Presence of CTX-M-1 group extended-spectrum-β-lactamase in dust from Dutch pig farms

Research Project Veterinary Medicine University of Utrecht

A. H. P. Hoffmann

Student number 3515494

December 2013 – April 2014

Supervisor:

Heike Schmitt

Institute for Risk Assessment Sciences,

Faculty of Veterinary Medicine, University of Utrecht

Abstract

A variety of plasmid-mediated β-lactamases have emerged in gram-negative bacteria in the last ten years, including extended-spectrum β-lactamases (ESBLs). ESBLs are the most influential resistance genes for cephalosporin resistance in *Enterobacteriaceae*. They represent a public health treat as they limit the choice of effective antimicrobial agents for human treatment, thereby increasing morbidity, mortality, longer hospital stays and higher costs. Among *Enterobacteriaceae* in livestock CTX-M is the most common ESBL type, and the subtype CTX-M-1 is most common in pigs in Europe. The most relevant human exposure route has not yet been identified. Next to, one possible exposure route could be dust. CTX-M has been already demonstrated in dust from broiler stables, but not in dust from pig stables. The purpose of this study is to determine whether CTX-M-1 genes can be detected in dust, and if so, how strongly EDC CTX-M-1 positive farms are associated with CTX-M-1 positive pig feces farms. In addition, the relevance of CTX-M-1 positive dust as exposure route for humans is evaluated by use of the results from individual human feces cultures. 123 compartment electrostatic dust fall collector (EDC) samples and 34 household EDC samples from fourty different farms were used in this study. After stomaching, freeze drying and DNA extraction, qPCR and gel electrophoresis, ten farms and one household were tested positive. Pig feces culture of these farms shows that twelve farms tested positive by culture and ten farms tested positive by PCR. CTX-M-1 positive EDCs were significantly more often found in farms in which feces was CTX-M-1 positive. Odds ratios of this association were 11.67 (2.22 – 61.28) for culture-positive feces, and 56.00 (6.78 – 462.66) for PCR-positive feces. Similar results were obtained when limiting the analysis to compartments in which both EDC and feces were analyzed. There was also a significant association between CTX-M-1 positive feces from farm inhabitants and farms with positive dust samples (odds ratio 25.96 (2.89 – 233.59)). Thus, an inhalable exposure route seems to be possible. On the other side, the association between CTX-M-1 positive feces from farm inhabitants and farms with positive pig feces samples was also significant (odds ratio 17.41 (1.96 – 155.04)), it is difficult to disentangle the role of direct animal contact from the role of airborne exposure. Further research is warranted to gain more insight into airborne exposure to ESBL.

Table of contents

1. Introduction

A variety of plasmid-mediated β-lactamases have emerged in gram-negative bacteria the last ten years. One of these includes extended-spectrum β-lactamases (ESBLs) (Liebana et al. 2013). ESBLs are the most influential mechanism for cephalosporin resistance in *Enterobactericeae*, particularly *Escherichia coli (E.coli)* and *Klebsiella pneumonia (K. pneumonia)* (Zhao and Hu 2013). ESBL producing *Enterobacteriaceae* give resistance to a variety of β-lactams, including penicillins, first-, second-, third-, and fourth generation cephalosporins, and monobactams by hydrolysis of the antibiotics (Liebana et al. 2013; Paterson and Bonomo 2005; Seiffert et al. 2013).

Reports from the European Antibiotic Resistance Surveillance System show that in hospitals in Europe, the rate of invasive *E. coli* and *K. pneumonia* isolates resistant to cefotaxime and ceftazidime (both cephalosporin's) has been increasing since 2000 (European Centre for Disease Prevention and Control 2011). They represent a public health treat because the choice of effective antimicrobial agents for human treatment is limited, increasing morbidity, mortality, longer hospital stays and higher costs (Liebana et al. 2013). This resistance is especially seen in clinical conditions. Nonclinical carries are mainly symptomless, but carriage can become problematic when developing into an infection.

Up to 1990, most ESBL producing *Enterobacteriaceae* identified in humans were of SHV- and TEM types. Currently, the CTX-M enzymes have become the most prevalent type of ESBL producing *Enterobacteriaceae* (Seiffert et al. 2013), particularly in *E. coli, K. pneumonia* and *P. mirabilis* (Zhao and Hu 2013). Nowadays, there are more than 110 different CTX-M-type ESBL producing *Enterobacteriaceae* recognized (Zheng et al. 2012) and they have been detected in at least 26 bacterial species. Although the dominant variants of CTX-Ms are geographically different, CTX-M-15 and CTX-M-14 are the most common variants detected worldwide in clinically important pathogens, followed by CTX-M-2, CTX-M-3 and CTX-M-1 (Zhao and Hu 2013). Conjugative plasmid-mediated horizontal transfer and clonal spread have contributed to their increased prevalence and have facilitated their transfer among different hosts around the world (Zhao and Hu 2013; Zheng et al. 2012).

Though the first ESBL producing *Enterobacteriaceae* in humans were identified during a *K. pneumonia* outbreak in German in 1982, one of the first clinical ESBL producing *Enterobacteriaceae* observations in animals dates back to 2000 when a SHV-12 producing *E. coli* isolate was found in a dog. The earliest description of poultry as carriers of ESBL producing *Enterobacteriaceae* dates from 2000 and 2001: CTX-M-14, SHV-12 and CMY-2 producing *E. coli* was found in feces of healthy broilers. Studies about the occurrence of various ESBL producing *Enterobacteriaceae* types in pigs and cattle followed soon. Less attention had been paid to the occurrence of ESBL-producing bacteria in companion animals for a long time, but recent investigations found ESBL producing *Enterobacteriaceae* especially in dogs, cats and horses. Initial data about the prevalence, in particular, of ESBL producing *E. coli* from companion animals indicate high carriage and infection rates. Diseased as well as healthy animals are affected, raising new animal welfare and public health issues (Ewers et al. 2012).

The total number of articles about the commensal colonization of extended-spectrum cephalosporin resistant gram negative organisms (ESC-R-GNO) in livestock increased exponentially in the last five years. According to the most recent European Food Safety Authority (EFSA) survey, the overall prevalence of extended-spectrum cephalosporin resistant *E.coli* (ESC-R-E) in pigs in the EU was 2.3%, with a range of 0% (Denmark and Estonia) to 3.8% (Hungary and Poland) in 2009 and these strains

were found with low prevalence (range 0.6-1.2%) in Austria, Denmark, France, Italy, the Netherlands, and Spain in 2007. Extended-spectrum cephalosporin resistant *Salmonella* (ESC-R-Sal) were only reported in Spain and Germany in 2009 (prevalence of 1% and 2%, respectively), while in 2007 this strains were also reported in Estonia (5.3%), Ireland (1.5%) and Italy (0.7%). Research about the prevalence in cattle and poultry show a similar increasing trend for cephalosporin resistance over the last few years in which most reports of ESC-R-*Enterobacteriaceae* (ESC-R-Ent) from poultry (Seiffert et al. 2013). The MARAN report of 2012 gives an indication about the prevalence of ESBL-producing *E.coli* in flocks in the Netherlands in 2011. At Dutch slaughterhouses fecal samples were taken from 100 flocks of slaughter pigs. This study reported that 68% of the flocks were positive for ESBL producing *E.coli* (MARAN 2012 2012).

Looking more specifically into the types of ESBL producing *Enterobacteriaceae* in food producing-animals, the most frequent encountered are the CTX-M-types (Seiffert et al. 2013). Eleven different subtypes of the CTX-M type have been described in food producing animals and food in Europe, in which CTX-M-1 currently is the most common (Liebana et al. 2013). In Europe, CTX-M-1 represented 28% of all ESBL producing *E.coli* types in companion animals, 28% in poultry and 72% in cattle/pigs, and this gene rarely reported in other regions and habitats. CTX-M-14 is less prevalent in livestock (4-7%) in Europe, and is absent in companion animals. CTX-M-15 was incidentally detected in poultry in Europe, while companion animals (15%) and cattle/pigs (8%) are frequently associated with this type (Ewers et al. 2012). Also SHV ESBL producing *Enterobacteriaceae*, in particular containing SHV-12 and SHV-2, have been frequently detected throughout the European Union. In addition, the most frequently detected TEM ESBL type is TEM-52, while TEM-20 is less common (Liebana et al. 2013).

While research in poultry has shown that farmers have a higher risk for ESBL producing *E.coli* carriage, the most relevant occupational exposure routes have not yet been identified (Dierikx et al. 2013). For example, it is unknown to what extent these germs can give a risk for humans trough inhalable exposure. Research has shown that modern pig farming in closed stable environments with high animal densities can create poor indoor air quality. High animal densities are typical in modern swine production leading to an increase in the load of airborne contaminants. These, in turn, can affect animal health detrimentally, and can represent occupational health risks (Chien et al. 2011; Duan et al. 2009; Kristiansen et al. 2012). It is known that microbial degradation of fecal matter and urine causes increased concentrations of volatile organic compounds, ammonia, and sulfides, while feed materials, epithelia cells, and dried feces create dust that carries absorbed microorganisms, viruses, endotoxins and mycotoxins. The most common cultured microorganisms in pig stable environments are gram-positive bacteria, which are dominated by *Staphylococcus*, *Micrococcus*, *Aerococcus* and *Enterococcus*. In addition, many fungal species are also detected in pig stable bioaerosols (Kristiansen et al. 2012).

The airborne spread of E. coli was studied in detail in Yuan et al, 2010. It was found that fecal *E. coli* could be aerosolized and spread to the outdoor air, especially downwind of the pig houses. They suggest that ambient air outside pig houses could be a threat to the health of neighboring human inhabitants (Yuan et al. 2010). In addition, research from 2005 gives information about the risk of exposure for humans for multi-drug resistant *Enterococcus*. In this study *Enterococcus*, coagulase-negative staphylococci, and viridans group streptococci were detected in the air of a concentrated swine feeding operation. This study suggests that the inhalation of air from these facilities may serve as an exposure pathway for the transfer of multidrug-resistant bacterial pathogens from swine to humans (Chapin et al. 2005).

Analysis of airborne inhalable dust is the most direct approach to study inhalable exposure. However, such measurements represent only the conditions during the measurement day. Settled dust may represent exposure over a longer time period and includes day to day variation in indoor activities and ventilation. Earlier research shows correlations between airborne dust and settled dust and gave reason to consider settled dust as a surrogate for inhalable microbial exposure (Frankel et al. 2012). It is possible to use electrostatic dust fall collectors (EDCs) to collect settled dust. An EDC consists of a custom-fabricated polypropylene sampler that has four electrostatic cloths attached to it to provide a sampling surface. Airborne dust settles on this surface and is captured by the electrostatic properties of the cloth. Benefits of EDCs are their easy usage, the fact that they can be sent by mail, their competitive price, their reproducibility and their general reliability as methods for the collection of airborne dust (Noss et al. 2008). In addition, it is a potential alternative to or a complement for vacuum dust sampling in large-scale epidemiological studies, because an assessment of aerial microbial diversity in a large sample of farms and stables is difficult to perform (Normand et al. 2009; Noss et al. 2008).

Up till now studies about the prevalence of ESBL producing *Enterobacteriaceae* genes in dust are rare. Laube et al, 2013, demonstrated CTX-M in *E.coli* in dust from broiler stables (Laube et al. 2013), but the abundance of CTX-M-1 in dust from pig farms is not known. The purpose of this study is to determine whether the most frequently occurring ESBL group in pigs, CTX-M-1, can also be detected in dust, and if so, how strongly CTX-M-1 positive EDC farms are associated with CTX-M-1 positive pig feces farms. In addition, the relevance of CTX-M-1 positive dust as exposure route for humans will be evaluated by use of the results from positive individual human feces samples by culture and CTX-M-1 positive EDCs from stables. For this, data from an earlier research will be used (results human feces culture and pig feces culture and PCR of pig feces samples, all from the same farms as used for the EDCs).

2. Material and methods

2.1 Sample collection

The samples used for this study come from the Bactopath intervention study. This study specifically looked at the effect of intervention measures on the presence of methicillin resistance *staphylococcus aureus* (MRSA) in animals and humans, by analyzing the farms before (t=0) and after interventions (t=6, 12, and 18 months). It also examined if antibiotic-resistant bacteria can be found in the environment. For this reason, EDCs were deposited for a period of two weeks in different compartments from stables on pig farms and households on this farms. These samples (t=0) are used in this study. The samples were collected in the period of March 2011 up to and including November 2011 and stored at -80°C. A total of fourty different pig farms participated in this study in which originally four EDC samples were taken from different compartments on each farm and one EDC sample was taken from the household on each farm. In total there are 123 compartment samples and 34 house samples collected (some samples failed for different reasons).
The Bactopath intervention study contains data on the presence of CTX-M-1 in pig feces from different compartments from these farms and in feces samples from humans who lived on these farms (both pig and human feces were analyzed by culture and PCR). Ten pens are sampled in different compartments for each farm, and for each pen sampled, feces from six animals was mixed. The people gathered their own feces sample after a short instruction. These results are also included.

2.2 Stomaching EDCs

It started with a step of stomaching to remove dust from the EDCs. For this, the EDC was transferred to a stomacher bag and 10 ml pyrogenfree water + 0.05% Tween20 was added to the bag. The bag was placed for 10 minutes in the stomacher. The EDC was squeezed and the remaining fluid was removed with a sterile pipet and transferred to a 50 ml Greiner tube. Then the bag was replenished by 10 ml pyrogenfree water + 0.05% Tween20 and placed in the stomacher for a second time for 10 minutes and after that the remaining fluid was transferred to the same Greiner tube as described above. The Greiner tube with fluid was stored over night at -80°C. Next to the samples of the farms, ten EDC samples taken from different compartments of the experimental farm of the Faculty of Veterinary Medicine and fourteen blanco EDCs were extracted. This gave a total of 181 samples.

2.3 Freeze drying samples

The samples were freeze-dried to remove the extraction liquid. Therefore the lid of the 50 ml Greiner tubes was a little bit unscrewed before placing the samples in the freeze dryer. The samples remained for 3-4 days in the freeze dryer and were then stored at -80°C until DNA isolation.

2.4 DNA extraction

DNA was extracted from the dust samples using the Machery-Nagel NucleoSpin® 8 Plant II kit and according the protocol of the kit. Small changes were made in this protocol. First, a maximum of 40 mg of dust was transferred to a beat-beating tube with 500 mg glass beads in order to prevent overloading the kit. When the dust could not be weighed due to very small amounts, the whole Greiner tube was taken to the next step. Cell lysis was accomplished by adding 500 µl PL1 and 10 µl RNAse A in samples with an amount of dust less than 20 mg, and 1000 µl PL1 and 20 µl RNAse A in samples with an amount of dust between 20 and 40 mg. This was added to the Greiner tube or the bead beating tube depending in which the sample was. The lysate in the Greiner tubes was transferred to a bead beating tube (with 500 mg glass beads) when all the dust was resolved. The samples were bead beaten for 45 seconds at maximum speed (6.0 g) and were then heated for 30 minutes at 65°C. Then a lysate was created by centrifugation of the bead beating tubes for 20 minutes at 6000 rfc. To adjust the binding conditions the lysate was pipetted from the samples in an MN square-well Block and the same amount as the lysate of Binding Buffer PC was added in the square-well block in the sample and was mixed by pipetting it three times. Afterwards, the lysate was transferred to the binding strips. Then the DNA was bound to a silica membrane with vacuum -0.2 to -0.4 bar. After this step the silica membranes were washed by adding 400 µl PW1 and vacuum -0.4 bar. This step is repeated for samples at the baseline when there was more than 20 mg dust. After that 700 µl PW2 was added to the samples and vacuum -0.4 bar was turned on. This step was done twice for all samples. The vacuum step took one minute each time. After washing the membranes they were dried for 10 minutes by vacuum. Hereafter DNA was eluted with 100 µl prewarmed buffer PE, incubated 2 minutes at room temperature and then vacuum -0.4 bar for 2 minutes was turned on. At the end 20 µl aliquots were made and stored together with the current samples at -80°C (the complete protocol is added to the Annex). In total, there was roughly extracted 40 µl DNA per sample.

2.5 qPCR

Before starting with the samples, experimental PCRs had shown that a solution of 1:100 for samples from stables and 1:10 for household samples gave the most reliable results due to inhibition by a 1:10 dilution for stable samples (Ct higher than 3.3). For this reason the samples were diluted in these proportions. Master mixes for qPCR were prepared in the flow cabinet within the PCR lab. The total amount of mastermix needed was calculated using a pre-made Microsoft Excel file. The total volume per reaction was 10 µl and this contained 1.4 µl MQ water; 0.30 µl of RV and FW primer with a target concentration of 300 nM; 5 µl of SYBR-Green mix and 3 µl extracted DNA. In this lab 7 µl of

mastermix was loaded onto a 384 wells plate. After this, 3 µl of each DNA sample was added to the wells in triplicate in the DNA lab. qPCR was performed with the Bio-Rad CFX manager and a specific primer set and protocol were used shown in Table 1.

Two 384 wells plates were used for all the 171 samples. At this moment, there were no longer 181 samples, because ten samples from the experimental farm from the Faculty of Veterinary Medicine were used for small experiments for the optimization for this research. At the place of these samples NTCs (MQ from PCR lab) were used. A set of diluted standards were added to each PCR. These had a known concentration of the gene of interest and were used to quantify the amount of genes of interest in the DNA samples. The calibration curve of CTX-M-1 was obtained from a plasmid extraction of the CTX-M-1 PCR product of an ESBL producing *Enterobacteriaceae* positive strain (*E. coli 38.52,* kindly provided by C. Dierikx), cloned into pGEM-TEasy. Standard curves were determined in CFX software and further calculations were performed using Microsoft Excel to determine the amount of gene copies per sample.

Primer set	Sequence	Annealing	Protocol
CTX-M-1 fw CTX-M-1 rv	GCG-TGA-TAC-CAC-TTC-ACC-TC TGA-AGT-AAG-TGA-CCA-GAA-TC	57.5°C	3 min 95; 40 x 95 15 sec; ann 60 sec (Xu et al. 2005)

Table 1: Properties of used primer set.

2.6 Verification of PCR products

Melting curves and an agarose gel were used to identify the PCR product. Samples with one or more positive amplification curve and melt peak in the range 87.0-88.0°C (the expected temperature for the peaks was 87.5) were repeated with PCR (this includes the samples who were in triple positive). In addition, samples from plate one in which amplification was below the threshold line, but the amplification curves in at least two from three PCR replicates increased at the end, were also repeated. This was done because plate one had on average a one to two point higher Ct than plate two. The dilution used for these samples was 1:10 and 1:100 for samples from stables and 1:10 for household samples and these DNA samples were added in triplicate in the wells plate. For the repeated PCR new CTX-M-1 FW and RV primers were used, new dilutions of stable and household samples were made due to an insufficient amount of remaining dilutions and a standard curve and thirteen NTCs in triple were applied.
From all the DNA samples in the last PCR plate, one PCR replicate with a good amplification curve and a correct melt peak was selected. These PCR products were verified on an agarose gel in TBE buffer. A 2% agarose gel was used and 2 µl loading dye and 5 µl PCR product was loaded onto the gel. In six wells Gene Ruler 50 bp SM0371 was added instead of DNA. The gel ran for 2 hours at 100 Volt and was stained with a solution with 2 µl SybrGold and 50 ml TBE buffer in a plastic fold for 30 minutes on an orbital shaker and were protected from light. Pictures were taken from the gel using UV-light.

2.7 Data analysis

Bio-Rad CFX software was used to process the raw data from the qPCR and this data was exported to Microsoft Excel for further analysis. Data from one qPCR round was stored in a single Excel file. The results of the gel electrophoreses were also added in a single Excel file. All the data, including data from the Bactopath intervention study about human/pig feces samples and data of positive/negative EDCs with PCR and gel electrophoresis were put together in a survey file.

2.8 Statistics

Potential associations were evaluated by odds ratios, a test that is based on the hypothesis that there is no link between the different observations. This hypothesis can be confirmed or refuted (H0: there is no relation between the different obervations, H1: there is a relation between the different observations). The odds ratio was calculated by using the formula: OR= (a/b)/ (c/d) = (a*d)/ (b*c).
An odds ratio result from 0 to 1 indicates a reduced risk of exposure to the environmental factor and an odds ratio between 1 and infinite indicates an increased risk by exposure to the environmental factor. In this case there is not an exposure to an environmental factor but another test with positive or negative test results.
Confirming or refuting the hypothesis was done by calculating the 95% confidence interval and the p-value with an online odds ratio calculator (MedCalc March 16, 2014). The 95% confidence interval describes how precise the estimate of the odds ratio is and describes with a probability of 95% the interval in which the true odds ratio lies. There is a significant association if the value of the null hypothesis (there is no association: OR=1) is outside the confidence interval. The p-value is the probability that the value found in the statistical test is really true and not based on coincidences. The smaller the p-value, the less likely that there is a coincidence, which leads to better credibility of the test. The comparison between the tests is significant if the detected p-value is less than the limit of 5% ($p<0.05$) and non-significant if the p-value is greater than 5% ($p>0.05$). If the p-value is lesser than 0.05 the hypothesis H0 will be rejected and if the p-value is greater than 0.05 this hypothesis will be accepted.

3. Results

Data of one antibiotic resistant group, CTX-M-1, was collected from fourty different pig farms and fourty households. First the results of the EDCs will be discussed, then the existing results of culture and PCR of feces samples and finally possible associations will be discussed.

3.1 Results EDCs

In the first PCR plate there were nine samples with at least one good amplification curve and a melt peak at 87.0-88.0°C and four samples in which in two of the three PCR replicates the amplification curves increased at the end. No NTCs were deployed.
In the second plate there were six samples with at least one good amplification curve and a melt peak at 87.0-88.0°C. Of the thirty NTCs none of them showed an amplification curve.
The third PCR plate contained above samples, including NTCs. The primers used for this third plate were freshly purchased, still, the difference between Ct values from this plate and earlier plates was very small. The Ct value of 1:10 and 1:100 dilutions differed by 3.3, as expected. The results from plate three are thus comparable with the results of the samples on plate one and two. However, there are samples in which the amplification curves raised on plate three where it did not happen in plate one because of the too late increasing amplification curves. Additionally, there are samples from stables that have good amplification curves and melt peaks with a dilution of 1:10 and do not have it by a dilution of 1:100 (and the converse also). Four NTCs gave a Ct on this plate, but examination of the melt peaks showed that these are unspecific products.

The results of the gel confirmed the data from plate three of the PCR. Only PCR products with a melt peak at 87.0-88.0°C gave positive results on gel. Finally, fifteen samples tested positive by gel electrophoreses of which one sample was a household sample. These samples had a clear band on the CTX-M-1 fragment (260 base pairs), shown in Figure 1. Table 2 gives an overview of all farms that tested positive on PCR and tested positive by gel electrophoresis. It shows the number of positive replicates from stable and household samples that have been tested twice in triplicate for 1:100

dilution stable samples and 1:10 household samples. 1:10 stable samples were only in triple deployed in the third PCR plate. The average Ct was calculated for the samples on plate three to be able to compare. Two of these samples are still doubtful, because they had only one positive PCR replicate in a dilution that tested positive by gel electrophoresis. These samples are shown in Table 3. These two samples are not convincing enough and are no longer seen as positive in this report. In general, all added blanco EDCs tested negative.

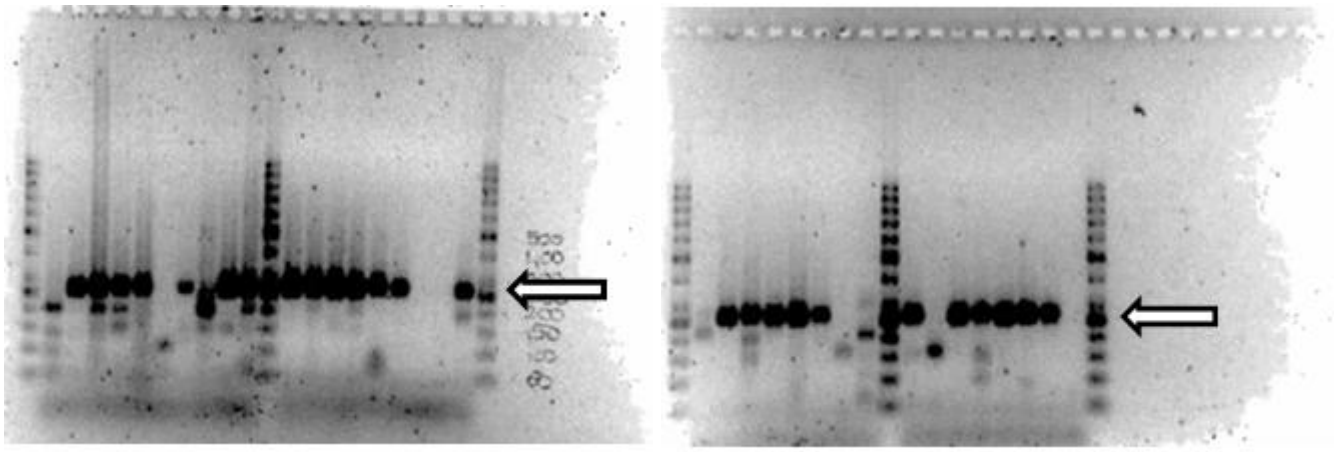

Figure 1: Result of the gel electrophoresis. In the illustration left the 1:10 dilutions with left, right and in the middle the Gene Ruler. In the illustration right the 1:100 dilutions with left, right and in the middle also the Gene Ruler. The household samples did not have a dilution of 1:00.

Dilution 1:100					Dilution 1:10				
BNR	EDC	Positive replicates	Average Ct plate 3	Gel +/-	BNR	EDC	Positive replicates	Average Ct plate 3	Gel +/-
3	1	6/6	31,16	+	3	1	3/3	27,88	+
6	1	5/6	31,19	+	6	1	3/3	33,55	+
6	3	1/6	33,60	+	6	3	2/3	30,72	+
6	4	6/6	30,03	+	6	4	3/3	33,15	+
10	1	1/6	36,38	-	10	1	3/3	31,75	+
14	2	6/6	32,79	+	14	2	3/3	29,15	+
15	2	1/6	34,43	-	15	2	3/3	33,24	+
16	1	6/6	31,13	+	16	1	3/3	27,89	+
18	1	3/6	34,30	+	18	1	3/3	29,93	+
23	1	6/6	30,78	+	23	1	3/3	27,11	+
32	1	6/6	31,62	+	32	1	3/3	28,87	+
33	2	2/6	36,78	+	33	2	3/3	30,20	+
					23	H	5/6	30,31	+

Table 2: All farms that seems like positive on PCR and tested positive by gel electrophoresis.

Dilution 1:100					Dilution 1:10				
BNR	EDC	Positive replicates	Average Ct plate 3	Gel +/-	BNR	EDC	Positive replicates	Average Ct plate 3	Gel +/-
9	1	1/6	36,40	+	9	1	0/3	33,88	-
33	4	1/6	NA	-	33	4	1/3	33,37	+

Table 3: Samples which positivity is doubtful.

Because of the low prevalence and low quantities of the gene we decided to collect only qualitative data. If one or more compartments have tested positive for CTX-M-1 in one farm, the farm was labeled as positive. From the households there was only one sample present, so a household was labeled positive if this sample tested positive in the tests.
From the fourty farms in total, ten farms tested positive for CTX-M-1 with certainty (BNR 3, 6, 10, 14, 15, 16, 18, 23, 32, 33). On each farm one compartment tested positive, except for BNR 6 (three compartments were positive). In total there were twelve positive compartment EDCs out of 123 investigated compartment EDCs that were involved in this study (9.76%).
From the 34 households, one household tested positive for CTX-M-1: BNR 23 (2.94%), a farm that already was found positive for CTX-M-1 in dust in one compartment in the stable (Figure 2).

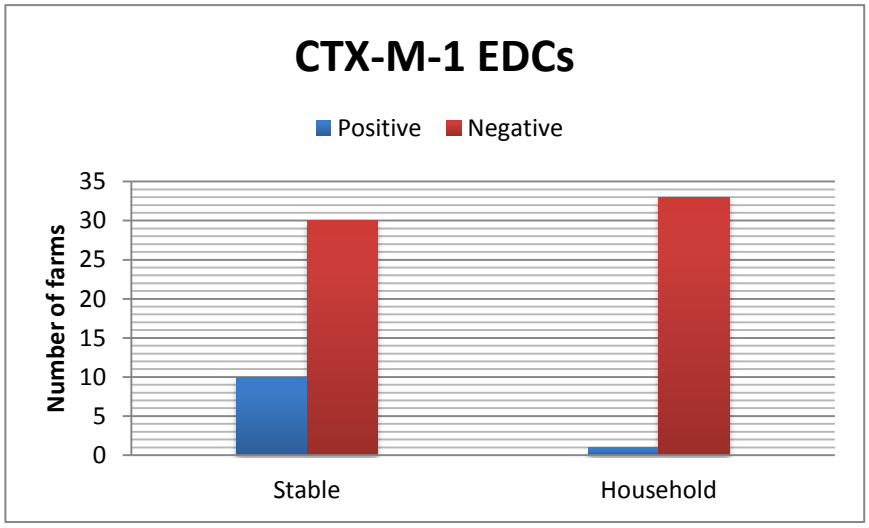

Figure 2: Number of CTX-M-1 dust positive and negative farms with samples from stables and households.

3.2 Results culture feces

Data on the presence of specific ESBL genotypes in isolates obtained from pig feces from ten different pens per farm were already available and analyzed. If one or more pens were tested positive for CTX-M-1, the farm was labeled as positive. Besides pig feces, also humans on these farms were tested by means of a feces sample. If one or more person was tested positive, the farm was labeled as positive for human carriage (PCR feces samples same principle).
Twelve farms tested positive for CTX-M-1 (BNR 3, 4, 5, 6, 8, 10, 13, 14, 23, 27, 32, 33). The amount of positive pens differs between the positive farms. There were four farms that had one positive pen; one farm four positive pens; two farms five positive pens; two farms seven positive pens; two farms nine positive pens and one farm had ten positive pens. In total there were sixty positive pens out of 398 investigated pens (15.08%).
Four farms tested positive for human CTX-M-1 carriage (BNR 1, 6, 14, 32). At two farms (BNR 6 and 32) two persons tested positive. Of these six persons there were four farmers who lived on the farm and had long working hours in the stables. In addition, one person was a child who lived on the farm and spent 20 hours weekly in the stable. The sixth person was a partner from a positive tested farmer and she came for 20 hours weekly in the stables. In total there were six positive individual human feces samples out of 136 human feces samples by culture (4.41%) (Figure 3).

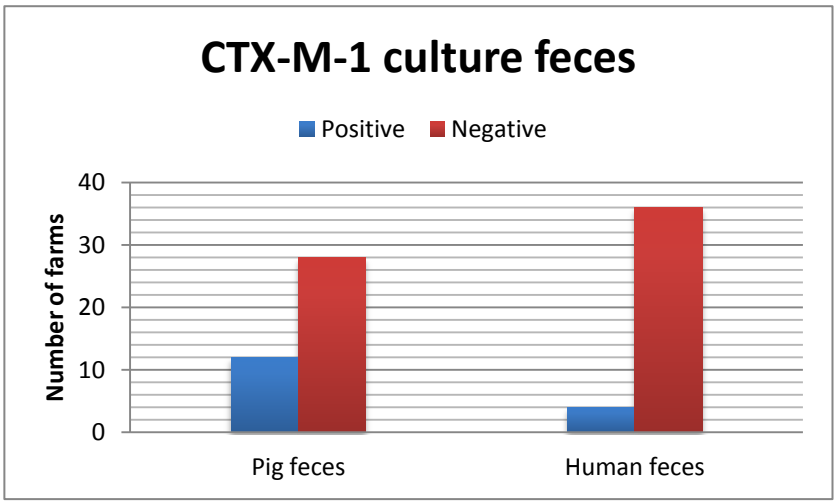

Figure 3: Number of CTX-M-1 positive and negative farms by culture pig feces samples and human feces samples.

3.3 Results PCR feces

Feces samples from five different pens per farm (first five out of above ten samples) were checked for the presence of CTX-M-1 by using qPCR.

In total, ten farms tested positive for CTX-M-1 (BNR 2, 3, 6, 10, 13, 14, 18, 23, 32, 33). Two farms had one positive pen; three farms two positive pens; four farms three positive pens and one farm four positive pens. In total there were 24 positive pens out of two hundred investigated pens (12%).

Four farms tested positive for human CTX-M-1 carriage (BNR 1, 5, 6, 32) (Figure 4). Three from the four persons were already positive by culture, the child and two farmers. The fourth person was a daughter in law from a farmer, residing elsewhere, and there was no information available from her contact with pigs. In total there were four positive individual human feces samples out of 136 human feces samples by PCR (2.94%).

Table 4 gives an overview of all the farms that were tested positive in at least one test. The results of EDCs, culture pig and human feces and PCR pig and human feces are processed.

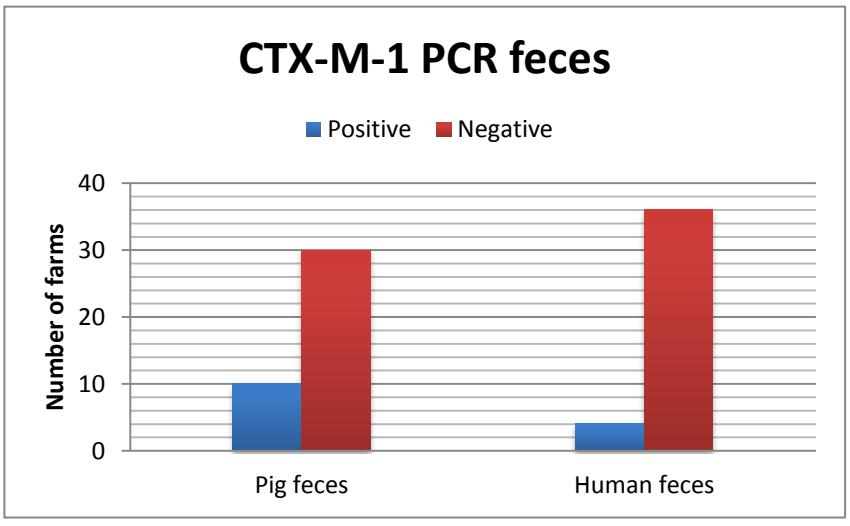

Figure 4: Number of CTX-M-1 positive and negative farms by PCR pig feces samples and human feces samples.

BNR	EDC	Culture pig feces	PCR pig feces	Culture human feces	PCR human feces
1	0/5	0/10	0/5	1/6	1/6
2	0/5	0/10	1/5	0/5	0/5
3	1/5	9/10	2/5	0/0	0/0
4	0/5	1/10	0/5	0/1	0/1
5	0/5	1/10	0/5	0/7	1/7
6	3/5	5/10	3/5	2/4	1/4
8	0/5	1/10	0/5	0/2	0/2
10	1/5	4/9	4/5	0/2	0/2
13	0/5	10/10	2/5	0/3	0/3
14	1/5	9/10	3/5	1/3	0/3
15	1/5	0/10	0/5	0/4	0/4
16	1/5	0/10	0/5	0/6	0/6
18	1/5	0/10	2/5	0/0	0/0
23	1/5 + H	5/10	3/5	0/2	0/2
27	0/5	1/10	0/5	0/5	0/5
32	1/5	7/10	1/5	2/5	1/5
33	1/5	7/10	3/5	0/0	0/0
% positive from total:	9.76 (stable) 2.94 (household)	15.08	12.00	4.41	2.94

Table 4: Farms that were tested positive in at least one test.

3.4 Associations

Associations between CTX-M-1 carriage in EDC and in pigs/humans were investigated. The principles earlier described about when a farm was seen as positive are also used for these calculations. Figure 5 and Figure 6 illustrate the relation of CTX-M-1 in stable EDCs with pig feces culture and pig feces PCR. Positive associations were found for both associations, as shown in Table 5.

Specifically, a significant association was found between a positive EDC from a stable and a farm that is positive by culture of pig feces. The odds ratio was 11.67, the range of the 95% confidence interval 2.22 – 61.28 and the p-value 0.0037.

There was also a significant association between a positive EDC from a stable and a farm that is positive by PCR of pig feces. In this case the odds ratio was 56.00, the range of the 95% confidence interval 6.7782 – 462.6571 and the p-value 0.0002.

The comparison between positive individual human feces samples and farms positive by EDCs and pig feces culture show more significant associations.

A significant association was found between a positive individual human feces sample and a farm that is positive by a stable EDC. The odds ratio was 25.95, the range of the 95% confidence interval 2.88 – 0.0037 and the p-value 0.0037.

A significant association was also found between a positive individual human feces sample and a farm that was positive on culture of pig feces. The odds ratio was 17.41, the 95% confidence interval 1.96 – 155.04 and the p-value 0.0104.

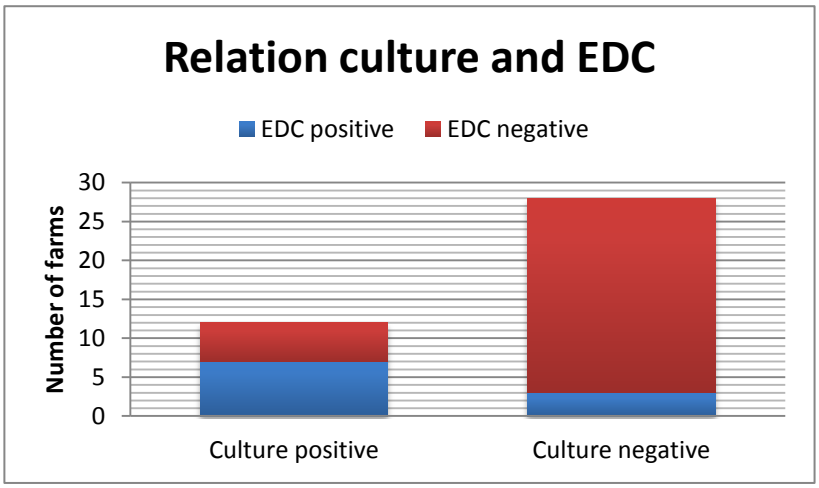

Figure 5: Number of CTX-M-1 positive and negative farms by culture pig feces. And within the amount of CTX-M-1 positive and negative farms by dust from EDCs.

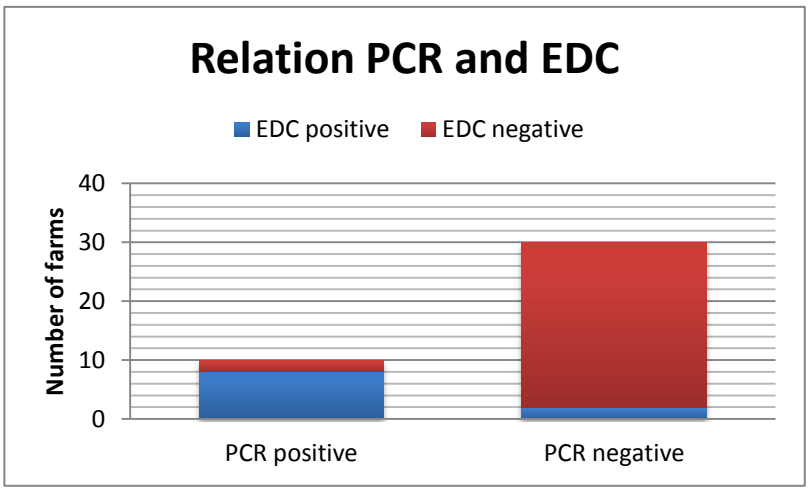

Figure 6: Number of CTX-M-1 positive and negative farms by PCR of pig feces. And within the amount of CTX-M-1 positive and negative farms by dust from EDCS.

No significant associations were found between a positive household EDC and a farm that is positive by pig feces culture and the association between a positive individual human feces culture and a positive household EDC. In the first, the odds ratio is undefined because a number is divided by 0 in the calculation. For the second association, there is a high odds ratio, 39.08, that gives the indication that there is an association. But the 95% confidence interval is not significant, namely 0.17 – 91.08 and the p-value was significant, namely 0.39. So, in general there are insufficient data for these associations.

The housing compartment where the EDCs were deposited was compared with the pens where the feces samples were taken. If the places were similar, it could be examined whether there is an association between these two. The database revealed that the places where the EDCs were deposited were not described in sufficient detail. From fourty stable EDCs it was known in which compartment they were located. The EDCs, each located in a different compartment, were compared with the results of the pens in these compartments. If one or more pens were positive on culture, the compartment was considered as positive. The odds ratio is calculated based on this (Table 5).

There was a significant association between a positive EDC in a compartment and a positive pen in the same compartment. However, this dataset is restricted to fourty compartments for which the compartment containing the EDC was known, while there were 160 EDCs in total.
This association cannot be viewed between positive EDCs in different compartments and the results of the PCR of pens in these compartments because only five of ten pens have been investigated with the PCR in which the location of the EDC did not always matched with the investigated pens or the location of the EDC itself was unknown.

EDC stables – culture pig feces						
	EDC +	EDC -	Total		Odds ratio	11.6667
Culture +	7	5	12		95% confidence interval	2.2212 – 61.2795
Culture -	3	25	28		P-value	0.0037
Total	10	30	40			
EDC stables – PCR pig feces						
	EDC +	EDC -	Total		Odds ratio	56.0000
PCR +	8	2	10		95% confidence interval	6.7782 – 462.6571
PCR -	2	28	30		P-value	0.0002
Total	10	30	40			
Individual human feces culture – EDC stables						
	Culture +	Culture -	Total		Odds ratio	25.9524
EDC +	3	7	10		95% confidence interval	2.8883 - 233.5875
EDC -	1	29	30		P-value	0.0037
Total	4	36	40			
Individual human feces culture – culture pig feces						
	Culture + human	Culture - human	Total		Odds ratio	17.4138
Culture + pig	3	9	12		95% confidence interval	1.9559 – 155.0396
Culture - pig	1	27	28		P-value	0.0104
Total	4	36	40			
EDC households – culture pig feces						
	EDC +	EDC -	Total		Odds ratio	Undefined
Culture +	1	11	12		95% confidence interval	-
Culture -	0	28	28		P-value	-
Total	1	39	40			
Individual human feces culture - EDC households						
	Culture +	Culture -	Total		Odds ratio	39.538
EDC +	0	2	2		95% confidence interval	0.1716 – 91.0752
EDC -	6	128	134		P-value	0.3904
Total	6	130	136			
EDC compartments – culture pens compartments						
	EDC +	EDC -	Total		Odds ratio	11.0000
Pen +	2	3	5		95% confidence interval	1.1153 - 108.4926
Pen -	2	33	35		P-value	0.0400
Total	4	36	40			

Table 5: Statistical analysis with odds ratios.

4. Discussion

The results show that CTX-M-1 can be detected in dust from pig stables, but in addition, also in one household dust sample. Several associations were observed which will be discussed below.

CTX-M-1 EDCs were significantly more often found in farms with CTX-M-1 positive pig feces (culture and PCR). Both associations were very strong, but the PCR-based odds ratio was even higher. The reason for difference may have to do with the difference in detection limits between qPCR and culture. The theoretical detection limit for qPCR is around 33.000 genes per gram dust. The following values were used for this calculation: 1 gene is demonstrated in 3 µl DNA and in total we had 40 µl DNA after DNA isolation out of 40 mg dust and DNA was diluted 100 times. Assuming that some 50 mg feces have been used for culturing in the analysis of fecal swabs of pigs, a theoretical detection limit would be in the order of 20 bacteria with CTX-M-1 per gram feces, and thus is culture about 1000x more sensitive than qPCR. Thus, the presence of farms with culture-detectable CTX-M-1 in feces, but without PCR-detectable CTX-M-1 in EDCs reduces the odds ratio of CTX-M-1 in EDCs versus culturable bacteria with CTX-M-1 in feces. The fact that positive farms by EDC have many positive pens by culture reflects also the higher detection limit for PCR.
Another reason why a positive EDC from a stable not always tested positive on culture or PCR from pig feces (or conversely) can also be related to the fact that the place where the EDC was deposited not always was the same as were the pig feces samples were taken. This will be discussed in more detail later on in this chapter.
Above association was also seen at a smaller level, when the analysis was limited to compartments in which both EDC and pig feces samples were analyzed. The statistical results did not indicate that a comparison of samples at local level were more correlated than at farm level. The odds ratio for both was 11.00. It was expected that local levels would be more correlated, perhaps, the small number of samples that were suitable for this comparison affects this.

In order to study the relevance of inhalation of CTX-M-1 positive dust as an exposure route for humans, odds ratios for the association between positive CTX-M-1 individual human feces samples and farms positive for CTX-M-1 by EDCs were studied. Feces from farm inhabitants was significantly more positive on farms positive by dust samples. An inhalable exposure route seems thus to be possible. But, we also found that feces from farm inhabitants was significantly more positive on farms that were positive by pig feces culture. This could represent a direct contact route, but this association can maybe also be explained by the fact that dust became infected from positive pig feces and human became infected from this positive dust. The different routes that were significant are shown in Figure 7. The main point of this figure is that we cannot prove a causal relationship. Human carrier ship can arise either from positive pig feces by a direct contact route or from positive dust by an exposure route.

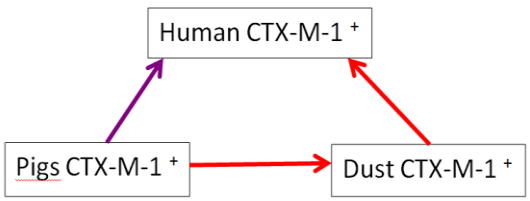

Figure 7: Significant associations in this research.

This research looked also to the association if positive CTX-M-1 household EDCs can be found more on farms with pig feces positive for CTX-M-1 by culture, but this odds ratio was undefined. The association between positive human inhabitants lived on farms that have a positive EDC household gave a odds ratio of 39.54 and the p-value was significant, but the 95% confidence interval was not significant. It seems that there is an association, but due to insufficient data this is not discussed.

As said earlier, there is research that showed correlations between airborne dust and settled dust and that gave reasons to consider using settled dust as a surrogate for inhalable microbial exposure (Frankel et al. 2012). The positive associations between stable EDCs and positive stables on culture and PCR show a possibility for airborne transmission. Also the significant association between positive EDC stables samples and human tested on feces culture positive reflects this. Nevertheless, it is difficult to relate dust content to a quantitative airborne human uptake. To prove that EDCs could be used as a surrogate for inhalable exposure (so that you may equate), investigations comparing dust sampling by EDC and with filter systems are needed. If there is a significant correlation, it can be assumed that the amount of bacteria in dust can be equated to the inhalable exposure for humans. Frankel, et al, 2013, have already carried out a similar study for indoor microbial exposure (including cultural fungi and bacteria, endotoxin and total inflammatory potential of dust samples from homes), but they have not mentioned the amount of bacteria on EDCs and other studies on this have not been found. Further research for this is therefore necessary to evaluate the effect of exposure to dust in the future.

During the study there were several choices made that may affect the total amount of DNA in the samples. First, the EDC samples were stored for extended periods of time in the -80°C freezer (the first data that were known about putting samples in the -80°C freezer were from July 2011). Nascimento, et al, 2013, evaluated the effect of temperature and time storage on the microbial detection of oral samples by Checkerboard DNA-DNA hybridization. This study showed as result that either total, individual microbial counts (species detection by DNA hybridization) and the total microbial incidence had lower values for samples processed after 12 months of storage, irrespective for temperatures tested (do Nascimento et al. 2014). According to these finding, Ivanova, et al, 2013, have reported that for PCR success, the temperature is a crucial factor to obtain high quality DNA in PCR reaction after long-time samples storage (Ivanova and Kuzmina 2013). The samples in this current study were stored at -80°C temperature, so that the temperature may have little effect on the total amount of DNA. However, the samples were stored for a period of nearly two and a half years before they were used for this study, so if the results from Nascimento were extrapolated to our samples, it could be that the time of storage could has a negative impact on the total amount of DNA and can have led to false-negative results in this research.

The choice for the dilutions for samples from stables (1:10) could also have led to false-negative results. Before starting the PCR it was tried to determine the best dilution for the use of this PCR. This is achieved by randomly choosing sixteen stable samples and four house samples. From these samples 1:10 and 1:100 dilutions were tested together with undiluted samples (of the same samples) in a PCR (primer set 16S in order to get an estimate about the amount of bacterial DNA). For farm dust samples, marked inhibition was seen in 1:10 dilutions, while 1:100 dilutions resulted in appropriate amplification. For this reason, the dilution 1:100 was chosen for stable samples and 1:10 for house samples. When higher diluted DNA is used for PCR, inhibition is reduced, but the target DNA is also diluted, possibly beyond the detection limit. In the gel electrophoresis probable CTX-M-1 positive stable samples from both 1:10 and 1:100 dilutions were tested. It appears that there were three stable samples positive at a dilution of 1:10, but negative at a dilution of 1:100 and there was also one sample positive at 1:100 and negative at 1:10. This demonstrated that the choice for a dilution is not so easy, because the samples gave different results on the various dilutions.

There were large amount of adjustments made in the protocol for DNA isolation with the main reason that the used kit was unsuitable for the total amount of dust we extracted from de EDCs. The user manual from the NucleoSpin® 8 Plant II kit indicated that 20 mg dried sample had to be used.
A higher weight is preferred because this often gives the most DNA and therefore a higher detection limit. Experiments were done with different amounts of dust (20-80 mg) up to and including DNA isolation. To see at which weight the greatest amount of bacterial DNA was present after DNA extraction, a Picogreen staining was performed. In addition, gel electrophoresis was implemented to check if the DNA still had proper lengths after the DNA isolation. These experiments showed that most DNA was isolated from samples with 40 mg dust. According to the supplier, the kit could be used for material with a dry weight of maximal 40 mg. After weighting the dust it was directly transferred to a beat beating tube, so there was one transfer step less in which DNA may be lost (this was not applicable for samples that could not be weighed). In the protocol for DNA isolation the volume PL1 and RNAse were doubled for samples between 20-40 mg dust to be able to retain the optimum function as described in the user manual of the kit. Because of the (probably) greater amount of DNA on the silica membrane it was chosen to wash with PW1 twice instead of once, and it was considered sufficient to wash with PW2 once. There was one time eluted with PE just because the concentration of DNA will be much lower when this step will be repeated.

It seemed to be very difficult to analyze the association between EDCs from a compartment and pen samples from the same compartment. For this reason we looked first at farm level (farm positive or negative in a test) and only afterwards at pen level. For 40 EDCs it was known in which compartment the EDCs were taken (in these compartments also pens were sampled otherwise no comparison can made). The difficult part in this was that we knew from which specific compartment an EDC came from, but that this specific compartment can have several stables. As an example, in a maternity department there can be multiple farrowing stables and this information from the EDC wasn't present. For that reason, pens from a specific compartment in one farm were considered as one group, irrespective in which stable from this compartment the pens were taken and in which stable from this compartment the EDC was located. This makes the association somehow less reliable. More reliable would be to know in which stable from a specific compartment the EDC was deposited and that in this stable feces samples from different pens were taken. Unfortunately, we cannot make this reliable comparison.

In conclusion, CTX-M-1, the most prevalent ESBL producing *Enterobacteriaceae* group in pig farming, can be detected in dust from stables and households. CTX-M-1 positive EDCs were significantly more often found on farms with CTX-M-1 positive pig feces (both culture and PCR). Feces from farm inhabitants was significantly more positive on farms with positive stable dust samples and on farms with positive pig feces samples. However, due to the correlation of EDC and pig feces results, it is difficult to establish a causal relationship between either direct contact or airborne exposure and human carriage. Further research is warranted to gain more insight into airborne exposure to ESBL.

References

Chapin A, Rule A, Gibson K, Buckley T, Schwab K. 2005. Airborne multidrug-resistant bacteria isolated from a concentrated swine feeding operation. Environ Health Perspect 113(2):137-142.

Chien YC, Chen CJ, Lin TH, Chen SH, Chien YC. 2011. Characteristics of microbial aerosols released from chicken and swine feces. J Air Waste Manag Assoc 61(8):882-889.

Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A, Smith H, Mevius D. 2013. Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing escherichia coli in dutch broilers and broiler farmers. J Antimicrob Chemother 68(1):60-67.

do Nascimento C, dos Santos JN, Pedrazzi V, Pita MS, Monesi N, Ribeiro RF et al. 2014. Impact of temperature and time storage on the microbial detection of oral samples by checkerboard DNA-DNA hybridization method. Arch Oral Biol 59(1):12-21.

Duan H, Chai T, Liu J, Zhang X, Qi C, Gao J et al. 2009. Source identification of airborne escherichia coli of swine house surroundings using ERIC-PCR and REP-PCR. Environ Res 109(5):511-517.

European Centre for Disease Prevention and Control. 2011. Antimicrobial resistance surveillance in europe 2010. annual report of the european antimicrobial resistance surveillance network (EARS-net). Stockholm, Sweden: ECDC 2011.

Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing escherichia coli from livestock and companion animals, and their putative impact on public health: A global perspective. Clin Microbiol Infect 18(7):646-655.

Frankel M, Timm M, Hansen EW, Madsen AM. 2012. Comparison of sampling methods for the assessment of indoor microbial exposure. Indoor Air 22(5):405-414.

Ivanova NV, Kuzmina ML. 2013. Protocols for dry DNA storage and shipment at room temperature. Mol Ecol Resour 13(5):890-898.

Kristiansen A, Saunders AM, Hansen AA, Nielsen PH, Nielsen JL. 2012. Community structure of bacteria and fungi in aerosols of a pig confinement building. FEMS Microbiol Ecol 80(2):390-401.

Laube H, Friese A, von Salviati C, Guerra B, Kasbohrer A, Kreienbrock L et al. 2013. Longitudinal monitoring of extended-spectrum-beta-lactamase/AmpC-producing escherichia coli at german broiler chicken fattening farms. Appl Environ Microbiol 79(16):4815-4820.

Liebana E, Carattoli A, Coque TM, Hasman H, Magiorakos A, Mevius D et al. 2013. Public health risks of enterobacterial isolates producing extended-spectrum b-lactamases or AmpC g-lactamases in food and food-producing animals: An EU perspective of epidemiology, analytical methods, risk factors, and control options. Food Safety 56(1 april):1030-1037.

MARAN 2012. 2012. Monitoring of antimicrobial resistance and antibiotic usage in animals in the netherlands in 2010/2011. .

MedCalc ess. March 16, 2014. Odds Ratio. http://www.medcalc.org/calc/odds_ratio.php ed.

Normand AC, Vacheyrou M, Sudre B, Heederik DJ, Piarroux R. 2009. Assessment of dust sampling methods for the study of cultivable-microorganism exposure in stables. Appl Environ Microbiol 75(24):7617-7623.

Noss I, Wouters IM, Visser M, Heederik DJ, Thorne PS, Brunekreef B et al. 2008. Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment. Appl Environ Microbiol 74(18):5621-5627.

Paterson DL, Bonomo RA. 2005. Extended-spectrum beta-lactamases: A clinical update. Clin Microbiol Rev 18(4):657-686.

Seiffert SN, Hilty M, Perreten V, Endimiani A. 2013. Extended-spectrum cephalosporin-resistant gram-negative organisms in livestock: An emerging problem for human health? Drug Resist Updat 16(1-2):22-45.

Xu L, Ensor V, Gossain S, Nye K, Hawkey P. 2005. Rapid and simple detection of blaCTX-M genes by multiplex PCR assay. J Med Microbiol 54(Pt 12):1183-1187.

Yuan W, Chai TJ, Miao ZM. 2010. ERIC-PCR identification of the spread of airborne escherichia coli in pig houses. Sci Total Environ 408(6):1446-1450.

Zhao WH, Hu ZQ. 2013. Epidemiology and genetics of CTX-M extended-spectrum beta-lactamases in gram-negative bacteria. Crit Rev Microbiol 39(1):79-101.

Zheng H, Zeng Z, Chen S, Liu Y, Yao Q, Deng Y et al. 2012. Prevalence and characterisation of CTX-M beta-lactamases amongst escherichia coli isolates from healthy food animals in china. Int J Antimicrob Agents 39(4):305-310.

Annex

Protocols material and methods.

Protocol for DNA extraction of EDCs.

1. Transfer EDC to stomacher bag.
2. Add 10 ml of pyrogenfree water + 0.05% Tween 20.
3. Place in stomacher for 10 min.
4. Squeeze and remove remaining fluid with a sterile pipet and transfer it in a 50ml Greiner tube.
5. Replenish with 10ml of pyrogenfree water + 0.05% Tween 20.
6. Place in stomacher for 10 min.
7. Squeeze and remove remaining fluid in the 50ml Greiner tube.
   - the total volume should be +/- 16ml.
8. Freeze the fluid under angle over night at -80°C.

Protocol for freeze-drying the samples.

1. Unscrew the lid of the 50ml Greiner tubes a little bit (instead of using parafilm or tissue).
2. Place the samples in the freeze dryer.
3. After 2-4 days collect the freeze-dried samples from the freeze dryer and store them in the -80°C until DNA isolation.

Protocol Machery-Nagel NucleoSpin 8 Plant II (using a vacuum).

Needed: Starter kit A (containing Column Holders A and NucleoSpin Dummy strips) & NucleoVac 96 vacuum manifold.

- Store RNAse A at 4°C, the rest at room temperature.
- Check if Buffer PW2 and RNAse A were prepared according to section 3.
- Add ethanol 96% to buffer PW2 before first use.
- Add MQ to RNAse A (1.25ml to 15mg). Solution is stable for 3 months at 4°C.
- Set incubator or oven at 65°C.
- Equilibrate Buffer PE to 70°C.

1. Add 500ul PL1 and 10ul RNAse in the samples with an amount of dust less than 20ug, and add 1000 ul PL1 and 20ul RNAse A in samples with an amount of dust between 20 and 40 mg. It depends on whether the dust could be weighed if it is added in a bead beating tube or a Greiner tube.
2. Transfer the lysate of samples in a Greiner tube to a bead beating tube (2.0ml tube with 500 mg glass beads).
3. Bead beat for 45-60 seconds at maximum speed.
4. Heat the bead beating tubes with the samples for 30 minutes at 65°C.
5. Centrifuge the bead beating tubes 20 min at 6000 g.
6. Add the lysate of the samples in a MN square Well Block and add the same amount as the lysate of PC in the MN square Well Block. Mix by pipetting 3x.
7. Transfer lysate to NucleoSpin Plant II Binding Strips (insert appropriate number of NucleoSpin Plant II Binding Strips into a Column Holder A).
   - Close any unused openings of the Column Holder A with NucleoSpin Dummy Strips.
   - Insert spacers MTP/Multi-96 Plate, notched side up, into the grooves located on the short sides of the manifold. Insert the waste reservoir into the center of the manifold.
8. Bind DNA to silica membrane of the NucleoSpin II Binding Strips. Vacuum -0.2 to -0.4 bar.

9. Wash and dry the silica membrane by adding 400ul PW1 and vacuum -0.4 bar and repeat this for a second time for samples at the start of more than 20mg. Add 700ul PW2 and vacuum -0.4 bar and repeat this step also for a second time. 1 min each step.
   - After washing, dry the membrane. Remove the waste reservoir and the MN wash plate. Apply vacuum for 10 more minutes to dry the membrane.
10. Elute DNA with 100ul prewarmed (70°C) PE, incubate 2 minutes at room temperature and vacuum at -0.4 bar.
11. Make a 20ul aliquot and store at -80°C.