Occurrence and faecal shedding of extended-spectrum beta-lactamase-producing *Escherichia coli* in sows and their offspring

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Abstract

The aim of the present study was to monitor the presence of extended-spectrum beta-lactamase (ESBL) producing *E. coli* on farm A with the history of previous use of ceftiofur in suckling pigletsand to analyse the risk factors of selection and dissemination of ESBL producers in the production herd. In the year of 2014, a total of 411 samples (rectal swabs or faeces)from pigs of various age categories (sows, gilts and suckling piglets) were collected. The sampling was performed more than 24 months after the ban of ceftiofur on the farm.The sows and gilts were sampled repeatedly before and after farrowing. All collected samples were directly cultivated on MacConkey agar (MCA) containing cefotaxime (2 mg/l) and obtained sub-cultures were tested for ESBL production by double disc synergy test. According to our results, all gilts were negative for ESBL-producing *E. coli* in the introduction period, however, the excretion of ESBL-producing *E. coli* was observed before and after delivery. Most of the new-born piglets from positive sows and gilts shed ESBL-producing *E. coli* early after birth. All tested ESBL-producing isolates were resistant to multiple antimicrobials, suggesting that antibiotics from other groups used for therapy co-select for ESBL producers in pigs on the studied farm. Intestinal colonization of lactating sows and their offspring as well as survival of ESBL-producing *E. coli* in the farm environment should be recognised as important risk factors of circulation and long-time persistence of ESBL producers in the herd.

Ceftiofur, new-born piglets, persistence, ESBL

Antimicrobial agents used for therapy and prophylaxis of bacterial infections in food-producing animals constitute the risk for selection of antimicrobial resistance in enteric commensal bacteria. *Escherichia coli* is a dominant part of normal intestinal flora in humans and animals as well as an important pathogen causing various infections. It also represents an important reservoir of antibiotic resistance determinants. The increasing prevalence of *E. coli* resistant to major antimicrobials in food-producing animals has become global public health concern (Seiffert et al. 2013).

Extended-spectrum cephalosporins, especially those of the 3rd and 4th generation, are among the most important used antimicrobial agents in human and veterinary medicine. Cephalosporins are widely prescribed against various enteric infections in farm and pet animals (Hornish and Kotarski 2002).The resistance of enteric bacteria totheir antimicrobials is mostly caused by the bacterial production of extended-spectrum beta-lactamases (ESBL) or AmpC beta-lactamases (Smet et al. 2010). These enzymes are classified into several groups, among which CTX-M enzymes are the most prevalent, successively disseminating in animals and humans all over the world (D'Andrea et al. 2013). ESBL-producing organisms exhibit resistance to multiple antibiotics apart from beta-lactams, thus the antimicrobial agents of various groups used for the therapy are able to co-select the ESBL producers (Pitout and Laupland 2008).

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ESBL-producing *E. coli* isolates have been increasingly documented in pig farms in Europe (EFSA 2011). One of the main factors for the selection and spread of ESBL-producing *E. coli* among food-producing animals is the excessive use of cephalosporins. Several studies proved a link between the use of 3rd and 4th generation cephalosporins and selection of ESBL-producing *E. coli* in poultry (Dutil et al. 2010), cows (Tragesser et al. 2006; Dolejska et al. 2011) and pigs (Jørgensen et al. 2007; Lutz et al. 2011; Agersø et al. 2012; Andersen et al. 2015). The occurrence of ESBL-producing commensal *E. coli* in swine is alarming due to the potential risk of transfer of antibiotic resistance determinants to human microbiome via the food chain (Denkel et al. 2016). Increasing prevalence of ESBL-producing *E. coli* led to a voluntary ban or significant reduction of cephalosporin use in pig production in some European countries (EMA 2015).

The aim of the study was to evaluate the circulation of ESBL-producing *E. coli* isolates in sows and their piglets on farm A two years after a voluntary withdrawal of ceftiofur that had been used as part of the control program against *Streptococcus suis* in sucking piglets, and to determine the occurrence of ESBL-producing *E. coli* in gilts originating from a multiplier herd on farm B where cephalosporins had never been used. The colonization by ESBL producers was monitored before and after their introduction into the herd on farm A.

Materials and Methods

Farm description, study design and sampling

Farm A is a conventional farm with one site operation housing 1300 sows in 2014. The use of ceftiofur for the metaphylactic purposes was banned on the farm in December 2012, more than 24 months before this study was conducted. Antimicrobials used on the farm included tiamulin, amoxicillin and a combined therapy by macrolides (tilmicosin, aivlosin) and chlortetracycline.

Rectal swabs or faeces were taken from sows, gilts and piglets. All samples were refrigerated and analysed in the laboratory within 24 h.

Rectal swabs from a total of 100 pregnant sows of second and higher parity (farm A) were taken a few days before farrowing. For sows that were positive for ESBL-producing *E. coli*, samples were also taken after the farrowing or after 7 days. For that purpose, faeces samples were collected in order to quantify the excretion of ESBL-producing *E. coli*. Samples were taken from litters of the positive sows; in total, rectal swabs of 72 piglets aged one to eight days were examined.

Gilts originating from a multiplier herd at farm B with no history of cephalosporins were sampled before and after their introduction into the sow herd at farm A. Rectal swabs from 110 gilts during the isolation period on farm A were collected. Thirty-five randomly selected pregnant gilts were sampled approximately 107 days after their introduction to the herd, a few days before farrowing. Gilts that were found positive for ESBL-producing *E. coli* were sampled also after the farrowing and tested for excretion of ESBL-producing *E. coli*. Rectal swabs were taken from 55 randomly selected one to seven-days old suckling piglets of the positive gilts.

Isolation of ESBL-producing *E. coli*

The samples of rectal swabs were cultivated directly on MacConkey agar (Oxoid, Basingstoke, UK) supplemented with cefotaxime (Sigma-Aldrich, Prague, Czech Republic) at a concentration of 2 mg/l (MCA_{CEF}). Species identification of one presumptive colony per sample per bacteria of interest was made using the Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) system (Bruker Daltonics, Brehmen, Germany). Mass spectra were estimated using the software FlexControl–microflex v 3.3 (Bruker Daltonics, Brehmen, Germany) and MaldiBiotyper v 3.0 (Bruker Daltonics, Brehmen, Germany). The resistant colonies identified as *E. coli* were tested for production of ESBL using double-disc synergy test (CLSI 2008). The ESBL-producing strain *E. coli* 172DI/B was used as a positive control.

Faeces samples from sows and gilts were first processed semi-quantitatively to estimate the counts of cefotaxime-resistant coliform. They were inoculated on MCA_{CEF} using a 1-µl disposable plastic loop by the streak plate method into four quadrants. The loop was discarded between each sector. Quantification was expressed as 1+, 2+, 3+, 4+ or 5+ based on the number of quadrants that demonstrated bacterial growth. Growth of a few isolated colonies limited to quadrant 1 was categorized as 1+, growth of many colonies limited to quadrant1 was categorized as 2+, growth limited to quadrants 1 and 2 was categorized as 3+, growth in quadrants 1, 2 and 3 was categorized as 4+ and bacterial growth that extended to all 4 quadrants as 5+ (Van Saene et al. 2012). Viable counts of cefotaxime-resistant coliforms in randomly selected faeces samples were determined. First, 1 g of faeces was diluted 10 × in buffered peptone water, followed by homogenization of the sample by vigorous vortexing. These 10^{-1} homogenates were again diluted 10 × (10^{-2} dilution) and 100 × (10^{-3} dilution) using peptone saline,

and 100 µl of all three dilutions were streaked on MCA_{CEF}. Cefotaxime-resistant lactose-positive colonies were counted to determine mean bacterial densities expressed as CFU/g.

Antimicrobial susceptibility testing

Selected ESBL-producing *E. coli* isolates were further tested for antimicrobial susceptibility using the disk diffusion method and the following discs according to CLSI criteria (CLSI 2008): amoxicillin/clavulanic acid (AMC; 30 µg), ampicillin (AMP; 10 µg), cefoxitin (FOX; 30 µg), ceftazidime (CAZ; 30 µg), cephalothin (CEP; 30 µg), chloramphenicol (CHL; 30 µg), ciprofloxacin (CIP; 5 µg), gentamicin (GEN; 10 µg), imipenem (IMP; 10 µg), nalidixic acid (NA; 30 µg), streptomycin (STR; 10 µg), trimethoprim-sulphamethoxazole (SXT; 25 µg), compound sulphonamides (S3; 300 µg) and tetracycline (TCY; 30 µg) (Oxoid, Basingstoke, UK). We used CLSI susceptibility testing guideline breakpoints to categorize isolates into susceptible and resistant category (CLSI 2008).*Escherichia coli* ATCC25922 (American Type Culture Collection) was used for quality control of the disk diffusion method.

Results

ESBL-producing *E. coli* isolates were found in rectal swabs of 25 (25%, n=100) pregnant sows. The sows that were found positive were sampled repeatedly shortly after the delivery and ESBL-producing *E. coli* isolates were identified in 96% animals in counts ranging from 10^1 to 10^6 CFU (mean 10^3) per gram of faeces. Litters of 16 positive sows were sampled and ESBL-producing *E. coli* isolates were found in 15 of them (Table 1). Faeces samples were collected from three to 11 piglets per litter, in total 72 piglets aged one to seven days were examined. ESBL producers were isolated from 45 (63%, n = 72) new-born piglets. Eight litters were excluded from the study because of the presence of cross-fostering piglets.

Gilts (farm B with no history of cephalosporins) examined during the isolation period did not carry ESBL producers. However, during the second sampling shortly before the farrowing and approximately three months after their introduction into the herdon farm A, ESBL-producing *E. coli* isolates were found in 12 (34%, n = 35) animals. Most gilts (11 out of 12) excreted ESBL producers in counts ranging from 10^3 to 10^5 CFU (mean 10^5) per gram of faeces (Table 1). ESBL producers were detected in all but one litters of the positive gilts. Five to seven piglets per litter were examined and a total of 42 (76%, n = 55) piglets carried ESBL-producing *E. coli*. Two litters were excluded because of cross-fostering piglets.

The results of antibiotic susceptibility testing of 107 randomly selected ESBL-producing *E. coli* are summarized in Table 2. Resistance to ampicillin and cephalotin associated with ESBL phenotype was found in all isolates. Majority of the isolates (94.3%) showed resistance to streptomycin followed by resistance to sulphonamides (82.2%), tetracyclines (80.4%), trimethoprim-sulphamethoxazole (53.3%) and chloramphenicol (45.8%). Resistance to amoxicillin/clavulanic acid, cephalosporins (cefoxitin, ceftazidime, cephalothin), ciprofloxacin, gentamicin and imipenem was not found. All isolates showed multi-resistance profile defined as resistance to two and more substances from different antimicrobial groups. Resistance to four antimicrobial agents including streptomycin, sulphonamides, tetracycline and chloramphenicol was the most common type identified in 23.3% isolates. The second most prevalent phenotype was resistance to substances from five antimicrobial groups (streptomycin, sulphonamides, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol) detected in 19.6% ESBL-producing *E. coli* isolates. No significant differences in resistance profiles and prevalence among isolates from sows, gilts and their piglets were observed (data not shown).

Discussion

The prophylactic and therapeutic use of antibiotics is known to be the cause of the high prevalence and long-term persistence of resistant strains of *E. coli* in food-producing

Table 1. Excretion of ESBL-producing *E. coli* in faeces of positive sows and their offspring.

Category	No. of sow	Counts in faeces after farrowing*		Piglets positive/examined
		Streak plating method	CFU/g faeces	
Sows	1	negative	10^1	NT
	2	1+	10^1	6/11
	3	1+	10^2	10/10
	4	1+	10^1	NT
	5	1+	10^1	NT
	6	1+	nd	0/8
	7	1+	10^2	1/8
	8	1+	10^2	NT
	9	1+	10^2	NT
	10	1+	10^3	2/3
	11	2+	10^3	NT
	12	2+	10^2	NT
	13	1+	10^2	2/3
	14	1+	10^3	2/3
	15	2+	10^3	2/3
	16	2+	10^2	2/3
	17	1+	10^3	NT
	18	2+	10^5	3/3
	19	4+	10^1	3/3
	20	1+	10^6	2/3
	21	4+	10^5	2/3
	22	4+	nd	2/3
	23	3+	10^5	3/3
	24	4+	10^4	3/3
	25	3+	10^3	NT
Gilts	1	3+	10^4	NT
	2	3+	10^4	1/7
	3	negative	10^3	5/6
	4	3+	10^4	4/6
	5	5+	10^4	2/6
	6	3+	10^4	5/5
	7	3+	10^4	5/5
	8	5+	10^5	5/5
	9	5+	10^3	NT
	10	3+	10^3	5/5
	11	5+	10^5	5/5
	12	3+	10^4	5/5

*Bacterial growth on MCA_{CEF} using the streak plating method was determined as follows: Growth of a few isolated colonies only in quadrant 1 was categorized as 1+, growth of many colonies limited to quadrant 1 was categorized as 2+, growth limited to quadrants 1 and 2 was categorized. as 3+, growth in quadrants 1, 2 and 3 as 4+ and bacterial growth that extended to all 4 quadrants was categorized as 5+; nd, not determined.
[+]NT, litter was not tested because of cross fostering piglets.

Table 2. Resistance phenotypes to selected antimicrobials in ESBL-producing *E. coli* from pigs.

Resistance phenotype	No. of isolates	Resistance to antimicrobials					
		S	S3	TCY	SXT	CHL	NA
1	21	■	■	■	■	■	
2	16	■	■	■	■		■
3	1	■	■	■	■	■	■
4	2	■	■	■	■		
5	7	■	■	■			■
6	1	■	■	■		■	
7	25	■		■			
8	10	■	■		■		
9	12	■	■				
10	2	■	■				■
11	3	■		■	■		
12	1	■	■			■	
13	4	■					
14	1	■			■		
15	1				■	■	
Total	107	106	88	86	57	49	20

Resistance is indicated by black boxes; S - streptomycin; S3 - suplhonamide compounds; TCY - tetracyclines; SXT - trimethoprim-suphamethoxazole; CHL - chloramphenicol; NA -nalidixic acid

animals. Various levels of ESBL producers have been documented in the pig production system mainly due to the differences in antibiotic policies on farms, including the use of higher generation cephalosporins. Cavaco et al. (2008) proved in their experimental study in pigs that cephalosporins (ceftiofur and cefquinom) have a high selective effect on ESBL-producing *E. coli*. In Denmark, ESBL-producing *E. coli* in pigs was detected on 79% of the farms with high consumption of cephalosporins compared to 20% on farms with no consumption of cephalosporins (Hammerum et al. 2014). In our study, ESBL-producing *E. coli* isolates were found in 25% sows and 34% gilts despite the fact that cephalosporins were banned on farm Amore than 24 months prior to the study. In Denmark, a significant effect was recognized on reduction of ESBL-producing *E. coli* prevalence on pig farms, in slaughtered pigs as well as pork meat approximately six months after the ban of cephalosporin use in pig production in the year of 2010 (Agersø et al. 2012; Agersø and Aarestrup 2013).

Similar to our study, other authors have also reported that the withdrawal of cephalosporins from pig farms did not lead to the immediate reduction of resistant *E. coli* in the respective herds (Andersen et al. 2015). High prevalence of CTX-M-producing *E. coli* was demonstrated in up to 71% sows and 97% piglets on Danish farms one year after the ban of cephalosporins (Hansen et al. 2013). However, these were farms with the history of high-level consumption of cephalosporins. It has been proposed that factors other than cephalosporins are likely involved in the persistence of ESBL producers on the farm. These may include the use of antibiotic from other groups, purchase of pigs from herds with history of cephalosporin use or contamination of feed, water and sludge by ESBL-producing isolates (Marshall and Levy 2011). Andersen et al. (2015) documented that the use of antimicrobials other than cephalosporins on pig farms is associated with higher probability of finding ESBL-producing *E. coli*. This association may be a result of co-selection by antimicrobials from other groups, since most of the isolates obtained

from both sows and piglets in our study showed multi-resistance profiles. Resistances to tetracyclines and sulphonamides were the most common, including the combination with trimethoprim. These antimicrobials are indicated for the therapy of various bacterial infections in sows or piglets and may have resulted in co-selection and maintenance of ESBL-producing *E. coli* in the herd. Genes associated with ESBL phenotype in *E. coli* are often carried by transferrable plasmids along with genetic determinants conferring resistance to the above mentioned antimicrobials, therefore the use of multiple antimicrobial agents main impact the maintenance of ESBL plasmids (Carattoli 2013).

In contrast to other studies that were mainly focused on ESBL isolates in the intestine of slaughtered pigs, here we document ESBL-producing *E. coli* in a sow herd including their offspring. Our results indicate the circulation and long-term persistence of ESBL-producing *E. coli* in the herd even in the absence of selective pressure of cephalosporins. ESBL-producing isolates were likely selected in piglets treated by ceftiofur prior to the withdrawal followed by the transferand establishment in the gut microbiota of sows. Gilts originating from the multiplying herd on farm B with no history of cephalosporins were proved to be free of ESBL-producing isolates before their introduction to the herd on farm A. ESBL-producing isolates were found in rectal swabs of gilts three months after their introduction as well as in their faeces shortly after the farrowing. Our results indicate that sows of higher parity were the source of ESBL isolates not only for their offspring but also for the newly introduced gilts.

Interestingly, ESBL-producing *E. coli* were found in higher faecal densities in the introduced gilts compared to sows of second or higher parity. Moreover, most offspring of gilts and sows shed ESBL-producing *E. coli,* suggesting that the colonized dams and the farm environment contaminated by their faeces are plausible sources of these strains for the piglets. We assume that the colonization of the gut of new-born piglets by ESBL-producing *E. coli* happens shortly after the farrowing since these strains were found in the intestine of 12-hour-old offspring. Hansen et al. (2013, 2014) reported significant changes in carriage proportion, faecal counts and diversity of ESBL-producing *E. coli* in pigs during the production cycle. Reduction of prevalence of ESBL-producing *E. coli* accompanied by the decrease of mean faecal counts and strain diversity from piglets to finishers was observed. They suggested that observed differences reflect age-related physiological changes in the pig intestinal tract and as well as management procedures such as change in diet and antimicrobial treatment that are specific for each production stage.

In conclusion, results of this study showed long-term persistence of ESBL-producing *E. coli* in sow herd despite the ban of ceftiofur. Intestinal colonization of milking sows of the second and higher parity and their offspring as well as survivability of ESBL-producing *E. coli* in the farm environment should be recognised as important risk factors of their circulation and long-term persistence in pig production system.

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