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Prevalence and genetic diversity of enteropathogenic *Yersinia* spp. in pigs at farms and slaughter in Lithuania

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ABSTRACT

The prevalence of enteropathogenic *Yersinia* spp. in pigs at farms and slaughter in relation to potential farming risk factors in Lithuania was examined. Pig faeces and carcase swab samples from 11 farms were studied at slaughterhouses. Nine of the 11 farms were visited again 3–5 months later, and pooled feacal samples and environmental samples were collected. Pathogenic *Yersinia enterocolitica* was found in 64% and *Yersinia pseudotuberculosis* in 45% of the sampled pig farms. All obtained isolates belonged to bioserotypes 4/O:3 and 2/O:3, respectively. Low biosecurity level was associated with a high prevalence of *Y. enterocolitica* on farms. Characterization with PFGE of 64 *Y. enterocolitica* and 27 *Y. pseudotuberculosis* isolates revealed seven and two different genotypes, respectively. Dominant enteropathogenic *Yersinia* spp. genotypes were obtained in both pig feacal and carcase samples. The high contamination of pig carcases (25%) with enteropathogenic *Yersinia* spp. may be an important factor contributing to the high incidence of human yersiniosis in Lithuania.

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are important foodborne pathogens (Bottone, 1999; Nuorti et al., 2004). Yersiniosis is one of the three leading foodborne zoonoses in Lithuania, and an increase in the number of human cases over the last decade has been reported (Anon., 2007, 2012). The incidence of 12.86 per 100,000 population in Lithuania was the highest among European Union (EU) member states in 2010 (Anon., 2012). Several studies have linked outbreaks of human yersiniosis to the consumption of contaminated foods, including pork meat and vegetables, as well as water (Fredriksson-Ahomaa and Korkeala, 2003; Nuorti et al., 2004). Pigs are of particular importance in *Yersinia* spp. epidemiology, as they are the main carriers and source of human enteropathogenic *Y. enterocolitica*, especially bioserotype 4/O:3, and *Y. pseudotuberculosis* bioserotype 2/O:3 (Bottone, 1999; Niskanen et al., 2008). Pig carcases can be contaminated with enteropathogenic *Yersinia* spp. during slaughter, and contamination usually occurs from faeces and tonsils (Laukkanen et al., 2008, 2009).

Studies have shown that the prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* in pigs varies considerably among farms (Andersen et al., 1991; Letellier et al., 1999; Gürtler et al., 2005; Niskanen et al., 2008). Some studies suggest that *Y. enterocolitica* is more common in conventional than organic production (Nowak et al., 2006; Virtanen et al., 2011) and on high-capacity farms than low-capacity farms (Laukkanen et al., 2009). However, more recent study suggests that no significant difference exists between production types or production capacities (Laukkanen et al., 2010). The prevalence of *Y. pseudotuberculosis*, by contrast, was higher in organic production than in conventional production and on conventional farms with high rather than low production capacity (Laukkanen et al., 2008). Thus, many factors may affect the prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* on different pig farms. Identification of these factors is crucial for establishing adequate control measures to minimize the prevalence of enteropathogenic *Yersinia* spp. at both the farm and slaughterhouse levels. Despite the impact of yersiniosis on human health in Lithuania, the prevalence and genetic diversity of enteropathogenic *Yersinia* spp. have not been investigated. This study examined the prevalence of *Y. enterocolitica* and *Y. pseudotuberculosis* in pigs on farms and at slaughter in Lithuania, the genetic variability of enteropathogenic *Yersinia* spp., and potential risk factors contributing to the higher prevalence of these pathogens in pigs.

Eleven pig farms located in seven of 10 counties in Lithuania, representing intensive pig production regions, were investigated for the prevalence of enteropathogenic *Yersinia* spp. in 2009–2010. Tested farms produce approx. 25% of the total pigs (800,000) grown in Lithuania each year. Fattening pigs aged

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6 months were sampled at slaughterhouses and farms. Pig feacal and carcase swabs were collected from two slaughterhouses at which the same slaughtering technique was used. Sample collection was performed as described in our previous study (Novoslavskij et al., 2010). In total 110 feacal and 55 carcase swab samples (10 feacal and five carcase swab samples per farm) were collected from pigs originating from 11 farms. Three to five months later nine of these 11 farms were visited for sampling to examine on farm prevalence of *Yersinia* spp. Thus, 90 pooled feacal samples (10 pooled samples per farm) from fattening pigs and 45 environmental samples (five samples per farm) were collected. At each farm, fresh feacal samples collected from the floor of five fattening pigs per pen were pooled together into a sterile plastic bag (5×1 g) and suspended in 50 ml of PMB (Phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts). Environmental samples were collected by swabbing the surface of the floor between pens (two samples per farm), the tread surface of boots of farm workers (one sample per farm), and the surfaces of rodent traps (two samples per farm). Sample collection was performed using a 7.5 cm sterile gauze square moistened with 10 ml of 0.1% peptone water, which was then transferred into bottles containing 90 ml of PMB. All samples were transported at 4–6 °C to the laboratory on the day of collection.

Detection of enteropathogenic *Yersinia* spp. was performed using the cold enrichment method (21 days at 4 °C) in PMB according to Korte et al. (2004) and Niskanen et al. (2002) with further identification using the API 20E test (BioMèrieux, Marcy l'Etoile, France).

Confirmation of *Y. enterocolitica* and *Y. pseudotuberculosis* isolates was performed with multiplex PCR targeting *virF*, *ail*, *rfb*, *16SrRNA* and *wzz* genes according to Thisted Lambertz and Danielsson-Tham (2005), with minor modifications as described previously (Novoslavskij et al., 2010). *Y. enterocolitica* O:3 DSM 13030 and *Y. pseudotuberculosis* III HH 146–36/84 strains were used as positive controls. A negative control of sterile water instead of the DNA template was also prepared.

Biotyping of *Y. enterocolitica* and *Y. pseudotuberculosis* isolates was performed according to Wauters et al. (1987) and Tsubokura and Aleksić (1995), respectively. Serotyping was done using a slide agglutination test with commercial antisera O:1–O:6 for *Y. pseudotuberculosis* and O:3 for *Y. enterocolitica* (Denka Seiken, Tokyo, Japan).

In total, 64 *Y. enterocolitica* and 27 *Y. pseudotuberculosis* isolates were characterized by Pulsed-field gel electrophoresis method (PFGE) according to Fredriksson-Ahomaa et al. (1999). One to three isolates for each *Yersinia* spp. positive sample were studied. Typing by PFGE was carried out as described by Fredriksson-Ahomaa et al. (1999) and Niskanen et al. (2002). Restriction patterns were analyzed visually and with BioNumerics software version 5.1 (Applied Maths, Sint Martens-Latem, Belgium).

Associations between enteropathogenic *Yersinia* spp. and farm factors were evaluated according to data collected using a questionnaire submitted to veterinarians and the first author's personal on-farm observations. The questionnaire focused on production type, management practices, pest and pet monitoring and control, and farm hygiene (Table 1). Farm biosecurity level was assessed according to the presence or lack of disinfection barriers for farm incoming transport, disinfection barriers for worker's footwear near the entrance to piggeries and a fence around the farm. Biosecurity was rated high only if all these measures were present.

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A two-stage procedure (univariable and multinomial logistic regression) was applied to assess the relationship between explanatory variables on the questionnaire and the number of *Y. enterocolitica* and *Y. pseudotuberculosis*-positive samples found on farms. Correlations between farm factors and the number of positive *Y. enterocolitica* and *Y. pseudotuberculosis* feacal samples collected on farms were also calculated.

Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* were found in 64% and 45% of sampled pig farms, respectively, in Lithuania. A farm was considered positive when at least one feacal sample was positive for *Y. enterocolitica* or *Y. pseudotuberculosis*. The number of contaminated pig pens among different positive farms varied from 10% to 70% for *Y. enterocolitica* and from 10% to 40% for *Y. pseudotuberculosis* (Table 2). Environmental samples from the floor, boots, and rodent traps collected at eight of nine tested farms were negative for enteropathogenic *Yersinia* spp. One floor swab sample from farm B and one from worker's boots were positive for *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively. No seasonality in shedding enteropathogenic *Yersinia* spp. in our study was tested. Other studies have shown the farm prevalence of enteropathogenic *Yersinia* spp. in pigs varies and could reach 100% depending on the country, and the selected farms and detection methods used (Ortiz Martínez et al., 2009, 2011). In this study, the farm prevalence of enteropathogenic *Yersinia* spp. in pigs (64%) and the detection rate of *Y. enterocolitica*-positive (18% and 22%) and *Y. pseudotuberculosis*-positive (10% and 9%) feacal samples collected at slaughterhouses and farms in Lithuania are similar to those reported in neighboring countries such as Latvia and the Leningrad region of Russia (Ortiz Martínez et al., 2009; Terentjeva and Berzins, 2010). We were unable to detect enteropathogenic *Yersinia* spp. on 36% of farms (4/11, farms: C, E, G and J). This finding is consistent with other studies that propose that enteropathogenic *Yersinia*-negative herds can exist despite high prevalence rates (Pilon et al., 2000; Ortiz Martínez et al., 2009; Terentjeva and Berzins, 2010). While enteropathogenic *Yersinia* spp. are known to be present in pig tonsils, lymphatic nodes and digestive tract, levels in these sites vary with age. In infected herds, young pigs start to shed in their faeces at 12–14 weeks, with maximal sheding at 19–20 weeks (Gürtler et al., 2005; Nesbakken et al., 2006). At slaughter age (26 weeks) pigs can carry enteropathogenic *Yersinia* spp. in their tonsils without shedding the agent in the faeces (Niskanen et al., 2008; Laukkanen et al., 2009). On this basis and given the age of pigs at the time of sampling in this study, false-negative detection may have occurred.

Samples from pig carcases were collected after the carcase inspection when tonsils and tongues are already removed. Swab sampling of the area of the removed tonsils was done in addition to sampling of tonsils to enable evaluation of carcase cross-contamination. Twenty-five percent of carcases tested in this study were contaminated with enteropathogenic *Yersinia* spp. This rate is higher than reported in some other studies (Fukushima et al., 1989; Frederiksson-Ahomaa et al., 2000; Nesbakken et al., 2003; Gürtler et al., 2005). Although, one recent study reported the rate of enteropathogenic *Y. enterocolitica*-contaminated pig carcases was as high as 26% (Laukkanen et al., 2010). The high prevalence of enteropathogenic *Yersinia* spp. on pig farms and poor slaughter hygiene resulting in carcase contamination may represent a significant public health risk, particularly in light of the high rates of human yersiniosis in Lithuania.

In total, 63 (*virF*, *ail*, *rfb*, and *16SrRNA*-positive) and one (*virF*-negative; *ail*, *rfb*, and *16SrRNA*-positive) *Y. enterocolitica* and 27 (*virF* and *wzz*-positive) *Y. pseudotuberculosis* isolates were recovered from samples collected at slaughterhouses and pig farms. All *Y. enterocolitica* isolates were confirmed as bioserotype 4/O:3 and all *Y. pseudotuberculosis* isolates as bioserotype 2/O:3. These results support the finding of other studies indicating that *Y. enterocolitica* 4/O:3 is the most common bioserotype circulating in the Baltic region and in continental Europe (Ortiz Martínez et al., 2009, 2011).

From 64 *Y. enterocolitica* isolates (collected from 54 positive samples), seven and three PFGE patterns were obtained using *NotI*