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PREVALENCE OF *YERSINIA ENTEROCOLITICA* AND *YERSINIA PSEUDOTUBERCULOSIS* IN SLAUGHTERED PIGS WITHIN 5 MONTHS PERIOD IN LITHUANIA

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Summary. The aim of the present work was to determine the prevalence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* bacteria in slaughtered pigs from Lithuanian pig farms using multiplex Polymerase Chain Reaction (PCR) method. A total of 105 microbiological samples (70 faecal samples and 35 carcass swab samples) were collected and examined from slaughtered pigs from seven different Lithuanian pig farms. *Yersinia* spp. bacteria were isolated using cold enrichment method (21days at 4ºC) in PMB broth with further planting on CIN agar. The results of our study showed that using multiplex PCR method pathogenic *Yersinia* spp. was found in slaughtered pig samples delivered from 6 of 7 (86%) different farms. Four tested pig herds were contaminated with both pathogens *Y. enterocolitica* and *Y. pseudotuberculosis*, while the remaining herds were contaminated only with *Y. enterocolitica*. *Yersinia* spp. were identified in 21 (30%) of faecal and in 7 (20%) of carcass swab samples respectively. *Yersinia enterocolitica* was more prevalent in faecal samples (19%) than in carcass swab samples (17%) and *Y. pseudotuberculosis* was found in 3% of carcass and 11% of faecal samples, respectively. All obtained isolates of *Y. enterocolitica* were identified as serotype O:3. No other pathogenic bacteria serotypes were found in our study.

To our knowledge, no report of *Yersinia* spp. isolation and identification from slaughtered pigs from Lithuanian pig farms in Lithuania was made before. The results obtained in the present study could serve for future investigations of the *Yersinia* spp. infection, mainly focusing on the possible contamination routes at the pork production and possibility of prevention at farm level.

Keywords: *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, PCR, prevalence, pigs.

YERSINIA ENTEROCOLITICA IR *YERSINIA PSEUDOTUBERCULIOSIS* BAKTERIJŲ PAPLITIMAS LIETUVOJE SKERDŽIAMŲ KIAULIŲ BANDOSE 5 MĖN. LAIKOTARPIU

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Santrauka. Tyrimo tikslas buvo dauginės polimerazės grandininės reakcijos (dauginė PGR) metodu nustatyti *Yersinia enterocolitica* ir *Yersinia pseudotuberculosis* bakterijų paplitimą Lietuvoje užaugintų kiaulių bandos ir skerdenose. Ištirti 105 mikrobiologiniai mėginiai (70 išmatų, 35 skerdenos tamponavimo) kiaulių, užaugintų septyniose skirtingose Lietuvos kiaulių fermose. *Yersinia* spp. bakterijos išskirtos šaltuoju pagausinimo metodu PMB terpėje (21 diena 4ºC temperatūroje), vėliau – sėjant ant CIN agaro. Tyrimo metu nustatyta, kad dauginės PGR metodu *Yersinia* spp. bakterijos identifikuotos paskerstų kiaulių mėginiuose, surinktuose šešiose (86 proc.) kiaulių fermose. Keturiose kiaulių fermose rasta ir *Y. enterocolitia,* ir *Y. pseudotuberculosis* bakterijų, kitose – tik *Y. enterocolitica*. *Yersinia* spp. identifikuotos 21 (30 proc.) išmatų ir septyniuose (20 proc.) skerdenos tamponavimo mėginiuose. *Y. enterocolitica* bakterijų dažniau rasta išmatų (19 proc.) nei skerdenų mėginiuose (17 proc.). *Y. pseudotuberculosis* bakterijų rasta 3 proc. skerdenos ir 11 proc. išmatų mėginių. Visos *Y. enterocolitica* bakterijos priskirtos serotipui O:3, tačiau neradome jokių kitų šios bakterijos patogeninių serotipų.

Mūsų duomenimis, Lietuvoje iki šiol nėra žinoma apie *Yersinia* spp. bakterijų išskyrimą ir identifikavimą iš Lietuvoje išaugintų ir paskerstų kiaulių. Tikimės, kad šio tyrimo rezultatai bus naudingi atliekant tolimesnius tyrimus, susijusius su patogeninių *Yersinia* spp. bakterijų užsikrėtimo rizika kiaulienos gamybos grandinėje bei galimomis prevencinėmis priemonėmis kiaulių fermose.

Raktažodžiai: *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, PGR, paplitimas, kiaulės.

Introduction. The genus *Yersinia* belongs to the family *Enterobacteriaceae*. Of the 12 species that comprise the genus *Yersinia*, three (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) are thought to be a serious pathogens for humans. *Y. pestis* is the causative agent of the bubonic plague, and *Y. pseudotuberculosis* and *Y. enterocolitica* are considered serious intestinal pathogens for many animal species as well as for humans

(Bottone, 1999; Niskanen *et al.,* 2008; Platt-Samoraj *et al.,* 2006; Sprague and Neubauer, 2005; Tennant *et al.,* 2005). In 2008, a total of 8346 confirmed cases of yersiniosis were reported from 27 European Union countries, corresponding to an EU incidence of 1.8 cases per 100 000 population. Yersiniosis is one of the three most prevalent foodborne zoonoses in humans with the incidence of 15.9 per 100 000 population in Lithuania. The increase of human yersiniosis cases is observed in Lithuania during last five years. Moreover, the incidence of 15.9 per 100 000 population in 2008 was one of the biggest among European Union member states (The EFSA Journal, 2010).

According to EFSA statistical analysis *Y. enterocolitica* was the most common species reported in human yersiniosis outbreaks in 2008. Bacteria was isolated from 91.9% of all confirmed cases and *Y. pseudotuberculosis* only represented 1.8% of all isolates, while no information was provided about the remaining 6.3% *Yersinia* species (The EFSA Journal, 2010).

Y. enterocolitica and *Y. pseudotuberculosis* have a nearly worldwide distribution and the organisms are found in the environment, including soil and water, and also in the intestinal tract of mammals and birds (Kämpfer, 2000; Kaneko and Hashimoto, 1981). *Y. enterocolitica* is frequently found in pigs, cattle, sheep, and even poultry (Fratamico *et al.* 2005). In Europe, pigs are considered the main reservoir of human pathogenic strains of *Y. enterocolitica* (Fredriksson-Ahomaa *et al.* 2006), while some authors report that wild birds are thought to be the main reservoirs of pathogenic *Y. pseudotuberculosis* strains (Fukushima and Gomyoda, 1991; Schiemann, 1989). Pigs are often asymptomatic carriers of the bacterium. *Y. enterocolitica* and *Y. pseudotuberculosis* are frequently found in tonsils and intestinal contents of clinically healthy pigs at slaughterhouses around the world (Fredriksson-Ahomaa *et al.,* 2006; Laukkanen *et al*., 2008). Some authors reported that *Y. enterocolitica* was isolated from 7.4% to 18% in faecal samples (Simonova *et al.,* 2008; Asplund *et al.,* 1990; Mafu *et al.,* 1989). Pig carcass contamination with *Y. enterocolitica* can vary up to 21% (Frederiksson Ahomaa *et al.,* 2000; Adesiyyun and Krishnan, 1995; Nesbakken *et al.,* 2003). The prevalence of *Y. pseudotuberculosis* has varied in different studies from 0.6% to 7% in faecal samples (Chiesa *et al.,* 1993; Fukushima *et al.,* 1987; Laukkanen *et al.,* 2008) and from 0.3% to 3% in carcass samples (Fukushima *et al.,* 1989; Laukkanen *et al.,* 2008).

Y. enterocolitica bacteria are divided into 6 biotypes (1A, 1B, 2, 3, 4 and 5) (The EFSA Journal, 2007) and 60 serotypes (Wannet *et al.* 2001). Most *Y. enterocolitica* strains associated with human yersiniosis belong to bioserotypes 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3 (Fredriksson-Ahomaa and Korkeala, 2003). It is worth to mention that high similarity between pig and human strains has been demonstrated with several DNA-based methods (Fredriksson-Ahomaa *et al.,* 2006). All *Y. pseudotuberculosis* strains (serotypes I to V) are potentially pathogenic for humans (The EFSA Journal, 2007).

The aim of the present study was to determine the prevalence of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* bacteria in slaughtered pigs from Lithuanian pig farms using multiplex Polymerase Chain Reaction (PCR) method.

Materials and methods. *Sampling.* The pig samples were collected at the slaughterhouses from May till September in 2009. A total of 105 microbiological samples were collected (70 faecal samples (10 samples per farm) and 35 carcass swab samples (5 samples per farm)). Swine faecal and carcass samples from seven different Lithuanian pig farms (in total 15 samples per farm) were collected at the slaughter line after meat inspection before chilling. Faecal samples were taken from the large intestine using sterile plastic spoon and 1 g of each sample was transferred into tubes containing 10 ml of PMB (Phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts according to International Organization for Standardization protocol ISO/DIS 10273). Carcass samples were collected by swabbing thoracic and pelvic cavities of both halves of the carcass with a 7.5 × 7.5-cm sterile gauze square moistened with 10 ml of 0.1% peptone water. Carcass swabs were transferred into bottles containing 90 ml of PMB. Samples were stored cold during transportation and delivered to the laboratory on the same day.

Isolation of Y. enterocolitica and Y. pseudotuberculosis. Samples were analyzed at the laboratory of the Department of Food Safety and Animal Hygiene, Lithuanian Veterinary Academy, Kaunas, Lithuania. Detection of *Yersinia* spp. was done using cold enrichment method (21 day at 4ºC) in PMB (Phosphate-buffered saline supplemented with 1% mannitol and 0.15% bile salts) and subsequent plating on a cefsulodin-irgasan-novobiocin (CIN) agar (Yersinia selective agar base, Liofilchem, Via Scozia, Italy). Alkali treatment (0.5 ml of the sample was mixed with 4.5 ml of 0.25% KOH solution for 20 s) was made before streaking onto CIN agar to reduce background contaminating flora. Further a 100 µl aliquot was plated on CIN agar and all plates were incubated at 30ºC for 20 h. To recover *Y. pseudotuberculosis*, a further incubation of the agar plates at 22°C for 24-48 h was used. Up to five whole small (diameter, <1 mm) colonies with typical a bull's eye appearance (deep red centers surrounded by outer transparent zones) on each CIN agar plate were taken and streaked onto BHA agar plates (Brain heart infussion agar, Liofilchem, Via Scozia, Italy) for pure culture. Agar plates were incubated at 28ºC for 24 h. All obtained isolates were tested for urea hydrolysis using a urea agar slant, and urea positive isolates were chosen for further identification using PCR reaction.

Template DNA isolation for PCR. Bacterial DNA was prepared using boiling method with sterile 0.9% NaCl. After growing the bacteria for 24h on BHA agar plates, a loop full of bacterial culture was taken and suspended in 1 ml of sterile 0.9% NaCl liquid. A tube was mixed well and centrifuged for 2 min at 8000 rpm with further supernant removing. A procedure was repeated twice and a quantity of 200 µl of 0.9% NaCl was added to the tube. The suspension was heated at 95ºC for 10 min, cooled on ice, mixed well and then centrifuged for 3 min at 14000

rpm. Supernant was transferred into a new tube. Extracted template DNA were used immediately for PCR amplification or stored at -20ºC until examination.

Detection and identification of Y. enterocolitica and Y. pseudotuberculosis by PCR. Multiplex PCR method was used for identification of *Yersinia* spp. as described by Thisted Lambertz and Danielsson-Tham (2005) with minor changes. A shorter 9A-ail gene sequence -TTA TCA ATT GCG TCT GTT AAT GTG TA- and 10A-ail gene sequence -ATC GAG TTT GGA GTA TTC ATA TGA AG- was used. Also instead of Pr2A-yst and Pr2c-yst genes sequences we used YeI-16SrRNA (-ATA CCG CAT AAC GTC TTC G-) and YeII-16SrRNA (-TTC TTC TGC GAG TAA CGT C-) genes as targets (Arnold *et al.,* 2004).

The PCR amplification was performed in a final volume of 25 µl containing 10 X reaction buffer (MBI Fermentas), 25 mM magnesium chloride (MBI Fermentas), 10 mM dNTP (MBI Fermentas), 5 U/µl of Taq DNA polymerase (MBI Fermentas), 5 µM of each of the oligonucleotide (Sigma Genosys, USA) and 6.9 µl of template DNA. The PCR amplification was performed on a PTC-100 Programmable thermal controller (MJ Research Inc., USA) at 94°C for 3 min, 30 cycles at 94°C for 30 s, 55°C for 60 s, 72°C for 60 s and the final amplification at 72°C for 5 min. A negative control with sterile water instead of DNA template was also prepared. PCR products were analyzed by electrophoresis: 10 µl volume of each PCR product was loaded onto a 1.3 % Top Vision LE GQ Agarose gel (MBI Fermentas) containing 0.05 µl/ml. of ethidium bromide solution. The gel was visualised on an UV board. The GeneRulerTM 100 bp DNA Ladder (MBI Fermentas) was used as the molecular size marker. Expected presence of DNA fragments indicating positive results for pathogenic *Yersinia* spp. bacteria is given in Table 1.

Table 1. **Primers used for identification of *Y. enterocolitica* and *Y. pseudotuberculosis***

Primer	Target-Gen	*Y. enterocolitica* O:3	*Y. enterocolitica* O:9,O:8,O:5,27	*Y. pseudotuberculosis*	Amplicon length (bp)	Primer sequence (Reference)
11A-virF 12A-virF	pYV-Plasmid	+	+	+	700	Thisted Lambertz *et al.*, (2005)
9A-ail 10A-ail	*ail* Gen chrom.	+	+	+	454	Strauch (personal communication, 2009)
rfbCa rfbCb	O3-Antigen chrom.	+	no	no	405	Weynants *et al.,* (1996)
Ypf-20210-wzz Ypf-20538-wzz	*wzz* Gen chrom.	no	no	+	418	Bogdanovitch *et al.,* (2003)
Yel-16SrRNA Yell-16SrRNA	16Sr DNA chrom.	+	+	no	328	Arnold *et al.,* (2004)

Identification of *Y. pseudotuberculosis* was done using the same protocol as described above only using specific primers for *Y. pseudotuberculosis* identification: forward primer Ypf-20210-wzz (-GGT GAT GAG CAA GTT CAA G-) and reverse primer Ypr-20538-wzz (-GCT AAA TCC ACT GCT CGC TG-) (Bogdanovitch *et al.,* 2003) instead of additional rfbCa and rfbCb primers.

Storage of pathogenic bacteria colony's recovered from samples. After subculturing the bacteria on BHA agar a loop full of bacteria was transferred to tube containing 1 ml. of BHI (Brain heart infusion broth, Liofilchem, Via Scozia, Italy) supplemented with glycerol with final 30% concentration. All samples were stored at minus 70ºC.

Results. In total 105 samples, comprising feacal samples and carcass swab samples from seven different pig farms were tested for present of *Y. enterocolitica* and *Y. pseudotuberculosis*. Pathogenic *Yersinia* spp. strains were identified in 21 (30%) of faecal and in 7 (20%) of carcass swab samples. *Y. enterocolitica* was more frequently found in faecal samples (19%) than in carcass swab samples (17%). *Y. pseudotuberculosis* was identified in 8 of 70 tested faecal samples and only in 1 of 35 tested carcass swab samples. In total 32 pathogenic *Yersinia* spp. strains were found in our study. The list of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* strains isolated from the feaces and carcass swabs samples are given in Table 2.

Table 2. **Presence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in faecal and carcass samples**

Sample material	n	No. (%) of *Y. enterocolitica* positive samples	No. (%) of *Y. pseudotuberculosis* positive samples
Faecal samples	70	13 (19)	8 (11)
Carcass swab samples	35	6 (17)	1 (3)
Total	105	19 (18)	13 (12)

All *Y. enterocolitica* isolated strains were identified as serotype O:3. No other pathogenic bacteria serotypes were found in this study. Pathogenic *Yersinia* spp. bacteria were found in 6 of 7 of tested pig farms. *Y. enterocolitica* was found in all positive pig farms and *Y. pseudotuberculosis* was found in four (57%) of seven tested farms. In any sample both pathogenic species were found.

Discussion. Pigs, which are often asymptomatic carriers of *Y. enterocolitica* and *Y. pseudotuberculosis* strains, are considered the main reservoir of the bacterium (Platt Samoraj *et al.,* 2006; Niskanen *et al.,* 2008). The organisms are present in the oral cavity, especially in the tonsils, submaxillar lymph nodes, and in the intestine and feaces of pigs. Most frequently bacteria are isolated from pig tonsils and less from pig carcass and feaces (Fredriksson-Ahomaa *et al.,* 2006; Niskanen *et al.,* 2008). Contamination of carcasses of pigs with pathogenic *Yersinia* during slaughter process from tonsils, intestinal content and feaces may occur. This factor increases the possibility of *Y. enterocolitica* and *Y. pseudotuberculosis* accessing the food chain and multiplying in food products due to its ability to grow in low temperatures (Fredriksson-Ahomaa *et al.,* 2000; Laukkanen *et al.,* 2008).

Y. enterocolitica was found in (86%) of our tested pig herds, and *Y. pseudotuberculosis* was found in four (57%) of seven tested farms. Four tested pig herds were contaminated with dual infection of *Y. enterocolitica* and *Y. pseudotuberculosis*, while the remaining herds were contaminated only with *Y. enterocolitica*. Prevalence of *Y. enterocolitica* among different tested pig herds varied 0%-70% in faecal and 0%-60% in carcass swab samples. Whereas prevalence of *Y. pseudotuberculosis* varied 0%-40% in faecal and 0%-20% in carcass swab samples. Compare to other studies prevalence of pathogenic *Y. enterocolitica* can range from 35% to 70% of herds and 4% to 100% of individual pig (Robins-Browne, 1997).

Two PCR protocols for pathogenic *Yersinia* spp. were used in this study. First PCR protocol can identify three groups of food-borne pathogenic *Yersinia* spp. isolates: a) pathogenic *Y. enterocolitica* O:3, b) pathogenic *Y. enterocolitica* serogroups other than O:3, and c) preliminary *Y. pseudotuberculosis* (Thisted Lambertz and Danielsson-Tham, 2005). Seconcd PCR reaction for *Y. pseudotuberculosis* confirmation was performed using Ypf-20210-wzz and Ypf-20538-wzz genes (Bogdanovitch *et al.,* 2003). The confirmation rate was 100% for preliminary *Y. pseudotuberculosis* from first PCR.

All *Y. enterocolitica* isolated strains were identified as serotype O:3. No other pathogenic bacteria serotypes were found in our study. It is worth to mention that *Y. enterocolitica* serotype O:3 is isolated more than 90% during human yersiniosis outbreaks (The EFSA Journal, 2010).

A relatively high prevalence of *Y. enterocolitica* (17%) in pig carcass swabbing samples was found in our study. The reason of such high prevalence can be cross-contamination possibility during the slaughter process. Slaughter techniques and slaughter hygiene may influence the contamination rate (Andersen, 1988). Also in this study the large area of carcasses was sampled (30 × 20 cm) in comparison to other studies (Simonova *et al.,*, 2007) and this factor could also influence relatively high number of positive for *Yersinia* carcass samples.

The results obtained in the present study shows a high *Yersinia* spp. infection rate in slaughtered pigs from different pig farms in Lithuania. To our known no investigation focused on prevalence of *Yersinia* spp. in Lithuanian slaughtered pigs was made before. Therefore, there is an increased importance of further investigations on the epidemiology of the *Yersinia* spp. infection, mainly focusing on the possible contamination routes at the pork production and possibility of prevention at farm level.

Conclusions. The results of our study showed that 86% of pig herds from different pig farms tested at slaughterhouses was contaminated with *Yersinia* spp. indicating the high prevalence of these human pathogenic herds bacteria in pig. Four tested pig herds were contaminated with both pathogens *Y. enterocolitica* and *Y. pseudotuberculosis*, while the remaining herds were contaminated only with *Y. enterocolitica*. More often *Y. enterocolitica* and *Y. pseudotuberculosis* was found in feacal samples (19% and 11%), than in the carcass samples (17%, 3%, respectively).

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