germany so far, but in a comparable broiler study carried out by our research group, in which even higher prevalences were detected (Laube et al., 2013). Our results show that 37% of all 420 investigated individual faeces samples were tested positive for ESBL/AmpC-producing *E. coli*. Lower prevalences in the faeces of pigs, compared to our study were reported from Portugal and Switzerland (Goncalves et al., 2010; Geser et al., 2011). Higher prevalences were found in the study of Ramos et al. (2013), where 49% of the investigated individual faeces samples at slaughter were tested positive for ESBL-producing *E. coli*. Furthermore, in our study these resistant bacteria were frequently detected in samples taken from the animals' environment. Only the dust samples were consistently negative, which is in contrast to the broiler study of Laube et al. (2013) in which even 71.4% of the dust samples were tested positive for ESBL/AmpC-producing *E. coli*. Notably is the variation of the detection frequencies of these resistant bacteria in individual faeces at the different times of investigation within the fattening period. This is in line with Hansen et al. (2013) where carriage prevalences also varied during the production cycle and implies that the point of time chosen for screenings may influence the results. In addition, in our study randomly chosen animals were sampled at every visit, which could have influenced the detection frequencies as well. The bacterial count of suspected ESBL/AmpC-producing *E. coli* in individual faeces decreased significantly during the fattening period in this study. This is in line with the results from the longitudinal study of Hansen et al. (2013), which showed a significant reduction in faecal counts of *bla*$_{CTX-M}$-producing coliforms during the production cycle of Danish pigs. Furthermore, both studies also had similar findings just before slaughter where bacterial counts were detected at their lowest. Katouli et al. (1995) showed that changes occur in the composition of the intestinal flora of pigs during the production cycle. Therefore the decrease in the prevalence and counts could be due to age-related changes in the gut microbiome, which might result in the replacement of *bla*$_{CTX-M}$-producing *E. coli* by other strains (Hansen et al., 2013). Furthermore, the results of this study show differences in the detection frequency of ESBL/AmpC-producing *E. coli* amongst the different pig farms: There are those of continuous high prevalence, those of low prevalence and other farms with prevalences in between. The high detection rate of ESBL/AmpC-producing *E. coli* in individual and pooled faeces samples as early as at the first visit shows that the pigs were colonised with these resistant bacteria already before. These results raise the question when and how the animals get "colonised" w ith these bacteria in the first place: By vertical transmission as a piglet or at a later stage in the flatdeck by horizontal transmission? Hansen et al. (2013) proved that finishers are more likely to be *bla*$_{CTX-M}$-positive if they had been positive as piglets.

The use of beta-lactam antibiotics, especially cephalosporins, might be another factor for the selection of ESBL/AmpC-producing bacteria in pigs as shown in a previous study of Jorgensen et al. (2007). Also Cavaco et al. (2008) demonstrated that certain beta-lactams, including amoxicillin, used in pig production select for *bla*$_{CTX-M}$-producing *E. coli* strains in the intestinal flora of pigs. In addition, non-beta-lactam antibiotics might play a role in the selection of beta-lactamase genes. Funk et al. (2006) showed that gram-negative isolates from pigs exposed to subtherapeutic chlortetracycline in feed were more likely to be resistant to ceftriaxone than isolates

TABLE 3: *Detection frequencies of ESBL/AmpC-producing Escherichia coli (after preenrichment) in environmental samples of 7 investigated pig farms at three stages within the fattening period*

	ESBL/AmpC-positive/negative samples at three sampling dates per farm							
	(%) ESBL/AmpC-positive samples for all farms/all samplings*	1	2	3	4	5	6	7
Pooled faeces	47.6 (21)	+ – +	+ – +	– – –	+ + +	– – –	+ – +	– + –
Boot swabs	47.6 (21)	– + –	+ + –	– – –	– + +	+ – –	– + +	– + +
Pooled dust	0 (21)	– – –	– – –	– – –	– – –	– – –	– – –	– – –
Environmental swabs	5.9 (68)	o o –	o o +	– – –	– + –	– – –	– – +	– – –

* Numbers in parentheses are numbers of samples taken from all farms; Each sign is representing one sampling date (1st, 2nd, 3rd); + = positive for ESBL/AmpC-producing *Escherichia coli*; – = negative for ESBL/AmpC-producing *Escherichia coli*; o = sample not taken or further investigation of this sample was not possible

from untreated pigs. Other studies on the other hand reported that treatment of pigs with non-beta-lactam antibiotics (chlortetracycline and tylosin) had no effect on the prevalence of antimicrobial resistance (Wagner et al., 2008). The animals in this study were treated with different antimicrobial substances (Tab. 1), especially during the pre-fattening period and thus before our samplings, which may have contributed to the ESBL/AmpC-prevalences. Amoxicillin was the only beta-lactam antibiotic, applied only in pig farms 1, 2 and 5 of our study. Farm 2 applied amoxicillin twice and had a high occurrence of ESBL/AmpC-producing *E. coli* throughout the entire fattening period as well as a high faecal count of these bacteria. Hansen et al. (2013) assume that *bla*$_{CTX-M}$-producing *E. coli* may persist in pigs for a long time in the absence of any direct selective pressure, which would explain the occurrence of these resistant *E. coli* in the farms, where no beta-lactams were applied during the pre-fattening and finishing fattening period. However, since this was not the main objective of this study, the data of this study are too limited to demonstrate a significant association between the treatment with antibiotics and the prevalence of ESBL/AmpC-producing bacteria. Further studies should be carried out to investigate the true impact of antibiotic use and the possible effect of co-selection on the occurrence of these resistant bacteria. Moreover, other risk factors for the development of resistance should be mentioned such as environmental conditions (Mathew et al., 2003), herd health conditions and farm management (Schuppers et al., 2005).

ESBL/AmpC-positive faeces contaminate the entire stable environment. The lower detection rate of these bacteria observed in the boot swabs and environmental swabs at the first visit support this hypothesis as the barns are least of all contaminated at the beginning of the fattening period just after cleaning/disinfection of the barn. A contaminated environment on the other hand promotes the transmission of these microorganisms to the potential non-colonised animals. As even environmental swabs taken from watering places were tested positive for ESBL/AmpC-producing *E. coli* in this study, pigs might ingest these bacteria with the water and/or food. Another factor which might enhance the transmission of these resistant bacteria between animals are high animal numbers in pig barns. In our study, farm 4 with 760 pigs had the by far highest number of animals per investigated barn, followed by farm 6 and 2. The lowest number had farm 5 with only 80 pigs per barn (Tab. 1). Interestingly, farm 4 followed by farm 2 and 6 was the farm with the highest occurrence and farm 5 was the farm with the lowest occurrence of ESBL/AmpC-producing *E. coli*. In this context, Costa et al. (2009) suggest that high animal concentrations within broiler flocks might facilitate the transmission of resistant bacteria among animals.

Finally, the frequent occurrence of ESBL/AmpC-producing *E. coli* in pigs might represent an issue of food safety as it has been shown that carcass contamination of an individual pig is likely due to *E. coli* originating from the pigs own faeces or cross-contamination between pigs slaughtered at the same day (Wu et al., 2009). Omisakin et al. (2003) showed for cattle that animals which are shedding high levels of *E. coli* O157 increase the potential risk of meat contamination during the slaughtering process. In addition, Horton et al. (2011) suggested that "high-density shedders of CTX-M-producing *E. coli*" (≥1 x 10^4 cfu/g) pose an increased risk for contamination of the food chain. As in our study bacterial counts of all farms were at the sampling before slaughter below the faecal counts of "high-density shedders" it might be supposed that the risk of carcass contamination by the pigs investigated in this study is lower than by "high-density shedders" (Tab. 2). However, carcass contamination represents a risk and if the meat is not thoroughly cooked, a colonization of humans with ESBL-producing *E. coli* is possible (Ramos et al., 2013).

The results revealed that out of 228 suspected ESBL/AmpC-producing *E. coli* 179 (78.5%) were ESBL/AmpC-producers as proven by combination disc test, PCR and sequencing. However, further 31 *E. coli* isolates were phenotypically positive for ESBL (n = 8) and AmpC (n = 23), but not genotypically confirmed as ESBL/AmpC-producers by PCR (Tab. 4). As in this study the occurrence of a limited number of ESBL/AmpC-genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CMY}$) was tested by PCR, it is likely that other ESBL/AmpC-genes not tested in this study are responsible for the ESBL/AmpC phenotype of these isolates. *bla*$_{CTX-M}$ was by far the most detected gene in this study, which agrees with several other comparable studies (Goncalves et al., 2010; Ramos et al., 2013). Besides the single *bla*$_{CTX-M}$ gene the gene combination *bla*$_{CTX-M}$ & *bla*$_{TEM-1}$ was most common, which corresponds to other similar studies where this gene combination was also regularly detected (Goncalves et al., 2010; Tamang et al., 2013). In contrast to our study, Escudero et al. (2010) detected the *bla*$_{SHV-12}$ gene most often in *E. coli* isolates of pigs' faeces. Moreover, in the course of this study the novel gene *bla*$_{TEM-206}$ was found. Also in isolates from this study, which showed an AmpC phenotype, but none of the *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CMY}$ resistance genes were detected (*E. coli* from a boot swab and a pooled faeces sample, and a *Salmonella* Infantis isolate of a faeces sample), the *bla*$_{VIM-1}$ carbapenemase gene was found (Fischer et al., 2012, 2013).

TABLE 4: *Confirmed ESBL/AmpC-producers (using combination disc test, PCR and sequencing) out of 228 Escherichia coli isolated from MC+ agar*

		No. of *E. coli* isolates	*E. coli* isolates (n = 228) [%]
ESBL/AmpC-phenotype (β-lactam susceptibility patterns including clavulanic acid synergy tests)	**Total**	**210**	**92.11**
	ESBL-phenotype	186	81.58
	AmpC-phenotype	24	10.53
ESBL/AmpC-genotype (PCR amplification, sequencing)	**Total**	**179**	**78.51**
	Isolates with ESBL encoding genes	178	78.07
	single *bla*$_{CTX-M}$	110	48.25
	bla$_{CTX-M}$ & *bla*$_{TEM-1}$	65	28.51
	bla$_{SHV-12}$ & *bla*$_{TEM-1}$	1	0.44
	bla$_{SHV-12}$ & *bla*$_{CTX-M}$	1	0.44
	bla$_{CTX-M}$ & *bla*$_{SHV-12}$ & *bla*$_{TEM-1}$	1	0.44
	Isolates with AmpC encoding genes (*bla*$_{CMY}$ & *bla*$_{TEM-1}$)	1	0.44

These were the first described carbapenemase-producing *E. coli* and *Salmonella* spp. isolated from pigs so far.

In conclusion, this study showed different detection frequencies of ESBL/AmpC-producing *E. coli* in different pig farms and at different times of investigation during the fattening period. Significant decreases of faecal counts were detected in the course of the fattening period. These findings provide novel information about amounts and dynamics of ESBL/AmpC-producing *Escherichia coli* in the German pig production. To assess the herd status of pigs with respect to ESBL/AmpC-producing *Escherichia coli* pooled faeces and boot swab samples but not dust samples seem to be appropriate. A potential emission of these microorganisms and consequently a contamination of the farm vicinity cannot be excluded and should be discussed. Therefore, further investigations of possible transmission and emission pathways of these resistant bacteria and the occurrence of these in the surroundings of pig farms are currently carried out by our research group.

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