

Identification of Pathogenicity of *Yersinia enterocolitica* in Pig Tonsils Using the Real-Time PCR

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Abstract

The application of DNA-based methods enables to identify *Yersinia enterocolitica* carrying the *ail*-gene with a greater sensitivity compared to culture methods and biochemical tests used for detection of pathogenic *Y. enterocolitica* in animal and food samples. In this study, 100 samples of pig tonsils were examined, among which 17 were positive for the *ail* gene. Additionally, biochemical tests and RT-PCR showed that nine *Y. enterocolitica* isolates carried the *ail*-gene. Two *Y. enterocolitica* isolates of 1A biotype had the *ail* gene. The results demonstrated the usefulness of RT-PCR method applied for detection of potentially pathogenic, possessing the *ail* gene *Y. enterocolitica* in the material examined.

Key words: RT-PCR, *Yersinia enterocolitica*, pathogen specific gene (*ail*), biochemical testing, biotyping

Yersinia enterocolitica is a rod-shaped bacteria belonging to the family Enterobacteriaceae. *Yersinia* spp. contains 17 species. Three of them are pathogenic to humans, as follows: *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Thoerner *et al.*, 2003; Bolton *et al.*, 2013). The identification of *Y. enterocolitica* is based on the amplification of pathogen specific gene (*ail*) in the presence of the Internal Control (IC) with specific primers and probes labelled with fluorescent dyes. The main animal reservoir of *Y. enterocolitica* strains constitutes pig tonsils (Tennant *et al.*, 2003; Wang *et al.*, 2009). The bacteria attack the lymph tissues during infection due to a 70 kb virulence plasmid (pYV) that encode proteins that participate in infection of these tissues. *Y. enterocolitica* is an enteric pathogen of six biotypes: 1A, 1B, 2, 3, 4, and 5. Different bioserotypes are regarded to be pathogenic. The isolates of biotype 1A do not possess the virulence-associated pYV-encoded genes and most of chromosomal virulence markers, including the *ail* (attachment and invasion locus) gene.

Over the last years, there have been many molecular methods designed to improve and accelerate the detection procedures. The comparison of conventional and molecular detection methods of *Y. enterocolitica* showed a significantly higher sensitivity of the molecular methods. The most significant steps are: i) an efficient enrichment procedure for pathogenic *Y. enterocolitica* isolates in the appropriate medium and ii) a DNA preparation

protocol to remove any inhibitors of the subsequent molecular reactions (Bonardi *et al.*, 2014). Enrichment procedures enable the discrimination between viable and non-viable bacterial cells, thereby contributing to the diminished risk of false positive results (Lambertz *et al.*, 2007). The aim of this study was to show the usefulness of RT-PCR assay (PowerChek™ *Yersinia enterocolitica* RT-PCR Detection System (KogeneBiotech, Germany)) for fast detection of potentially pathogenic, possessing the *ail* gene *Y. enterocolitica* in pig tonsils.

Pig tonsils from 100 pigs, which were slaughtered in one abattoir in podlaskie voivodeship in Poland, were taken during 8 sampling visits. Pig tonsils came from pigs, which were slaughtered at the age of 150–170 days. Pigs were from 18 herds. Tonsil samples were aseptically cut into small pieces, and 10 g of tonsils were put into sterile stomacher bag. Samples were homogenized with 90 ml of 0.1% peptone water (Oxoid, UK) for 2 min in a stomacher (Colworth Stomacher 400, Seward Ltd, London, UK). 0.1 ml of such homogenate was plated on CIN agar plates by a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). CIN agar plates are cefsulodin-irgasan-novobiocin agar plates (*Yersinia* Selective Agar Base and *Yersinia* Selective Supplement, Oxoid, UK). CIN agar plates were incubated at 30°C for 24 h and investigated for characteristic *Yersinia* colonies using a stereomicroscope with Henry illumination (Olympus).

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To achieve the accurate assessment of the pathogenicity of *Y. enterocolitica* belonging to biogroup 1A with *ail* gene, the invasion and adhesion assay onto Hep-2 cells (human epidermis carcinoma cells, larynx) with 2006RAT and SDWL-003 were carried out. Two isolates of *Y. enterocolitica* with various virulence potentials were chosen as the reference isolates: weakly virulent (bioserotype 3/O:3, isolate NX-SA98-837) and highly virulent (bioserotype 1B/O:8, isolate Ye92010) (Table II). Invasion and adhesion assays were performed as described previously by Wang *et al.* (2008). Experiments were repeated three times. The assay for each isolate was repeated two times in one experiment. The results were analyzed by the T test; a critical P value of 0.05 was used as statistically significant.

The cultures grown in tryptic soy broth were taken for purification of DNA for RT-PCR analysis. The total reaction volume was 20 μ l, the volume of DNA was 5 μ l, Primer/Probe Mix was 4 μ l, 2X RT-PCR Master Mix was 10 μ l, PCR water (negative control) was 1 μ l. The fluorescent curves were analysed on HEX fluorescence detection channels (Table I). Analysing RT-PCR results assessed the presence or absence of *Y. enterocolitica* specific gene.

The biochemical tests were carried out to check the pathogenicity of *Yersinia* isolates. Nine out of 100 samples of tonsils gave positive results for pathogenic *Y. enterocolitica*. The growth on Bile Esculine Agar enabled to distinguish the pathogenic from non-pathogenic *Y. enterocolitica* isolates. A black halo around the colonies indicates a positive reaction. Any pathogenic *Y. enterocolitica* were found. Another test was carried out on Kligler Iron Agar. A butt was yellow and a slant was red what showed that the growth of *Y. enterocolitica* was observed. *Y. enterocolitica* is glucose positive and lactose negative, does not form H₂S or gas. The pathogenicity of *Yersinia* isolates was also tested on Urea Agar. Pink-violet or red-pink color of the slant indicated a positive urease reaction. An orange-yellow color indicated a negative urease reaction. As the slant was red-pink, the growth of *Y. enterocolitica* was observed, which indicated a positive urease reaction.

Table I
Detection of pathogenic *Yersinia enterocolitica* in pig tonsils with RT-PCR.

Number of samples	Source	Ct ranges in HEX channel for positive results	Ct ranges in HEX channel for negative results
Positive control	Control DNA	28.89	–
17	Pig tonsils	25.67–36.17	–
83	Pig tonsils	–	37.23–37.63
Negative control	PCR water	–	37.34

We used RT-PCR test to confirm the presence of potentially pathogenic *Y. enterocolitica* isolates possessing the *ail* gene in the material examined. The probe-based RT-PCR assay for detection of *ail*-positive *Y. enterocolitica* was found to be specific for all potentially pathogenic isolates present in pig tonsil samples (Table I). 17 out of 100 samples of tonsils gave positive results for pathogenic *Y. enterocolitica* as indicated a positive reaction with Ct values between 25.67 and 36.17. Remaining negative samples showed Ct values between 37.23–37.63. Relatively low Ct values (≤ 36.17) in the positive samples of pig tonsils for *ail* gene meant that tonsils were highly contaminated (Table I).

All the *ail*-positive isolates of *Y. enterocolitica* obtained using the culture method were subjected to biotyping. The API 20E encode isolates 2006RAT and SDWL-003 with numbers 1.155.723 and 1.114.721, respectively. In accordance to the code display results, they were all *Y. enterocolitica*. The isolates 2006RAT and SDWL-003 had typical biochemical profile for biotype 1A according to the biotyping schema. They were Tween-esterase positive, esculin positive, pyrazinamidase positive, indol positive, xylose positive and trehalose positive. The *ail*-positive isolate of *Y. enterocolitica* Ye92010 had typical biochemical profile for biotype 1B as follows: tween-esterase positive, esculin negative, pirazinamidase negative, indol positive, xylose positive and trehalose positive. The *ail*-positive isolate of *Y. enterocolitica* NX-SA98-837 had typical biochemical profile for biotype 3. It was Tween-esterase negative, esculin negative, pirazinamidase negative, indol negative, xylose positive and trehalose positive. We assessed biotypes of the isolates of *Y. enterocolitica* examined according to Wauters *et al.* scheme (1987) (Table II).

The adhesion and invasion properties of *Y. enterocolitica* biotype 1A containing the *ail* gene in comparison to biotype 3 were evaluated. The results of this test were presented in Table III. The results of the cell adhesion and invasion assay indicated the differences between 2006RAT, SDWL-003, a weakly virulent isolate NX-SA98-837 and a highly virulent isolate Ye9201. The isolates were arranged according to their ability of adhesion to Hep-2 cells. The highest number of NX-SA98-837 cells was able to adhere to Hep-2 cells and the lowest number of SDWL-003 cells was able to adhere to Hep-2 cells (Table III). The significant differences ($P < 0.05$) were observed for NX-SA98-837 and Ye92010 in comparison to SDWL-003. The gentamicin treatment in the invasion test revealed that number of live 2006RAT cells was significantly higher in comparison to Ye92010 cells. Moreover, no significant difference was observed for the survival of SDWL-003 cells. It means that 2006RAT was more invasive than SDWL-003 and highly virulent 1B/O:8 isolate Ye92010 (Table III).

Table II
Assessment of biotypes of *Yersinia enterocolitica* isolates according to Wauters *et al.* scheme (1987).

Strains	Biochemical tests									Biotype
	Lipase (Tween- esterase)	Esculin/ salicin 24 h	Indole	Xylose	Tre- halose/ NO ₃	Pyrazina- midase	β-D-Glu- cosidase	Voges- Proskauer	Proline peptidase	
2006RAT	+	+	+	+	+	+	+	+	V ¹	1A
SDWL-003	+	+	+	+	+	+	+	+	V ¹	1A
Ye92010	+	-	+	+	+	-	-	+	-	1B
NX-SA98-837	-	-	-	+	+	-	-	+	-	3

V¹ – variable

Table III
The adhesion and invasion properties of *Yersinia enterocolitica* biotype 1A containing the *ail* gene in comparison to biotype 3.

Isolate	Biotype	A number of bacterial cells adhering to Hep-2 cells	A number of bacterial cells surviving within Hep-2 cells
2006RAT	1A	5 000 ± 10 [†]	10 000 ± 12
SDWL-003	1A	1 000 ± 10	500 ± 7
Ye92010	1B	750 000 ± 23	4 500 ± 10
NX-SA98-837	3	2 100 000 ± 36	32 000 ± 16

[†] Standard deviation from three separate experiments P < 0.05

In our work we examined prevalence of pathogenic *Y. enterocolitica* isolates in pig tonsils using RT-PCR and biochemical tests. The higher percentage of pathogenic isolates was detected using RT-PCR. In RT-PCR, the isolates possessing the *ail* gene were classified as pathogenic, and in biochemical tests – the esculin-negative isolates were considered to be pathogenic. According to Wauters scheme (1987), the esculin-positive isolates belong to biotype 1A. The environmental isolates of biotype 1A are considered to be non-pathogenic because they lack the pYV plasmid and chromosomal virulence *ail* gene (Kraushaar *et al.*, 2011; Sihvonen *et al.*, 2012).

The majority of isolates isolated from food samples are not pathogenic. However, it is difficult to isolate pathogenic *Y. enterocolitica* that occur in low number in food samples and are accompanied by a high number of background flora. The application of RT-PCR enables to detect the pathogenic *Y. enterocolitica* in pig tonsils (Thisted and Lambertz, 2008). There are few well-determined chromosomal and plasmid-borne virulence factors present in pathogenic *Y. enterocolitica* and they are used as targets for RT-PCR (Falcao *et al.*, 2006; Wang *et al.*, 2010; Wang *et al.*, 2011). Expression of both chromosomal and plasmid genes is necessary to determine pathogenicity. One of the genes necessary to detect the virulence is *ail* gene present in pathogenic *Y. enterocolitica* (Lambertz *et al.*, 2008).

The detection rates of *ail*-positive *Y. enterocolitica* in pig tonsils were relatively low (Table II). The reason for the detection of numerous negative results could be low number of pathogenic strains present in pig tonsils (Fredriksson-Ahomaa and Korkeala, 2003). Another reason for the negative results could be background flora in the examined samples. Therefore, there is a necessity to carry out the selective enrichment step in order to eliminate the growth of background microflora because it decreases the sensitivity of the assay (van Damme, 2010). A good option is to use a selective enrichment medium CIN agar containing cefsulodin, irgasan and novobiocin which has been designed for RT-PCR detection of *Y. enterocolitica* (van Damme *et al.*, 2013a; 2013b). This medium contains selective components that inhibit the growth of background flora. The inclusivity and the exclusivity test show a potential in the applicability and reliability of the *ail* gene-based RT-PCR (Boyapalle *et al.*, 2001). The *ail* gene is chromosomally encoded and is inherited in comparison to the plasmid-encoded virulence factors that can be lost during the incubation because of instability of the virulence plasmid (Platt-Samoraj *et al.*, 2017).

The prevalence of this pathogen in naturally contaminated pig tonsils has previously been found to be high with RT-PCR. The results of this study showed that the presence of pathogenic *Y. enterocolitica* isolates in pigs slaughtered in the podlaskie voivodeship is relatively low. The microbiological contamination of carcasses pinpoints that good hygiene measures are required to control the spread of *Y. enterocolitica* isolates at abattoir especially during the evisceration and tonsils removal. Furthermore, the implementation of control programmes with the aim to reduce the number of carrier pigs at a farm level would be the best strategy to avoid the contamination at slaughterhouse. The culture method on CIN agar plates showed the growth of a high number of natural background microorganisms and it is not easy to distinguish presumptive pathogenic *Y. enterocolitica* from non-pathogenic ones. The biochemical tests are also not sensitive enough to detect the pathogenic isolates.

In summary, the effectiveness of detecting pathogenic *Y. enterocolitica* with PowerChek™ *Yersinia enterocolitica* RT-PCR Detection System has been described. The RT-PCR possesses a greater sensitivity in comparison to conventional methods for the detection of pathogenic *Y. enterocolitica* in animal and food samples. This method could find its application in meat processing plants as a reference method in the safety management system to monitor critical control points. Finding of two biotype 1A isolates possessing the *ail* gene widens the knowledge on the virulence of *Y. enterocolitica* biotype 1A. It means that some isolates of 1A biotype have also genes that are important in the pathogenesis of *Y. enterocolitica* infection.

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