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Short communication

Quantification, distribution and diversity of ESBL/AmpC-producing *Escherichia coli* on freshly slaughtered pig carcasses

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ABSTRACT

This study quantified cefotaxime-resistant *E. coli* (CREC) on nine different carcass areas of 104 freshly slaughtered pig carcasses. In 49% [95% confidence interval (95% CI): 29–69%] of the carcasses CREC could be isolated and enumerated (using Tryptone Bile Agar with X-Glucuronide supplemented with 1 mg/L cefotaxime). Proportions of positive samples varied between carcass areas from 1% [95% CI: 0–10%] (loin) to 23% [95% CI: 10–44%] (head). Maximum concentrations on positive samples ranged between $-0.6\ \log_{10}$ CFU/cm^2 (loin, elbow before evisceration) and $1.7\ \log_{10}$ CFU/cm^2 (head). The head was significantly more frequently contaminated than the loin ($p = 0.027$) and ham (3% [95% CI: 1–15%]). The foreleg was significantly more frequently contaminated (20% [95% CI: 13–30%]) than the ham. Combination disk diffusion assays revealed that 81% of the CREC isolates were extended-spectrum beta-lactamases (ESBL) producers, 13% were AmpC cephalosporinases (AmpC) producers and 2% ESBL and AmpC co-producers. Genotyping denoted $bla_{CTX\text{-}M\text{-}gr1}$ (63%) and bla_{TEM} (40%) as most present antibiotic resistance genes. Multiple gene combinations in one isolate and multiple combinations of genotypes and phenotypes among isolates of one sample were observed. These quantitative data can be used for intervention strategies to lower human exposure to CREC.

1. Introduction

The presence of *Enterobacteriaceae* producing extended spectrum beta-lactamases (ESBL) and AmpC cephalosporinases in food is of great concern to public health due to the risk for transmission of antibiotic resistant bacteria and resistant genes to humans (Ferri et al., 2017). ESBL/AmpC-producing *Enterobacteriaceae* are resistant against extended spectrum cephalosporins, which are widely used antibiotics in human and veterinary medicine (Brolund, 2014; Harris et al., 2015). Multiple studies isolated ESBL/AmpC-producing *Enterobacteriaceae* from food producing animals and have shown evidence that food producing animals contribute to the zoonotic spread of resistance against extended spectrum cephalosporinases (Lazarus et al., 2015). As such, the consumption of pork has been estimated to account for 4.5% and 12.5% of human exposure to ESBL/AmpC-producing *E. coli* in the Netherlands and Denmark, respectively (Carmo et al., 2014; Evers et al., 2017). A German study could also associate frequent consumption of pork (≥ 3 meals per week) with community acquisition of ESBL-producing *E. coli* (Leistner et al., 2013). The occurrence of pigs carrying ESBL-producing *E. coli* at slaughter shows great geographical differences with proportions ranging from 15.2% in Switzerland (fecal samples) to 23.4% in the UK (caecal samples) and 49% in Portugal (fecal samples) (Geser et al., 2011; Ramos et al., 2013; Randall et al., 2014). In a study by Van Damme et al. (2017), ESBL/AmpC-producing *E. coli* were found in 75% of the fecal samples and 47% of the tonsils of pigs at slaughter, in numbers up to 5.5 and $5.6\ \log_{10}$ CFU/g, respectively. However, little is known about the presence of ESBL/AmpC-producing *Enterobacteriaceae* on freshly slaughtered pig carcasses in Belgium and current information is based on qualitative data. Therefore, the aim of this study was to map the distribution and to quantify the presence of cefotaxime-resistant *E. coli* (CREC) on freshly slaughtered pig carcasses by performing sampling and testing in pig slaughterhouses in Belgium.

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2. Material & methods

2.1. Sampling

Between October 2015 and February 2016, seven Belgian pig slaughterhouses were each visited three times to collect swab samples from five randomly selected carcasses (Biasino et al., 2018). All involved slaughterhouses applied a similar slaughter procedure, as described by Swart et al. (2016). In short, 104 pig carcasses originating from 61 batches (average 1.7 pigs per batch) were sampled during 21 sampling visits. From each carcass, the elbow was sampled using a cellulose sponge (3 M, Diegem, Belgium) that was soaked in 25 mL Buffered Peptone Water (BPW; Bio-Rad Laboratories, Marnes-La-Coquette, France). The elbow swab was taken before evisceration, when the carcass entered the clean zone, each time alternating between the left and right carcass half among the different carcasses (e.g. for the first carcass the sample was taken from the left carcass half; for the second carcass the sample was taken from the right carcass half etc.). Moreover, the following nine areas (100 cm^2 each) were (separately) swabbed after evisceration and trimming of the carcass, but before cooling: elbow, head (nose bridges and ears), pelvic duct, sternum (breast cut and surrounding skin), belly, throat, distal part of the foreleg, medial side of the ham and the loin at the split surface. The above mentioned nine samples were taken from the opposite carcass half than the one from which the elbow swab was taken before evisceration to prevent sampling the same surface twice. All samples were transported under chilled conditions to the lab and were subjected to microbiological analysis within 6 h after collection. Upon arrival in the lab, samples were homogenized for 1 min in a stomacher (Colworth Stomacher 400, Steward Ltd., London, UK) prior to analysis.

2.2. Isolation and enumeration of CREC

In order to isolate and enumerate CREC, 1 mL of the initial suspension was distributed over two Tryptone Bile Agar with X-Glucuronide (TBX, Bio-Rad Laboratories, Marnes-La-Coquette, France) plates supplemented with 1 mg/L cefotaxime (Cefotaxime Sodium Salt, Sigma-Aldrich, Saint Louis, Missouri, USA) (TBX-CTX) by spread-plate method (Van Damme et al., 2017), resulting in a detection limit of $-0.6\ \log_{10}$ CFU/cm^2. After 24 h incubation at 44 °C, colonies with a typical *E. coli* phenotype (blue/green) were counted (ISO 16649-2:2001). A sample with at least one blue/green colony on TBX-CTX was regarded as CREC positive. A carcass was considered CREC positive if CREC were isolated from at least one of the sampled carcass areas. Up to twenty of these colonies per positive sample were individually subcultured overnight in 5 mL Tryptic Soy Broth (TSB, Bio-Rad Laboratories, Marnes-La-Coquette, France) of which subsequently 1 mL was added to 2 mL glycerol and stored at −20 °C.

2.3. Phenotypic identification of ESBL/AmpC-producing E. coli

The production of ESBL and/or AmpC-type-β-lactamases was determined phenotypically by a combination disk diffusion assay according to CLSI guidelines (M100-S22, 2012). Therefore, the isolates were cultivated from glycerol on Mueller-Hinton II agar (BD BBL, USA) and the following discs were used: boric acid (BA), cefotaxime (30 μg; CTX), cefotaxime (30 μg) combined with clavulanic acid (10 μg) (CTX + C), ceftazidime (30 μg; CAZ), ceftazidime (30 μg) combined with clavulanic acid (10 μg) (CAZ + C), cefepime (30 μg; FEP), cefepime (30 μg) combined with clavulanic acid (10 μg) (FEP + C) and cefoxitin (30 μg; CFO) (Rosco Diagnostica A/S, Taastrup, Denmark). The criteria used to screen for possible ESBL production were twofold. First, the disk zone of inhibition diameters for cefotaxime and/or ceftazidime with and without clavulanic acid were compared (≥ 5 mm increase in zone diameter in combination with clavulanic acid compared to the zone diameter without clavulanic acid). Second, the susceptibility to cefoxitin (> 19 mm) was assessed. Resistance to cefoxitin (< 19 mm) and an inhibitory effect of boric acid were the criteria applied to identify an AmpC phenotype. Isolates showing clavulanic acid synergy and resistance against cefoxitin were categorized as ESBL and AmpC co-producers. Isolates not complying to any of these categories were considered unusual. These isolates showed resistance against cefotaxime as they were isolated from TBX-CTX but the applied phenotypic characterization remained unable to categorize them.

2.4. Detection of β-lactamase genes by PCR

Genotypic identification of the ESBL/AmpC gene profiles of the isolates was performed as described by Dallenne et al. (2010) and Hasman et al. (2005). All isolates were cultivated from glycerol on TBX for 24 h at 44 °C and subsequently on Plate Count Agar (PCA, Bio-Rad Laboratories, Marnes-La-Coquette, France) for 24 h at 37 °C. One colony of each isolate was suspended in 100 μL H_2O and lysed using heat treatment (10 min at 95 °C). Subsequently, the lysates were centrifuged at 14500*g* (Centrifuge 5417C, Eppendorf, Hamburg, Germany) and stored at −20 °C. Isolates identified as ESBL-producing bacteria were analyzed with two multiplex PCRs targeting ESBL genes, i.e. TEM variants (including TEM-1 and TEM-2), SHV variants (including SH-1), OXA variants (including OXA-1,OXA-4, OXA-30), CTX-M group 1 variants (including CTX-M-1, CTX-M-3 and CTX-M-15), CTX-M group 2 variants (including CTX-M-2) and CTX-M group 9 variants (including CTX-M-9 and CTX-M-14) (Dallenne et al., 2010). Isolates identified as AmpC-type-β-lactamase-producing bacteria were subjected to one multiplex PCR targeting AmpC-type-β-lactamase genes ACC and CIT (Dallenne et al., 2010) as well as one single PCR targeting CMY-2 (Hasman et al., 2005). Isolates showing atypical behavior during the disk diffusion assay were examined with all four PCRs.

2.5. Statistical analysis

Qualitative and quantitative test results were recorded in an Excel 2013 (Microsoft® Corporation, Redmond, Washington, USA) spreadsheet. Bacterial counts were $\log_{10}$-transformed prior to analysis. All analyses were done using Stata 14.1 (Stata Corporation, College Station, Texas, USA). For the calculation of the 95% confidence interval (CI) of the proportion of positive samples (presence/absence of CREC), clustering per slaughterhouse was taken into account. When comparing the presence of CREC between the different carcass areas, a logistic regression analysis was performed with slaughterhouse included as group variable and Bonferroni corrections were applied for multiple testing. A significance level of 5% was used (Agresti, 2003).

3. Results

3.1. Distribution and quantification of CREC on freshly slaughtered pig carcasses

In total, CREC could be isolated from 51 of the investigated pig carcasses (49%; [95% CI: 29–69%]). On positive carcasses, CREC was found on 1 to 5 carcass areas (and accordingly, 1 to 5 samples). Table 1 gives an overview of the number of CREC positive samples per carcass area. The highest occurrence of CREC was seen on the head and the foreleg with 23% [95% CI: 10–44%] and 20% [95% CI: 13–30%] of the samples being positive, respectively. On the other hand, in only 1% (1/104; [95% CI: 0–10%]) of the loin samples and 3% (3/104; [95% CI: 1–15%]) of the ham samples CREC could be isolated. There were significantly more head samples positive for CREC compared to the loin ($p = 0.027$) and the ham ($p = 0.008$). Furthermore, the presence of CREC on foreleg samples was significantly higher than on the ham ($p = 0.024$). The maximum number of CREC ranged from $-0.6\ \log_{10}$ CFU/cm^2 (loin and elbow before evisceration) to $1.7\ \log_{10}$ CFU/cm^2 (head).