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Contamination of freshly slaughtered pig carcasses with enteropathogenic *Yersinia* spp.: Distribution, quantification and identification of risk factors

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ABSTRACT

A cross-sectional survey was undertaken to determine the overall prevalence of enteropathogenic *Yersinia* spp. in the tonsils, feces and on carcasses of pigs at slaughter. Moreover, factors associated with *Yersinia* contamination of freshly eviscerated pig carcasses were studied. *Yersinia enterocolitica* serotype O:3 was isolated from the tonsils and feces of 55.3% and 25.6% of pigs, and *Y. pseudotuberculosis* from 1.4% and 0.6%, respectively. The pathogens were also recovered from 39.7% of carcass surfaces post-evisceration. The highest prevalence was found at the mandibular region (28.9%), followed by the sternal region (16.4%), pelvic duct (7.8%), and split surface near the sacral *vertebrae* (6.9%). Regarding the quantification of the pathogen, the median concentration of pathogenic *Y. enterocolitica* was 4.14 $\log_{10}$ CFU/g in tonsils with countable numbers ($n = 143$) and 2.80 $\log_{10}$ CFU/g for fecal samples with countable numbers ($n = 26$). The quantitative load on the carcass surface was generally low as the majority of the carcass samples (97.0%) had *Yersinia* concentrations below the detection limit of enumeration (<1.30 $\log_{10}$ CFU/100 cm^2). The initial presence of *Y. enterocolitica* in the tonsils and/or feces was significantly associated with carcass contamination at all sampled areas. Other risk factors for carcass contamination are the splitting of the head together with the carcass, and incision of the tonsils during removal of the pluck. Small adaptations in slaughter practices and the training of slaughterhouse personnel to respect basic hygienic instructions may diminish carcass contamination with enteropathogenic *Yersinia*.

1. Introduction

Pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are responsible for around 7000 reported infections in humans annually in the European Union (EFSA and ECDC, 2013). Common manifestations of yersiniosis are enterocolitis, pseudoappendicitis and post-infectious reactive arthritis, but in some cases more severe infections, such as septicemia, can occur (Mischnik et al., 2012; Rosner et al., 2010; Zheng et al., 2008). Most infections are sporadic and are caused by *Y. enterocolitica* bioserotype 4/O:3, whereas *Y. pseudotuberculosis* infections account for less than 1% of yersiniosis cases (EFSA and ECDC, 2013). Contaminated raw or undercooked pork meat is particularly important in transmitting *Y. enterocolitica* to humans (Rosner et al., 2012). As such, Fosse et al. (2008) estimated that 77.3% of human yersiniosis cases are attributed to the consumption of pork.

Pigs have been identified as important reservoirs of human pathogenic *Y. enterocolitica*, particularly bioserotype 4/O:3 (Bucher et al., 2008; Virtanen et al., 2013). *Y. pseudotuberculosis* is also recovered from healthy pigs, though to a lesser extent than pathogenic *Y. enterocolitica* (Laukkanen et al., 2010; Novoslavskij et al., 2013). In pigs at slaughter, the pathogens are most commonly found in the tonsils (Bucher et al., 2008), in which concentrations up to 10^6 yersiniae per gram can be found (Van Damme et al., 2010; Vanantwerpen et al., 2014). Infected pigs may also carry pathogenic *Y. enterocolitica* in their intestinal content, and mesenteric and submaxillary lymph nodes, although the isolation rate is lower than for tonsils (Gürtler et al., 2005; Nesbakken et al., 2003). These infected tissues have been known to contaminate pork carcasses with enteropathogenic yersiniae during slaughter (Borch et al., 1996; Laukkanen et al., 2008). As pigs are asymptomatic carriers of the pathogens, infected pigs and contaminated carcasses cannot be identified by current meat inspection methods.

For public health, pathogenic *Y. enterocolitica* are among the most important biological hazards related to pigs and pork (EFSA, 2011). In order to control the spread of the pathogen to carcasses, a better insight in the epidemiology of enteropathogenic yersiniae is required. Moreover, the concentration of *Yersinia* in pigs at slaughter has not been extensively studied, but may influence the probability of carcass contamination, which subsequently may influence the risk of human

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yersiniosis. There is a current lack of quantitative data on *Yersinia* present on pork carcasses, although such information is important for quantitative risk assessments. Identification of the risk factors responsible for carcass contamination is needed to understand how pathogenic *Y. enterocolitica* are disseminated during pig slaughter. However, no comprehensive study has yet been conducted to determine risk factors associated with carcass contamination during the evisceration process of pigs.

Therefore, the aims of this study were to determine the occurrence, contamination level and bioserotype distribution of enteropathogenic *Yersinia* spp. on freshly eviscerated pork carcasses and to identify risk factors associated with the presence of these pathogens on pork carcasses during slaughter.

2. Materials and methods

2.1. Sampling

2.1.1. Study design

Nine pig slaughterhouses were randomly selected out of the 22 slaughterhouses in Belgium that slaughter more than 100,000 fattening pigs per year (based on slaughter data from 2009). The annual number of slaughtered fattening pigs in the selected slaughterhouses varied from about 135,000 to 1,250,000. Each slaughterhouse was visited 4 times during the period of August 2010 to August 2011. All sampling visits were performed on Mondays and were evenly distributed over the 1-year-period to account for seasonal differences in prevalence. On each sampling day, ten pigs in one slaughterhouse were sampled during normal slaughter operations.

2.1.2. Collection of samples at the slaughterhouse

Tonsils, feces and carcass swabs from 360 pigs were sampled during 36 different visits. The first sampled pig on each sampling day was randomly selected within 15 min after the evisceration of the first pig slaughtered that day. The following nine pigs were sampled every 15 min. The sampled pigs originated from 191 different farms (range 1–14 pigs per farm). Each selected carcass was marked just before the start of evisceration and the corresponding batch number was recorded. The whole intestinal tract 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 excised aseptically after removal of the pluck and put in a sterile plastic bag. The corresponding carcass was swabbed after splitting, but prior to cooling. Four different areas, each of approximately 100 cm^2 were swabbed using cellulose sponges (3M™ Sponge-Stick, 3M, Diegem, Belgium): (1) the pelvic duct, (2) the split surface near the sacral *vertebrae*, (3) the sternal region (breast cut and surrounding skin), and (4) the mandibular region and, where possible, the masseter muscle, but avoiding the region of the tonsils. Each sponge was hydrated by adding 20 mL phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts (PMB) to the sample bag immediately before sampling. Alternating between each pig, all swabs were taken either from the right or left carcass half. All samples were transported to the laboratory under cooled conditions and processed immediately upon arrival.

2.2. Isolation and enumeration of enteropathogenic Yersinia spp.

A total of 2160 samples were analyzed for enteropathogenic *Yersinia* spp. using (i) direct plating, (ii) selective enrichment and (iii) cold enrichment as described previously (Van Damme et al., 2013). From tonsils and feces, an 11-g subsample was weighed aseptically and homogenized (Stomacher 400, Seward, Worthing West Sussex, United Kingdom) in 99 mL of PMB for 1 min. Swab sponges were stomached for 1 min just before starting the analyses. (i) For direct plating, 1 mL of homogenate was spread plated onto two cefsulodin–irgasan–novobiocin (CIN; Bio-Rad, Nazareth, Belgium) agar plates. For tonsils and feces, an additional CIN agar plate was inoculated with 100 μL homogenate using a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). (ii) For selective enrichment, 10 and 5 mL of PMB homogenate from tonsils/feces and carcass swabs were transferred into 90 and 45 mL of irgasan–ticarcillin–potassium chlorate (ITC Broth Base supplemented with 1 mg/l ticarcillin [Sigma Aldrich, Steinheim, Germany] and 1 g/l $KClO_3$ [Merck, Darmstadt, Germany]) broth, respectively, and incubated at 25 °C for 48 h. (iii) For cold enrichment, the remaining PMB homogenate was incubated at 4 °C for 7 and 14 days. After enrichment, ITC and PMB were streaked directly and/or after alkali treatment on CIN agar plates (Van Damme et al., 2013). All agar plates were incubated at 30 °C for 24 h for *Y. enterocolitica* and an additional 24 h at room temperature for the detection of *Y. pseudotuberculosis*. Plates were examined for characteristic *Yersinia* colonies using a stereo microscope with Henry illumination. Additionally, after direct plating suspect colonies were enumerated.

Presumptive positive colonies were characterized biochemically using urea broth and Kligler Iron Agar (Oxoid) and transferred into Tryptone Soy Broth (TSB) for further molecular identification. One hundred microliters of incubated TSB (24 h at 30 °C) was used to extract DNA using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, USA), following the manufacturer's instructions. *Yersinia enterocolitica* isolates were confirmed by two multiplex PCR assays. A PCR was used to detect the *ail* and *ystA* gene on the chromosome of pathogenic *Y. enterocolitica*, and the *virF* gene on the virulence plasmid of pathogenic *Yersinia* (Harnett et al., 1996). Isolates were regarded as pathogenic *Y. enterocolitica* when the PCR reaction was positive for the *ail* and *ystA* gene, regardless of the presence of the *virF* gene. Subsequently, a PCR was performed to detect the most common serotypes, targeting the *rfbC* gene in *Y. enterocolitica* serotype O:3 (Weynants et al., 1996) and the *per* gene in *Y. enterocolitica* serotype O:9 (Jacobsen et al., 2005). *Yersinia enterocolitica* strains that did not belong to serotype O:3 or O:9 based on PCR serotyping, were serotyped by slide agglutination. *Yersinia pseudotuberculosis* isolates were confirmed using a PCR assay targeting the *inv* gene (Nakajima et al., 1992) and serotyped by slide agglutination using antisera O:1–O:6.

2.3. Data collection and analysis

All statistical analyses were carried out using Stata/MP 12.1 (StataCorp, 2011). Test results were recorded as binary variables (presence/absence of *Y. enterocolitica*) per sample type in Excel spreadsheets. The survey design corrected prevalence estimates were calculated using batch as primary sampling unit and the annual number of pigs slaughtered per slaughterhouse as sampling weight.

Slaughterhouse differences were analyzed using a logistic regression, including batch as random effect. Differences between carcass sites were analyzed using a logistic regression, including carcass as random effect. For the analysis of seasonal variation, data were pooled in four seasons and examined by mixed-effects logistic regressions with slaughterhouse and farm as random variables whenever required. Bonferroni corrections were applied for multiple testing. Results for *Y. pseudotuberculosis* were excluded from the statistical analyses as the number of positive samples was too low.

A questionnaire was completed at the time of sampling to collect information about specific slaughter practices (Table 1). Univariate logistic regressions were used to examine the association between the presence of *Y. enterocolitica* serotype O:3 on each of the four sampled carcass sites as dependent variable and different slaughter practices and the presence of the pathogen in tonsils and rectum as independent variables. Only highly contaminated tonsils and feces (positive by direct plating) were retained in the risk factor analysis to limit the effect of contamination during slaughter. A backwards elimination process was used in which all variables, significant at $p < 0.05$ in the univariate analysis, were included in the initial multivariate model (including all