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Quantification of hygiene indicators and *Salmonella* in the tonsils, oral cavity and rectal content samples of pigs during slaughter

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ABSTRACT

The tonsils, oral cavity and faeces of 94 pigs at slaughter were sampled to assess the numbers of total aerobic bacteria, *Enterobacteriaceae* and *Escherichia coli* in the rectal content, tonsils and oral cavity of pigs at time of evisceration. Moreover, the prevalence, numbers and types of *Salmonella* spp. were determined. Mean numbers of *Enterobacteriaceae* in tonsils and the oral cavity differed between slaughterhouses. The proportion of *Enterobacteriaceae* relative to total aerobic bacteria differed between the different tissues, though large variations were observed between animals. *Salmonella* spp. were mostly detected in oral cavity swabs (n = 51, 54%), of which six samples were contaminated in numbers over 2.0 log CFU/100 cm^2. *Salmonella* spp. were also recovered from 17 tonsillar tissue samples (18%) and 12 tonsillar swabs (13%). Out of the 29 rectal content samples from which *Salmonella* was recovered (31%), most were lowly contaminated, in the range between −1 and 0 log CFU/g. The predominant serotypes were *S.* Typhimurium and its monophasic variant, which were recovered from 33 and 13 pigs, respectively. In most cases, the same serotypes and MLVA profiles were found in pigs slaughtered during the same day, thus suggesting a common source of contamination.

1. Introduction

Salmonella is a well-established foodborne pathogen causing gastro-enteritis worldwide. Contaminated pork is an important vehicle for the transmission of *Salmonella* to humans (Fosse et al., 2008; Pires et al., 2014) and pigs represent an important reservoir for this pathogen. Since many pigs carry *Salmonella* asymptomatically at time of slaughter, these pathogens can not be detected using routine *ante* and *post mortem* inspection, resulting in the spread to carcasses and meat during slaughtering and processing of the meat. An effective control of *Salmonella* thus requires a comprehensive pork carcass safety assurance, including preventive measures at slaughterhouse level. This control should focus on the prevention of microbial contamination through technology- and process hygiene-based measures (EFSA (European Food Safety Authority), 2011). As the transfer of pathogens during slaughter and meat processing is complex, mathematical modelling may contribute to improve the understanding of contamination with foodborne pathogens. Quantitative microbial risk assessment (QMRA) has emerged in the area of food safety as a comprehensive and systematic approach for addressing the risk of microbial hazards in the food chain and has been used to assess the impact of control strategies and interventions in the food chain (Duarte et al., 2016; Møller et al., 2015). Modelling the transfer of *Salmonella* during slaughter requires comprehensive quantitative data, which are often lacking. For instance, Nauta et al. (2013) addressed the need to study the variation in bacterial concentrations of *Salmonella* and *Escherichia coli* in faecal samples of pigs during slaughter to model faecal contamination of pork carcasses. Moreover, the authors concluded that our current understanding of the origin of the contamination of carcasses is inadequate to control *Salmonella* contamination of pork.

Total aerobic bacteria (TAB) and *Enterobacteriaceae* are used as criteria to assess process hygiene applied during the slaughter of pig carcasses (Commission Regulation (EC) 2073/2005). Moreover, *Enterobacteriaceae* and *Escherichia coli* are often used as an indicator for faecal contamination (Ray, 2001) and there is a positive association between *Enterobacteriaceae* and the presence of *Salmonella* on pre-chill pork carcasses and pork cuts (Corbellini et al., 2016; Ghafir et al., 2005; Prendergast et al., 2008). Nevertheless, it's difficult to predict *Salmonella* on pork carcasses based on

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Enterobacteriaceae numbers as there is a large variability in the probability of finding *Salmonella* between slaughterhouses and slaughtering days (Corbellini et al., 2016).

Fattening pigs may get infected with zoonotic pathogens on-farm during different stages of production (Farzan et al., 2010; Merialdi et al., 2008; Virtanen et al., 2012) as well as during transport and lairage in the slaughterhouse (Bolton et al., 2013; Milnes et al., 2009). Contamination of organs and carcasses may also occur during different stages of the slaughtering process, such as scalding, dehairing, and polishing (De Busser et al., 2013; Spescha et al., 2006). Nevertheless, without considering the origin of contamination or infection, the quantitative levels of pathogens in the faeces, oral cavity and tonsils of pigs at time of evisceration is of utmost importance since removal of the abdominal organs and the pluck set may considerably contribute to carcass contamination (Gill and Jones, 1998). Therefore, the aim of this study is to assess the presence and to quantify the contamination level of hygiene indicators and *Salmonella* spp. in the tonsils, oral cavity and rectal content of pigs at time of evisceration.

2. Material and methods

2.1. Sampling and sample preparation

The tonsils, oral cavity, and intestines from 94 fattening pigs were sampled in three Belgian pig slaughterhouses (A, B, and C) during 24 sampling visits between April 2014 and June 2015. The slaughterhouses were located in Flanders (northern part of Belgium), where over 90% of Belgian pig production is located. The total production of the three slaughterhouses varied between 500.000 and 1.425.000 pigs/year. The slaughter capacity varied between 300 and 600 pigs/hour and in each of the slaughterhouses, pigs were slaughtered on one production line. Samples were taken during normal slaughter activities and 3 to 4 pigs were sampled during each sampling visit. One to three pigs were sampled per batch and pigs belonged to 55 different batches, originating from 52 farms. Oral cavity swabs (inside of the cheek, approx. 100 cm^2) were taken just before evisceration when pigs entered the clean zone, alternating per pig between the left and right side (e.g. for the first pig the left inner side of the cheek, second pig right side, third pig left side, etc.) using cellulose sponges (Sponge-Stick, 3M, Diegem, Belgium). The intestinal tract of the same pig was collected immediately after evisceration and the rectum was closed with threads, after which it was excised and put in a plastic bag. Tonsils were aseptically excised after removal of the pluck and put on a sterile surface. The surface of the tonsils (approximately 30 cm^2) was swabbed using a sterile cotton wool stick (tonsillar swab sample), after which the tonsils were put in a sterile plastic bag. All samples were transported to the laboratory under refrigerated conditions and processed within 4 h after sampling.

Twelve grams of tonsillar tissue and 15 g of rectal content were homogenized in Buffered Peptone Water (BPW; Bio-Rad, Marnes-la-Coquette, France) (1:10 w:w) using a stomacher apparatus (Colworth Stomacher 400, Seward Ltd, London, UK). Oral cavity swabs and tonsil swabs containing respectively 50 mL and 15 mL of BPW were stomached prior to analysis.

2.2. Enumeration of indicator bacteria

To quantify total aerobic bacteria, *Enterobacteriaceae* and *E. coli*, 100 μL of the initial suspension (10^{-1}) and 100-fold serial dilutions (up to 10^{-3} for tonsils and 10^{-5} for the rectal content and the oral cavity) were inoculated using a spiral plating apparatus (Eddie Jet, IUL Instruments, Barcelona, Spain). Colonies were enumerated using a counting grid (according to ISO 4833-2:2013, Annex A). For swab samples (tonsils and oral cavity), 1 mL of the initial suspension was additionally inoculated on two agar plates by spread plating. Plate Count Agar (PCA; Bio-Rad) was incubated at 30 °C for 48 h to determine the number of total aerobic bacteria. Violet Red Bile Glucose (VRBG; Bio-Rad) agar was incubated at 37 °C for 24 h for the enumeration of *Enterobacteriaceae*. Tryptone Bile X-glucuronide medium (TBX; Bio-Rad) was incubated at 44 °C for 24 h to quantify β-glucuronidase-positive *E. coli*.

2.3. Detection and enumeration of Salmonella spp.

To detect and to enumerate *Salmonella*, one mL of each of the initial suspensions was spread plated on two Xylose Lysine Deoxycholate (XLD; Bio-Rad) agar plates. Additionally, 100 μl of the initial suspension was inoculated on another XLD agar plate using the spiral plating apparatus, after which the plates were incubated at 37 °C for 24 h. Typical *Salmonella* colonies were counted and up to five randomly selected colonies per sample were tested as described below. The number of *Salmonella* per gram or per cm^2 was calculated from the number of colonies that was counted, corrected according to the proportion of confirmed *Salmonella* isolates.

All samples were also tested semi-quantitatively by analysing different test portions for the presence of *Salmonella*. The detection method for *Salmonella* was based on ISO 6579:2002/Amd 1:2007 (Annex D). After pre-enrichment of the homogenate at 37 °C for 18 h, 0.1 mL of BPW (dispersed in three drops) was transferred onto Modified Semi Solid Rappaport Vassiliadis medium (MSRV; LabM, Lancashire, UK) and incubated at 41.5 °C for 24 h. For MSRV plates showing a migration zone, a 1-μl loop of the migration zone was streaked on an XLD agar plate, which was incubated at 37 °C for 24 h. One or two suspected colonies per plate were tested and confirmed as described below. For tonsils and rectal content samples, a 10-mL and 100-mL aliquot of the homogenate was tested, corresponding to 1 g and 10 g, respectively. Samples for which the 10-g portion (100 mL homogenate) was positive and the 1-g portion (10 mL homogenate) was negative, were assumed to be contaminated between 1 CFU/10 g ($= -1$ $\log_{10}$ CFU/g) and 1 CFU/1g ($= 0$ $\log_{10}$ CFU/g). Samples for which the 1-g portion was positive and direct plating was negative, were assumed to be contaminated between 1 CFU/g ($= 0$ $\log_{10}$ CFU/g) and 10 CFU/g ($= 1$ $\log_{10}$ CFU/g, the detection limit of the direct plating method). For oral cavity swabs, 4-mL and 40-mL of homogenate were tested (corresponding to 8 and 80 cm^2, respectively). For tonsillar swabs, 10 mL of homogenate was tested, which corresponds to 20 cm^2. Semi-quantitative estimation of the *Salmonella* numbers was performed as described for the tonsillar tissue and rectal content samples (see above).

2.4. Identification and serotyping of Salmonella

Suspected *Salmonella* isolates were tested biochemically (triple sugar iron, indole reaction and lysine decarboxylase test) and at least two presumptive isolates per sample volume tested were further characterized. All *Salmonella* positive isolates ($n = 331$) were characterized by enterobacterial repetitive intergenic consensus (ERIC) PCR and clustered together to select isolates for serotyping (Rasschaert et al., 2005). Thirty-eight representative isolates (at least two isolates per cluster) were serotyped by slide agglutination following the Kauffmann-White Scheme (Grimont and Weill, 2007). All *S. Typhimurium* or monophasic variants (4, [5],12:i:) of *S. Typhimurium* ($n = 244$) were differentiated using a PCR assay according to Tennant et al. (2010).

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