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PREVALENCE OF PATHOGENIC *YERSINIA ENTEROCOLITICA* IN MINCED MEAT, PIG TONGUES AND HEARTS AT THE RETAIL LEVEL IN THE CZECH REPUBLIC DETECTED BY REAL TIME PCR

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ABSTRACT

Yersiniosis is the third most frequently reported zoonosis in the European Union and *Yersinia enterocolitica* is the most common species causing human infections. Pigs are assumed to be the main reservoir of human pathogenic *Y. enterocolitica* with the presence of bacteria mainly in the tonsils and intestinal content. Undercooked pork and pork products have been suggested as the primary source of human yersiniosis. Nevertheless, data on the prevalence of pathogenic *Y. enterocolitica* in foodstuffs including pork products are very limited. A molecular based method (real time PCR) targeting the *ompF* gene (detection of *Yersinia* genus) and the *ail* gene (a chromosomally located virulence marker of *Y. enterocolitica*) was used to determine the prevalence of pathogenic *Y. enterocolitica* in minced meat and edible pork offal at the retail level in the Czech Republic. A total of 50 pig tongues, 50 pig hearts, and 93 samples of minced meat containing pork were purchased at nine retail outlets in Brno. High detection rates of *Yersinia* spp. were found in all types of samples (pig tongues, 80.0%; pig hearts, 40.0%; and minced meat, 55.9%). The highest prevalence of pathogenic *Y. enterocolitica* was found in pig tongues (40.0%), followed by pig hearts (18.0%) and minced meat samples (17.2%). Although from the point of view of food safety the merely molecular detection of DNA of the pathogenic bacteria could represent a false positive result, our results indicate the presence of pathogenic *Y. enterocolitica* in raw pork products at the retail level in the Czech Republic, which may pose a risk of consumer infection. Sufficient heat treatment and prevention of cross-contamination during preparation of food in the kitchen should be recommended.

Keywords: *Yersinia enterocolitica*; *ail* gene; *ompF* gene; real time PCR; pork products; retail; zoonosis

INTRODUCTION

In the European Union, yersiniosis was the third most frequently reported zoonosis in 2014, despite the significantly decreasing trend between 2008 and 2014. *Yersinia enterocolitica* was the most common species reported, having been isolated as the causative agent from 97.7% of the confirmed cases **(EFSA and ECDC, 2015)**. Clinical manifestations of human infection are usually fever, enterocolitis, pseudoappendicitis, and mesenteric lymphadenitis with diarrhoea, vomiting, and abdominal pain. Post-infection complications such as reactive arthritis or erythema nodosum can also emerge **(Galindo et al., 2011)**.

Pigs have been considered to be the primary reservoir for the pathogenic *Y. enterocolitica* that has been isolated especially from tonsils, tongues, or throats, and to a lower extent from faeces, which all can be a source of contamination for other parts of the carcasses during slaughter procedures **(Fredriksson-Ahomaa et al., 2000; Fredriksson-Ahomaa et al., 2001a; 2001b; Simonova et al., 2008; Van Damme et al., 2015)**. The European Food Safety Authority (EFSA) considers *Y. enterocolitica* as one of the most relevant biological hazards in the context of meat inspection of swine **(EFSA, 2011)**.

Eating of raw or undercooked pork and pork products (especially minced meat) has been strongly associated with human yersiniosis **(Tauxe et al., 1987; Grahek-Ogden et al., 2007)**. **Fosse et al., (2008)** estimated that 77.3% of clinical cases of yersiniosis in humans are connected with the consumption of pork and the same genotypes of *Y. enterocolitica* strains isolated from slaughterhouse environments, pork products in retail outlets and patients with yersiniosis support this hypothesis **(Fredriksson-Ahomaa et al., 2001a)**. Nevertheless, at present, there is no harmonised surveillance of pathogenic *Yersinia* in food and animals in the EU **(EFSA and ECDC, 2015)**.

Y. enterocolitica is a ubiquitous microorganism. However, not all strains recovered from food and environmental samples are pathogenic. From the point of view of health hazard, it is necessary to distinguish between pathogenic and non-pathogenic variants **(Fredriksson-Ahomaa and Korkeala, 2003; EFSA and ECDC, 2015)**. The use of traditional culture methods may lead to underestimation of pathogenic *Y. enterocolitica* in clinical, food and environmental samples. Pathogenic yersinia have seldom been isolated from pork or other foods except for edible pig offal, because of their usually small numbers in the samples, limited sensitivity of the culture media without the ability to distinguish between pathogenic and non-pathogenic strains and subsequent overgrowth of target organisms by background flora **(Fredriksson-Ahomaa and Korkeala, 2003;**

Laukkanen-Ninios et al., 2014; EFSA and ECDC, 2015).

More rapid, sensitive and specific DNA-based methods have provided a better estimation of the occurrence of pathogenic *Y. enterocolitica* in naturally contaminated samples even when the pathogen is initially present in low numbers **(Fredriksson-Ahomaa et al., 1999 and 2001c; Fredriksson-Ahomaa and Korkeala, 2003; Messelhäusser et al., 2011; Laukkanen-Ninios et al., 2014)**. However, subsequent isolation of *Y. enterocolitica* strains is needed for further strain characterization (especially for information on the biotype and serotype) and to assess its public health significance **(EFSA and ECDC, 2015)**.

The virulence of *Y. enterocolitica* results from a complex of plasmid- and chromosomally encoded genes. Because of easy loss of the virulence plasmid during laboratory handling, chromosomally located genes are more reliable target genes for PCR assays **(Fredriksson-Ahomaa and Korkeala, 2003; Galindo et al., 2011)**. The chromosomally located *ail* gene (attachment and invasion locus) is an essential virulence factor in strains of *Yersinia* spp. **(Miller et al., 1989)** and it is the most frequently used target to detect human pathogenic *Y. enterocolitica* **(Miller et al., 1989; Fredriksson-Ahomaa and Korkeala, 2003)**. An enrichment step prior to PCR is recommended to increase sensitivity and probability of detecting viable cells; false-positive results due to dead cells can be avoided **(Lambertz et al., 2007)**.

According to the EFSA report **(EFSA and ECDC, 2015)**, not enough information is available about the prevalence of human pathogenic *Y. enterocolitica* in foodstuffs at the retail level. The aim of this pilot study was to survey the prevalence of pathogenic *Y. enterocolitica* in edible pork offal (tongues and hearts) and minced meat at the retail level in the Czech Republic in order to estimate the risk of consumer infection via products of porcine origin.

MATERIAL AND METHODOLOGY

During the period of Juny 2014 to August 2015, a total of 50 pig tongues, 50 pig hearts, and 93 samples of individually packaged minced meat (50 pure pork and 43 mixed with 15 to 50% beef) were purchased from nine different butcher shops and supermarkets in Brno, Czech Republic. The samples were transported to the laboratory under refrigeration and were analysed immediately. A 25 g portion of each sample was cut into small pieces and homogenized in 225 mL PSB (phosphate buffered saline with sorbitol and bile salts, HiMedia, India) in a stomacher blender for 2 min and enriched at 25 °C for 18 – 20 h.

One millilitre of the enriched culture was centrifuged at 14,000 g for 5 min. The pellet was used for DNA isolation using the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) slightly modified to include mechanical homogenization with zirconia/silica beads (0.2 mm) in a MagNALyser instrument (Roche, Mannheim, Germany). An aliquot (5 μL) of extracted DNA was used as a template for home-made triplex real time PCR (qPCR) assay able to detect genus *Yersinia* (*ompF* gene) **(Stenkova et al. 2008**) and differentiate pathogenic *Y. enterocolitica* strains (*ail* gene) **(Lambertz et al., 2008)**. The previously published internal amplification control was used in the assay to eliminate false negative samples **(Slana et al., 2008)**.

RESULTS AND DISCUSSION

Based on a qualitative risk assessment of foodborne hazards associated with chilled pork carcasses, *Y. enterocolitica* was considered of high relevance **(EFSA, 2011)**. However, pigs are mostly asymptomatic carriers of pathogenic *Y. enterocolitica* without any signs of illness or macroscopic lesions. Thus, routine meat inspection practices cannot reveal infected pigs or contaminated carcasses and their products can enter the food chain.

In the Czech Republic, a growing number of cases of human yersiniosis were recorded in recent years **(Dr. Cestmir Benes, The National Institute of Public Health, Czech Republic, personal communication)**. However, no systematic monitoring of the occurrence of pathogenic *Yersinia* spp. in animals and in food is performed. In previous studies, pathogenic *Y. enterocolitica* has been recovered from smears from pig tongues (from 1.1 to 27%), tonsils (7.5%), rectal content (7.4%), and skin surface (2.8%) obtained from different slaughterhouses in the Czech Republic **(Aldová and Švandová, 1984; Aldová et al., 1990; Vázlerová and Steinhauserová, 2006; Simonova et al., 2008)**. However, no information is available about the presence of pathogenic *Y. enterocolitica* in pork products at the retail level.

In the present study, we have found a high prevalence of *Yersinia* spp. in all three types of collected samples (Figure 1) with the highest contamination level in pig tongues (80.0%), followed by minced meat (55.9%) and pig hearts (40.0%). However, as was shown previously, the majority of *Yersinia* isolates obtained from food and environmental samples are non-pathogenic without any clinical importance **(Fredriksson-Ahomaa and Korkeala, 2003)**.

This fact is in accordance with our results because the detection rate of pathogenic *Y. enterocolitica* in the examined samples was considerably lower compared with the contamination by *Yersinia* in general (Figure 1). The highest positivity was found in pig tongues (40.0%) and then in hearts (18.0%) and minced meat samples (17.2%). Using PCR methods, even higher contamination rates with pathogenic *Y. enterocolitica* in pig tongues at the retail level were detected in previous European studies: 44.9% in Bavaria (Germany) **(Messelhäusser et al., 2011)**, 83% **(Fredriksson-Ahomaa et al., 2001c)** and 92% **(Fredriksson-Ahomaa et al., 1999)** in Finland. Pathogenic *Y. enterocolitica* was found also in 50% of pig hearts from retail shops in Finland, however, only a small number of samples (n = 8) were investigated **(Fredriksson-Ahomaa et al., 2001c)**. In the abovementioned studies, the prevalence of pathogenic *Y. enterocolitica* was found to be higher with PCR than with culture methods, which indicates a higher sensitivity of the PCR method for detection of pathogenic *Y. enterocolitica* in naturally contaminated samples.

The high prevalence of *Y. enterocolitica* in pig tongues and other offal might be caused by cross-contamination by tonsil tissue during the slaughtering process **(Fredriksson-Ahomaa et al., 2000; 2001b; Messelhäusser et al., 2011)**. Preventing contamination completely is practically impossible because the tonsils are removed and hung together with tongue, liver, lung and heart on a hook after

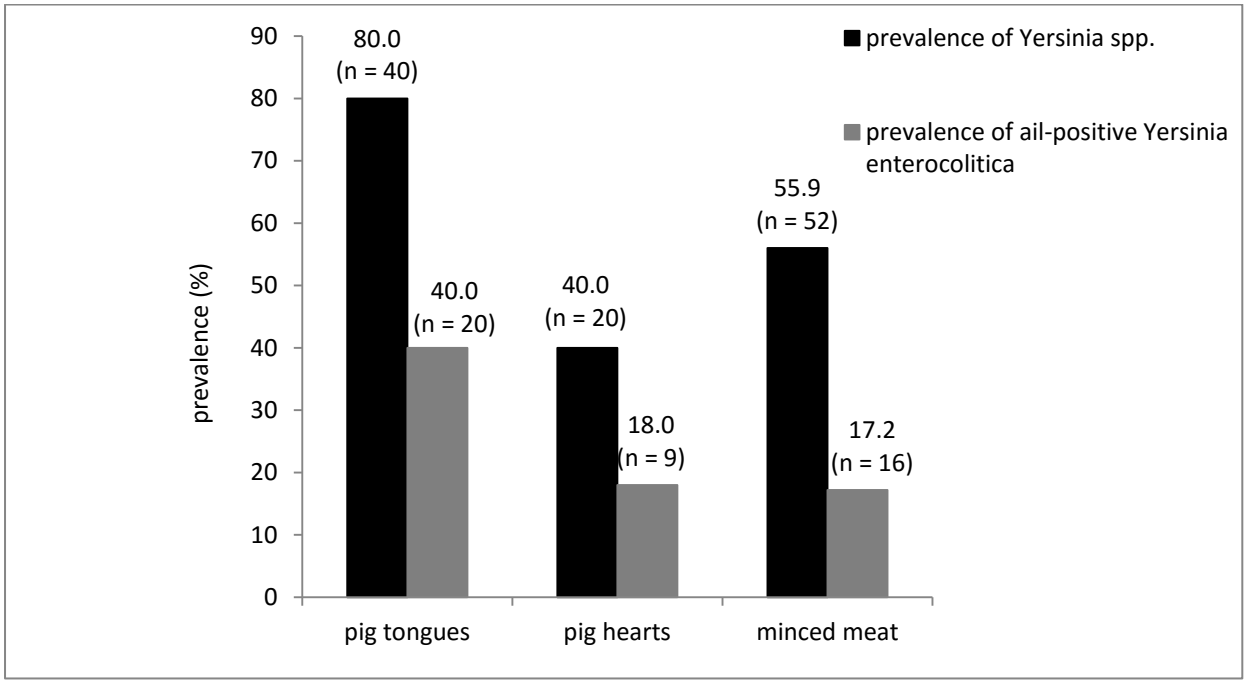

Figure 1 Prevalence of *Yersinia* spp. and pathogenic (*ail*-positive) *Yersinia enterocolitica* in pig tongues (n = 50), hearts (n = 50) and minced meat samples (n = 93) at the retail level using the real time PCR.

evisceration. The highest isolation rate (51%), in comparison to other raw pork products, of pathogenic *Y. enterocolitica* was also found in edible offal of slaughter pigs in southern Germany **(Bucher et al., 2008)**.

Van Damme et al., (2015) found that the initial presence of *Y. enterocolitica* in tonsils and/or in faeces of pigs at slaughter was significantly associated with carcass contamination and the findings of the same genotypes in tonsils, offal, and in minced pork support the assumption that tonsils are the primary source of contamination with pathogenic *Y. enterocolitica* at the slaughterhouse level **(Fredriksson-Ahomaa et al., 2000; Fredriksson-Ahomaa et al., 2001b)**. The blood of infected animals and rinse water can be another source of these bacteria for edible parts of the carcass and pathogenic strains were isolated also from the environment and from the air in a pig slaughterhouse **(Fredriksson-Ahomaa et al., 2000)**. Thus, the cross-contamination of carcases and offal of subsequently slaughtered non-infected pigs can also occur.

Minced meat represents another raw pork product with high risk of contamination by pathogenic *Y. enterocolitica*. Eating of raw ground pork and ground mixed meat was found to be strongly associated with human infection in Belgium **(Tauxe et al., 1987)**. In our study, 17.2% of individually packaged minced meat samples were found to contain pathogenic *Y. enterocolitica* using qPCR. Similarly, raw minced meat samples containing pork collected at the retail level in Finland **(Fredriksson-Ahomaa et al., 1999)** and in Sweden **(Lambertz et al., 2007)** were found to be relatively highly contaminated with pathogenic *Y. enterocolitica* using PCR methods (25% and 35% of samples, respectively). In the United States, 133 (38%) of 350 ground pork samples were found to be contaminated by pathogenic (*ail*-positive) *Y. enterocolitica* using qPCR assay. All samples investigated in that study were culture negative, which indicated only a low contamination level. Pathogenic *Y. enterocolitica* was also detected in 39% of cut meat samples (n = 155) collected at meat cutting plants before mincing in Finland studied with PCR **(Laukkanen–Ninios et al., 2014)**. On the other hand, in Germany, pathogenic *Y. enterocolitica* was detected only in 5 (4.9%) from 102 samples of pork minced meat using qPCR assay targeting the *ail* gene **(Messelhäusser et al., 2011)**.

In shops and then in kitchens *Y. enterocolitica* can easily contaminate other foods thorough direct contact with contaminated raw pork and edible offal or via contaminated hands or equipment during handling and preparation **(Fredriksson-Ahomaa et al., 2001a; 2001b)**. In our study, the detection of genomic equivalents of pathogenic *Y. enterocolitica* in pig tongues and/or hearts collected on the same day and in the same shop could be also the result of cross-contamination of offal stored together before sale. Moreover, because of the psychrotrophic character of *Y. enterocolitica*, these bacteria can persist and multiply in raw material and food during storage at refrigeration temperatures. This is of significant concern from the point of view of food safety and public health.

Sufficient heat treatment of raw meat and offal should eliminate *Y. enterocolitica*, but as was shown, bacteria can survive in core of the product, especially in foods high in fat content (e.g. some minced meat products) which can protect bacteria against the effect of high temperature **(Grahek-Ogden et al., 2007)**. Furthermore, subsequent cross-contamination of the heat-treated products can occur if good hygiene procedures are not followed.

The detection of only the DNA of the pathogenic bacteria in food could be dismissed as irrelevant with respect to food safety because of the possible presence of dead or damaged bacterial cells or free DNA alone **(Lambertz et al., 2007)**. However, if isolation of the pathogen is

difficult, as in the case of pathogenic *Y. enterocolitica*, positive PCR results indicate that it is present and this should be considered as a potential health hazard. In addition, an enrichment step prior to qPCR used in our study should increase the possibility of detecting only live bacteria **(Lambertz et al., 2007)**. Even if the bacteria were initially present in low numbers, their ability to survive and multiply in refrigerated meat and meat products increases the potential risk of infection to consumers.

CONCLUSION

Results of this pilot study indicate that raw pork products can be an important source of pathogenic *Y. enterocolitica* at retail level in the Czech Republic. Measures should be taken to prevent contamination of pig carcasses and edible offal during slaughter and good hygiene practices including proper cooking and prevention of cross-contamination at the household level should be adopted in order to minimize the spread of pathogenic yersiniae and risk of human infection. Further study is needed to obtain *Y. enterocolitica* isolates from the PCR-positive samples for their further characterisation in order to get important epidemiological information.

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