DETERMINING THE PREVALENCE OF *INV*-POSITIVE AND *AIL*-POSITIVE *YERSINIA ENTEROCOLITICA* IN PIG TONSILS USING PCR AND CULTURE METHODS[*]

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ABSTRACT

Background. Yersiniosis is believed to be the third most common intestinal zoonosis in the European Union, after campylobacteriosis and salmonellosis. *Yersinia enterocolitica* is the most common species responsible for human infections. Pigs are regarded as the biggest reservoir of pathogenic *Y. enterocolitica* strains, which are mainly isolated from pig tonsils. The aim of this paper is to examine the prevalence of *inv*-positive and *ail*-positive *Y. enterocolitica* in pigs which were slaughtered in a Polish abattoir.

Material and methods. Real-time PCR and culture methods were used to assess the prevalence of pathogenic *Y. enterocolitica* strains in pig tonsils. Real-time PCR was applied to detect *inv*-positive and *ail*-positive *Y. enterocolitica*. *Y. enterocolitica* was also isolated by applying direct plating, unselective (tryptic soy broth) and selective (irgasan-ticarcillin-potassium chlorate bouillon) enrichment.

Results. A total of 180 pigs were studied, of which 85% and 32% respectively were found to be infected with *inv*-positive and *ail*-positive *Y. enterocolitica*. The 92 *inv*-positive and *ail*-positive isolates, from 57 culture-positive tonsils, underwent bio- and serotyping. The most common was bioserotype 4/O:3, which was found in 53 (93%) out of 57 culture-positive tonsils. Strains of bioserotypes 2/O:5, 2/O:9 and 2/O:5.27 occurred in significantly lower numbers.

Conclusion. The prevalence of *inv*-positive and *ail*-positive *Y. enterocolitica* was found to be high in the tonsils of slaughtered pigs, using real-time PCR. The real-time PCR method for the detection and identification of pathogenic *Y. enterocolitica* is sensitive and specific, which has been verified by specificity and sensitivity tests using the pure cultures. Serotypes were distinguished from each other using PCR serotyping. The PCR method was essential in forming our conclusions.

Keywords: *Yersinia enterocolitica*, *inv* gene, *ail* gene, pig tonsils, biotyping, serotyping, the real-time PCR method, culture method

INTRODUCTION

Yersinia enterocolitica is a food-borne pathogen which can cause acute gastroenteritis and mesenteric lymphadenitis, which resembles appendicitis. Yersiniosis is thought to be the third most common intestinal zoonosis in the European Union, after campylobacteriosis and salmonellosis (Bucher et al., 2008; EFSA and

ECDC, 2016; Grahek-Ogden et al., 2007). Unfortunately, there is little epidemiological data concerning the assessment of pathogenic strains of *Y. enterocolitica* in animals in the European Union, as checking for this pathogen in animals is not obligatory in most countries (Fosse et al., 2008). Pig tonsils are known to be the biggest reservoir of pathogenic *Y. enterocolitica* strains (EFSA, 2011). The pathogenic strains have mostly been detected in pig tonsils, but they have also been isolated from environmental and food samples (Boyapalle et al., 2001; Fredriksson-Ahomaa and Korkeala, 2003; Lambertz et al., 2008). Moreover, there is a very strong resemblance between pig and human strains, which has been proven by DNA-based methods (Jacobsen et al., 2005).

However, there are some obstacles to isolating *Y. enterocolitica* strains from naturally contaminated tonsils or pork meat (Fredriksson-Ahomaa et al., 2000; Fredriksson-Ahomaa and Korkeala, 2003; Tauxe et al., 1987). Culture methods are insufficiently sensitive, which makes it impossible to differentiate pathogenic from non-pathogenic strains. What is more, it is essential to establish the pathogenicity of *Y. enterocolitica* strains, because most isolates from food and environmental samples are non-pathogenic. There are two genes which are chromosomally encoded and are responsible for the pathogenicity of *Y. enterocolitica.* They include the *ail* and *inv* genes. However, the *inv* gene can be present in both pathogenic and non-pathogenic strains. The *ail* gene determinates the pathogenicity of *Y. enterocolitica* strains. So the detection of only the *inv* gene is not sufficient for the detection of pathogenic strains. The detection of the *ail* gene is required to confirm the pathogenicity of *Y. enterocolitica* strains. The application of DNA-methods such as PCR techniques enables us to detect *inv*-positive and *ail*-positive *Y. enterocolitica* in natural samples, achieving a very high level of sensitivity and specificity (Zheng et al., 2008).

On the other hand, to achieve epidemiological data, the culture method should be carried out in combination with the PCR method to differentiate between *Y. enterocolitica* isolates. It is necessary to apply selective enrichment, especially for the isolation of *Y. enterocolitica* strains from food samples. There is no single procedure which is commercially available to collect all bioserotypes. The most frequently applied selective broth is the irgasan–ticarcillin–potassium chlorate (ITC) broth, which is used especially for the detection of bioserotype 4/O:3 (Fredriksson-Ahomaa et al., 2001a; Lambertz et al., 2007).

Bio- and serotyping have been widely applied to differentiate isolates. The biotyping scheme was suggested by Wauters et al. (1987) and universally accepted. Pathogenic isolates, including biotypes 1B, 2–5 can be discriminated from biotype 1A by using the pyrazinamidase test. Serotypes such as O:3, O:5 and O:8, which are responsible for human diseases, can also be met in non-pathogenic *Y. enterocolitica* strains. This means that a very precise biochemical characterisation for the correct identification of the strains is required, before and after serological typing, for every related species and biotype 1A strain which is present in food and environmental samples. Additionally, the subtyping of *Y. enterocolitica* strains which appear among the same bioserotype is a necessity in order to assess the animal reservoir or to estimate the disease outbreaks (Galindo et al., 2011).

The aims of this study were (1) to assess the prevalence of *inv*-positive and *ail*-positive *Y. enterocolitica* in pig tonsils at a slaughterhouse in Podlaskie voivodeship in Poland, using real-time PCR and culture methods, and (2) consequently to bio- and serotype the isolates to achieve more epidemiological information on *Y. enterocolitica* strains at the slaughterhouse.

MATERIAL AND METHODS

Sampling and sample preparation

Tonsils from 180 slaughtered pigs were taken from a slaughterhouse in Podlaskie voivodeship in Poland, in January and February 2017. The pigs were transported to the slaughterhouse from 12 different farms in the eastern region of Poland. Tonsils were removed aseptically immediately after evisceration, placed into sterile plastic bags and delivered to the laboratory under chilled conditions. Tonsil samples were aseptically cut into small pieces, and 10 g of tonsils were put into a sterile stomacher bag. Samples were homogenized with 90 ml of tryptic soy broth (Oxoid, UK) for 2 min in a stomacher (Colworth Stomacher 400, Seward Ltd, London, UK). This homogenate was used for the PCR and culture method investigations.

Detection of *inv*-positive and *ail*-positive *Y. enterocolitica* using the real-time PCR method

Real-time PCR was applied to detect *inv*-positive and *ail*-positive *Y. enterocolitica* after 18h-enrichment in tryptic soy broth at 30°C. DNA from a culture grown in trypton soy broth was taken for purification, for real-time PCR analysis. 1 ml of enrichment culture was pipetted into a 2 ml microcentrifuge screw-cap tube and was centrifuged at 13 000 × g for 5 min. Then the supernatant was discarded using a pipet. Care was taken not to disrupt the pellet. Then 200 µl of Fast Lysis Buffer (Syngen Biotech, Germany) was added to the bacterial pellet, the tube was tightly capped and the pellet was resuspended by vigorous vortexing. Then the microcentrifuge tube was placed into a thermal shaker (800 rpm) set to 100°C. The sample was heated for 10 min. The sample was removed and cooled to room temperature (15–25°C) for 2 min. The tube was centrifuged at 13 000 × g for 5 min. Then 100 µl of the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. This supernatant was subjected to further investigation by real-time PCR (Rotor Mx3000P Stratagene Agilent Technologies, USA), using PowerChek™ Yersinia enterocolitica Real-time PCR Kit Protocol. The kit was used to detect the specific sequence of *inv*- and *ail*-genes for *Y. enterocolitica*. This kit contains a Real-time PCR MasterMix, with enzyme components and the specific primer/probe set for the test by Real-time assay and for the Internal Control (IC) system for the reliability of results. This is a qualitative Duplex Real-time PCR test which includes the amplification of pathogen specific genes (*inv*, *ail*) and the Internal Control (IC) with specific primers and probes labelled with fluorescent dyes.

Table 1. PCR reaction mixture

Composition	Volume, µl
Primer/Probe Mix	4
2X Real-time PCR Master Mix	10
Template DNA (Control DNA or DNA isolated from food-borne pathogens)	5
TE buffer (PCR water, negative control)	1
Total	20

Table 2. The thermal profiles

Temperature, °C	Time	Cycle
95	10 min	1
95	15 s	40
60*	1 min	

*Detection of the fluorescence at this step.

The total reaction volume was 20 µl, the volume of DNA was 5 µl. The reaction mixture was prepared according to the Table 1. The Real-time PCR conditions are presented in Table 2.

Detection of *inv*-positive and *ail*-positive *Y. enterocolitica* using the culture method

Y. enterocolitica was isolated by applying direct plating, unselective and selective enrichment. 100 µl of the homogenate was plated on selective CIN (Yersinia Selective Agar Base and Yersinia Selective Supplement, Oxoid, UK) agar plates by a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). The homogenate was also enriched in unselective tryptic soy broth (Oxoid, UK) at 30°C for 18 h and subsequently 10 µl of 18 h-enrichment was streaked onto CIN agar plates. Another selective enrichment was applied. 1 ml of the homogenate was added to 9 ml of irgasan-ticarcillin-potassium chlorate bouillon (ITC, Oxoid, UK), then incubated at 30°C for 18 h and then 10 µl from the selective enrichment was streaked onto a CIN agar plate. All CIN agar plates were incubated at 30°C for 18 h. Suspected colonies resembling a "bull's eye" were checked for urea hydrolyse. Urea-positive isolates were identified with API 20E. The *inv*-positive *Y. enterocolitica* strains were confirmed by the real-time PCR method.

Biotyping and serotyping of *inv*-positive and *ail*-positive *Y. enterocolitica* isolates

92 *inv*-positive and *ail*-positive *Y. enterocolitica* isolates coming from 57 culture-positive tonsils were bio- and serotyped. The biotype was assessed by pyrazinamidase and tween activities, the ability to hydrolyse esculin, the ability to produce indole, and the the ability to ferment salicin, xylose and trehalose (Wauters et al., 1987). Serotyping was performed with slide agglutination with commercially available *Y. enterocolitica*

O:3, O:5, O:9 and O:27 (Argenta, Poland). Serotyping was also carried out by real-time PCR using a primer/probe set. Serotypes O:3 and O:9 were identified by the *wbbU* (Skurnik, 1999) and *per* (Jacobsen et al., 2005) genes respectively.

Assessment of specificity of the assay using the pure cultures

Specificity of the assay was checked by using 16 bacterial strains (Table 3). Pure cultures of *Yersinia* spp. were cultivated in trypton soy broth (TSB) broth at 30°C for 16–18 h. Bacterial DNA was extracted from 1 µl of overnight enrichment, which was transferred to 100 µl sterile distilled water and subsequently boiled for 10 min to lyse the bacteria. Two µl of the boiled bacteria suspension was used as template in PCR.

RESULTS AND DISCUSSION

In the study, pig tonsil samples were investigated, as they constitute the biggest reservoir of pathogenic *Y. enterocolitica* strains. *Y. enterocolitica* strains are found in numbers 10 times higher in pig tonsils than in pig faeces after slaughter. It means that tonsils are a more important source of carcass contamination than faeces in the slaughterhouse (Laukkanen-Ninios et al., 2014).

The prevalence of *inv*-positive and *ail*-positive *Y. enterocolitica* isolates present in tonsils coming from slaughtered pigs was found to be very high using real-time PCR. The prevalence rate was found to be 85% with a set of probe/primers (Table 4). The specificity and sensitivity test for the detection of *inv*-positive and *ail*-positive *Y. enterocolitica* was carried out on 16 strains. The assay was found to be specific for all potential pathogenic strains of *Y. enterocolitica* (Table 3).

This method has been used in many studies assessing the prevalence of pathogenic *Y. enterocolitica* strains in pig tonsils. The results of these studies showed that the detection rate seemed to be higher with the real-time PCR compared to culture methods (Boyapalle et al., 2001; Stenkova et al., 2008; Vázlerová and Steinhauserová, 2006). The real-time PCR assay was 1000–10,000 times more specific and more

Table 3. The specificity and sensitivity test using 16 strains

Species of *Yersinia*	Original strain no.	The real-time PCR
Y. enterocolitica (pathogenic bioserotype)	YE1	positive
Y. enterocolitica (pathogenic bioserotype)	YE3	positive
Y. enterocolitica (pathogenic bioserotype)	YE5	positive
Y. enterocolitica (pathogenic bioserotype)	YE7	positive
Y. enterocolitica (pathogenic bioserotype)	YE165	positive
Y. enterocolitica (pathogenic bioserotype)	KNG22703	positive
Y. enterocolitica (pathogenic bioserotype)	2516-87	positive
Y. enterocolitica (pathogenic bioserotype)	8081	positive
Y. enterocolitica (pathogenic bioserotype)	WA	positive
Y. enterocolitica (pathogenic bioserotype)	W22703	positive
Y. enterocolitica subsp. *palearctica* (pathogenic bioserotype)	105.5R(r)	positive
Y. enterocolitica subsp. *palearctica* (pathogenic bioserotype)	Y11	positive
Y. enterocolitica subsp. *palearctica* (pathogenic bioserotype)	8081	positive
Y. enterocolitica (nonpathogenic bioserotype)	HYE5283	negative
Y. enterocolitica (nonpathogenic bioserotype)	HYE9180	negative
Y. enterocolitica (nonpathogenic bioserotype)	T16.1M	negative

Table 4. Frequency of *inv*-positive and *ail*-positive *Y. enterocolitica* strains in 180 pig tonsil samples from pigs coming from 12 farms with various detection methods

Method	Number of positive samples	Percentage
The real-time PCR with a set of probe/primers	153	85
Culture method	57	32
Direct plating on CIN agar	42	23
Overnight enrichment in tryptic soy broth (non selective broth) and plating on CIN agar	38	21
Selective enrichment in ITC (irgasan-ticarcillin-potassium chlorate) broth and plating on CIN agar	42	23

reliable than the culture method or the traditional PCR assay (Boyapalle et al., 2001; Messelhäusser et al., 2011).

However, as the *inv*-gene can be present in both pathogenic and non-pathogenic strains, the confirmation of the pathogenicity of *Y. enterocolitica* was carried out by the detection other genes present in pathogenic strains, mostly the *ail*-gene. Momtaz et al. (2013) used other genes to detect pathogenic *Y. enterocolitica* strains. Among these were chromosomal virulence genes such as *inv*, *ail* and *ystA*, and the plasmid-encoded virulence factors such as *yadA* and *virF*. They used multiplex PCR assay and detected the chromosomal virulence genes, including *inv* (100%), *ail* (95.65%), and *ystA* (93.47%), and plasmid-encoded virulence factors including *yadA* (82.60%) and *virF* (58.69%), out of the total of 46 *Y. enterocolitica* serotype O:3 strains. The results of their studies were very similar to those carried out by Cocolin and Comi (2005) who isolated the 6 biotypes and 50 serotypes of *Y. enterocolitica* and proved that incidences of *Y. enterocolitica* serotype O:5.27 and biotype 3 were highest in chicken meat.

Momtaz et al. (2013) used both molecular and culture methods to characterize *Y. enterocolitica* isolates in chicken meat. Results indicated that 132 of the 720 (18.3%) chicken meat samples studied were infected

with *Y. enterocolitica*. Out of 132 samples positive for *Y. enterocolitica*, the prevalence of the 6 biotypes were found to be; 1A (0%), 1B (0%), 2 (18.18%), 3 (52.27%), 4 (17.42%), and 5 (12.12%), and the most prevalent serotypes were O:3 (34.84%), O:5.27 (59.84%), O:8 (5.30%), and O:9 (0%). Another study carried out by Fredriksson-Ahomaa and Korkeala (2003) showed that pigs are a major reservoir of pathogenic *Y. enterocolitica* of bioserotype 4/O:3, the most common type found in humans. However, Momtaz et al. (2013) indicated that that O:5.27/2 was the most frequent serobiotype of *Y. enterocolitica* isolated from chicken meat samples. Their study confirmed that the O:5.27/2 serobiotype of *Y. enterocolitica* caused the most human infections through consumption of contaminated chicken meat. They also confirmed that *ystA*, with an incidence of 93.47%, was one of the most prevalent virulence genes of the *Y. enterocolitica* that was isolated from chicken meat samples. Another study conducted by Favier et al. (2005) indicated that 38.65% of chicken meat samples were contaminated with *Y. enterocolitica*, which was higher than the results of Momtaz et al. (2013) (18.33%). All *Y. enterocolitica* 2/O:9 strains gave results related to virulence by phenotypic tests, and exhibited the genotype *virF*⁺*myfA*⁺*ail*⁺*ystA*⁺, whereas biotype 1A strains showed the genotype virF⁻myfA⁻ail⁺ystA⁺ ystB⁺. The study carried out by Zheng et al. (2008) indicated that the prevalence of virulence genes tested in 160 *Y. enterocolitica* isolates were *inv* (100%), *ail* (94%), *ystA* (93%), *ystB* (7.5%), *ystC* (5%), *yadA* (89%), and *virF* (82%) in patients with diarrhoea.

A study conducted by Lambertz and Danielsson-Tham (2005) proved that pigs were the main reservoir of food-borne pathogenic *Y. enterocolitica*. The researchers combined culturing and PCR methods for detection, and a multiplex PCR was developed for the detection of virulence genes (*yst*, *rfbC*, *ail*, *virF*). In all, 118 pork products (91 raw and 27 ready-to-eat) were collected. Pathogenic *Yersinia* spp. were detected by PCR in 9.89% (9 out of 91) of the raw pork samples but in none of the ready-to-eat products.

In our study, the final isolation rate of pathogenic *Y. enterocolitica* strains detected in pig tonsils was found to be 32% after direct plating, overnight enrichment and selective enrichment (Table 4). It was found that at least 10⁶ cfu/g of pathogenic *Y. enterocolitica*

Table 5. Types of biotypes and serotypes of *Y. enterocolitica* isolated from culture-positive tonsils

Species	Biotype	Serotype	Results of PCR that targets					Number of isolates	Number of culture-positive tonsils
			inv	*ail*	*rfb*C	*per*	*wbbU*		
Y. enterocolitica	4	O:3	+	+	–	–	+	75/92 (82%)	53/57 (93%)
Y. enterocolitica	2	O:5	+	+	–	–	–	8/92 (9%)	7/57 (12%)
Y. enterocolitica	2	O:9	+	+	–	+	–	3/92 (3%)	4/57 (7%)
Y. enterocolitica	2	O:5.27	+	+	–	–	–	6/92 (7%)	3/57 (5%)

strains are required to be detected in food. Selective enrichment was found to be more productive and to give a higher number of *Y. enterocolitica* cells in comparison to non-selective enrichment, which was demonstrated in this study (Aldová et al., 1990). It should be noted that selective enrichment enabled us primarily to recover isolates belonging to bioserotype 4/O:3 (Fredriksson-Ahomaa and Korkeala, 2003). It was also confirmed that ITC broth was designed for the detection and isolation of serotypes O:3 and O:9, but was not optimal for isolation of serotype O:9. The lowest prevalence of pathogenic *Y. enterocolitica* strains was observed when they were cultivated in non-selective enrichment broth, compared to the high prevalence rates found using the PCR method.

A total of 180 pigs investigated, of which 85% and 32% respectively were found to be infected with pathogenic strains, using PCR and culture methods. The 92 *inv*-positive and *ail*-positive isolates from 57 culture-positive tonsils were bio- and serotyped. The most frequent bioserotype was found to be 4/O:3, isolated from 93% (53/57) of the culture-positive tonsils. Moreover, tonsils also contained *inv*-positive and *ail*-positive *Y. enterocolitica* bioserotypes such as 2/O:5 and 2/O:9, which were isolated from 12% (7/57) and 7% (4/57) of the culture-positive tonsils (Table 4).

Among 92 *inv*-positive and *ail*-positive *Y. enterocolitica* isolates coming from 57 culture-positive tonsils, there were a few isolates belonging to biotype 4 which did not agglutinate with O:3, O:5 or O:9 antisera or agglutinated very poorly with O:3 antiserum and all six isolates of serotype O:5.27 did not visibly agglutinate with O:5. The next step was to carry out serotyping by PCR. All *inv*-positive and *ail*-positive *Y. enterocolitica* isolates which belonged to biotype

4 were *wbbU* positive and *per* negative, proving that these isolates belonged to serotype O:3. Three of the nine isolates belonging to biotype 2 were *per* positive and *rfb*C negative, confirming that these three isolates belonged to serotype O:9. The six isolates which belonged to biotype 2, which were both *rfb*C and *per* negative, agglutinated well with O:27 antiserum and belonged to serotype O:5.27 (Table 5).

The most common bioserotype was 4/O:3, which occurred in 53 (93%) out of 57 culture-positive tonsils. This bioserotype 4/O:3 commonly occurs in tonsils and is frequently isolated from slaughtered pigs (Fredriksson-Ahomaa et al., 2001b). It is responsible for human yersiniosis in most European countries. Isolates belonging to biotype 2 were also isolated from Polish pig tonsils. Biotype 2 was very rarely found in pigs. It is known to be common in ruminants (Table 5).

CONCLUSION

The prevalence of *inv*-positive and *ail*-positive *Y. enterocolitica* was found to be high in the tonsils of slaughtered pigs using real-time PCR. The real-time PCR method for the detection and identification of pathogenic *Y. enterocolitica* was found to be rapid and accurate. This method is highly sensitive and specific, which was confirmed by carrying out the specificity and sensitivity test for the detection of *inv*-positive and *ail*-positive *Y. enterocolitica* strains. The most common was bioserotype 4/O:3 which occurred in 53 (93%) out of 57 culture-positive tonsils. Strains of bioserotype 2/O:5, bioserotypes 2/O:9 and 2/O:5.27 occurred in significantly lower numbers. Serotypes were distinguished from each other by PCR serotyping. The PCR method was found to be essential in making conclusions.

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