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Original article

Detection of *Yersinia enterocolitica* species in pig tonsils and raw pork meat by the real-time PCR and culture methods

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Abstract

The aim of the present study was to establish a rapid and accurate real-time PCR method to detect pathogenic *Yersinia enterocolitica* in pork. *Yersinia enterocolitica* is considered to be a crucial zoonosis, which can provoke diseases both in humans and animals. The classical culture methods designated to detect *Y. enterocolitica* species in food matrices are often very time-consuming. The chromosomal locus _tag CH49_3099 gene, that appears in pathogenic *Y. enterocolitica* strains, was applied as DNA target for the 5' nuclease PCR protocol. The probe was labelled at the 5' end with the fluorescent reporter dye (FAM) and at the 3' end with the quencher dye (TAMRA). The real-time PCR cycling parameters included 41 cycles. A Ct value which reached a value higher than 40 constituted a negative result.

The developed for the needs of this study qualitative real-time PCR method appeared to give very specific and reliable results. The detection rate of locus _tag CH49_3099 – positive *Y. enterocolitica* in 150 pig tonsils was 85 % and 32 % with PCR and culture methods, respectively. Both the Real-time PCR results and culture method results were obtained from material that was enriched during overnight incubation. The subject of the study were also raw pork meat samples. Among 80 samples examined, 7 ones were positive when real-time PCR was applied, and 6 ones were positive when classical culture method was applied. The application of molecular techniques based on the analysis of DNA sequences such as the Real-time PCR enables to detect this pathogenic bacteria very rapidly and with higher specificity, sensitivity and reliability in comparison to classical culture methods.

Key words: Real-time PCR, *Yersinia enterocolitica*, locus _tag CH49_3099 gene, pork meat

Introduction

Yersinia enterocolitica belongs to Gram-negative bacteria which constitute a very heterogeneous group and include different bioserotypes, among which only a few provoke diseases in humans. Human yersiniosis is connected with the consumption of contaminated pork and water. The most widely symptoms include diarrhoea, abdominal pain, fever and nausea. *Yersinia enterocolitica* is considered to be the main cause of

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yersiniosis and an important foodborne pathogen responsible for provoking gastrointestinal diseases in humans (Bucher et al. 2008, Galindo et al. 2011). The most common transmission routes of bacteria include the route directly from pigs, via the consumption of contaminated pork products or indirectly from pets that were fed raw pork (EFSA 2011, Laukkanen-Ninios et al. 2014, EFSA and ECDC 2015, Van Damme et al. 2015). It is thought to be the third most common zoonosis in the European Union. Among *Y. enterocolitica* there can be distinguished 6 biotypes and more than 7 serotypes (Fredriksson-Ahomaa et al. 2001a). The pathogenicity of the bacteria is connected with the presence of a virulence plasmid, labelled as pYV, chromosomal invasion-associated genes like *ail* and *inv*, and the enterotoxin gene *yst*A (Fredriksson-Ahomaa et al. 2000, Grahek-Ogden et al. 2007). Pigs are considered to be the largest reservoir of *Y. enterocolitica*, and pork is known to be the most important risk factor responsible for provoking diseases in humans (Tauxe et al. 1987, Fredriksson-Ahomaa et al. 2001c, Fosse et al. 2008). The storage of pork meat in refrigeration conditions facilitates the bacterial growth because *Y. enterocolitica* belongs to psychrophile and is able to multiply at relatively cold temperatures (Fredriksson-Ahomaa et al. 2001b).

Classical culture methods of detection of *Y. enterocolitica* in food matrix require a long period of time lasting even for 4 weeks. A huge number of isolates obtained from food matrices are non-pathogenic (Aldová et al. 1990). Problems related to recovering pathogenic *Y. enterocolitica* refer to a relatively very low number of pathogenic strains in the samples and a significantly high number of accompanying background microflora. The application of molecular techniques like the real-time PCR enables to detect pathogenic *Y. enterocolitica* strains from food samples (Boyapalle et al. 2001, Messelhäusser et al. 2011). These methods use the specific sequences of nucleotides in DNA which code a few well-known chromosomal and plasmid-borne virulence determinants causing the pathogenicity of *Y. enterocolitica* (Miller et al. 1989, Simonova et al. 2008). Such sequences are used as a target for conventional PCR and the Real-time PCR. The expression of chromosomal and plasmid genes is needed for full pathogenicity, however, the virulence plasmid (pYV) is known to be difficult to maintain during culturing.

The main aim of this study was to design a set of a probe and primers to identify and quantify enteropathogenic *Y. enterocolitica* strains in raw pork meat and pig tonsils by applying the real-time PCR method. The assay is based on chromosomal locus _tag CH49_3099 gene, which only appears in pathogenic *Y. enterocolitica* strains, and its nucleotide sequence was applied as DNA target for the 5' nuclease PCR protocol. The probe was labelled at the 5' end with the fluorescent reporter dye (FAM) and at the 3' end with the quencher dye (TAMRA). Moreover, the results of this 5'-nuclease assay were compared with those obtained with classical culture methods using raw pork meat and naturally contaminated pig tonsils samples.

Materials and Methods

Bacterial strains. A number of 23 bacterial strains were subjected to the specificity test (Table 4). *Yersinia enterocolitica* bioserotype 8081 constituted the reference strain in all optimisation and sensitivity studies. All *Yersinia* strains were incubated on CIN (cefsulodin-irgasan-novobiocin) agar plate (Oxoid, UK) at 30C for 18-20 h. Colonies were taken by a sterile loopful and incubated in trypton soy broth (TSB) broth (Oxoid, UK) at 30C for 16-18 h (Oxoid, UK). Then such culture grown in TSB was taken for Real-time PCR analysis.

DNA extraction procedures. Culture grown in trypton soy broth was taken for purification of DNA for Real-time PCR analysis. One ml of enrichment culture was pipetted into a 2 ml microcentrifuge screw-cap tube and was centrifuged at 13 000 x g for 5 min. Then the supernatant was discarded using a pipet. The 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 x g for 5 min. After centrifugation, the supernatant was carefully transferred to a new tube and 2 µl of this supernatant was used as the template.

Designing of a real-time PCR assay for detection of locus _tag CH49_3099 gene present in pathogenic *Y. enterocolitica* strains. The primer/probe set targeting locus _tag CH49_3099 gene was designed on the base of 50-nucleotide sequence GACGATACCTTGGTATAGCAATCTATTTAGCACTGATGTGTCGGTTCCGG specific for pathogenic *Y. enterocolitica* species. The sequence of the gene was provided by GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number CP009846.1). Sequences unique to *Y. enterocolitica* were compared with those of closely related strains. The primer/probe set was designed using Primer Express Software v3.0 (Applied Biosystems, Foster City,

Table 1. Nucleotide sequences of the primers and the fluorescently labelled oligonucleotide probe used for the detection of pathogenic *Y. enterocolitica* strains.

Primer and probe	Sequence (5'→3') Region of gene from 3300786 to 3300855
Primers	
Forward	GACGATACCTTGGTATAGC
Reverse	ATAGCTGATGACTTTAT
Probe	CCGGAACCGACACATCAGTGCTAAATAGAT

The sequence of the gene was provided by GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number CP009846.1).

Table 2. The reaction mixture used for the probe-based real-time PCR for detection of locus _tag CH49_3099 gene present in pathogenic *Y. enterocolitica* strains.

Composition	Volume	End concentration
Primers F+R (2 μM each)	2.5 μl	200 nM
Probe (2 μM)	1 μl	80 nM
2x Real-time PCR Master Mix	12.5 μl	1 x
dNTP (400 M each)		200 μM (each)
Taq polymerase (50 U/ml)		1.25 U
Mg (6 mM)		3 mM
Template DNA (Control DNA or DNA isolated from food-borne pathogens)	2 μl	
TE buffer (PCR water, negative control)	7 μl	
Total	25 μl	

Table 3. The real-time PCR program used for the detection of locus _tag CH49_3099 gene present in pathogenic *Y. enterocolitica* strains with the thermocycler (Rotor Mx3000P Stratagene Agilent Technologies, USA).

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

* Detection of the fluorescence at this step.

CA, USA). The set was validated using NCBI BLAST (Basic Local Alignment Search Tool: www.ncbi.nlm.nih.gov/blast/). The sequences were as follows: forward primer 5'-GACGATACCTTGGTA TAGC-3'; reverse primer 5'- ATAGCTGATGAC-TTTAT-3'; probe 5'-FAM- CCGGAACCGACACAT CAGTGCTAAATAGAT-3'-TAMRA (Table 1). The amplicon size was 66 bases. The oligonucleotides were synthesised and purchased from Eurofins Genomics (Germany). The 5' hydrolysis probe was an internal fluorogenic probe labelled with 6-carboxyfluorescein (FAM) at the 5' end and quenched with 6-carboxytetramethylrhodamine (TAMRA) at the 3' end (Eurofins Genomics, Germany). The fluorescence was detected using an optical detection system installed in the thermocycler of Stratagene Mx3005P (Real-Time PCR Detection System, Agilent Technologies, USA). Fluorescence data were collected during the annealing/elongation step of each PCR cycle. The software automatically plots the relative fluorescence unit (RFU) versus the PCR cycle number. The threshold cycle (Ct), which expresses the amount of a particular nucleic acid sample, is the number of amplification cycles needed for the accumulated fluorescence to achieve a value essentially higher than the background. A Ct value which exceeds 40 meant a negative result.

PCR conditions. The 2x Real-time PCR Master Mix (Syngen Biotech, Germany) was used for this analysis (Table 2). The optimal primers and probe concentrations amounted to 200 nM and 80 nM, respectively. Two μl of the template was added to 23 μl of the master mix. The thermocycling conditions are presented in Table 3. The optimal annealing temperature of 60C was assessed using the gradient feature included in iQ^{TM}5 software.

Assessment of specificity of the assay using the pure cultures. Specificity of the assay was checked by

Table 4. The specificity test using 23 strains of *Yersinia enterocolitica*.

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
Y. kristensenii	2M25.1D	negative
Y. intermedia	5M25.1D	negative
Y. pestis	AYV161	negative
Y. rohdei	8M6.1D	negative
Y. pseudotuberculosis	IP32979	negative
Y. pseudotuberculosis	IP32981	negative
Y. pseudotuberculosis	IP32918	negative

using 23 bacterial strains (Table 4). Pure cultures of *Yersinia* spp. were cultivated in trypton soy broth (TSB) broth at 30C 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 this boiled bacteria suspension was used as template in PCR.

Assessment of sensitivity of the assay with pure culture and inoculated minced pork meat. Sensitivity of the assay with pure culture of *Y. enterocolitica* bioserotype 8081 was carried out to check the detection limit of the 5'-nuclease protocol. The culture was added to 10 ml of TSB, incubated at 30°C for 18 h, and then serially diluted (10-fold) in triplicate and enumerated in duplicate using CIN agar plates, and incubated at 30°C for 18-20 h. The DNA was isolated from 100 µl of each dilution. For inoculation analysis, four samples with 10 g of pork meat, that were checked to be negative for *Y. enterocolitica*, were added to 90 ml of tryptic soy broth (TSB) (Oxoid, UK) in sterile stomacher bags, inoculated with five different inoculation levels (Table 5). For each analysis an uninoculated sample was applied as a control and was found to be *Y. enterocolitica*-negative. The bags were then incubated overnight at 30°C. The overnight bacterial suspensions were analyzed with the Real-time PCR and culture methods. DNA was extracted from 100 µl of the overnight bacterial suspension. The culture method was based on direct plating on selective CIN agar plates after overnight enrichment in TSB.

Pig tonsil and pork samples. Naturally contaminated tonsil (150) and pork (80) samples were analyzed with the Real-time PCR and culture methods. Ten grams of each sample was homogenised with 90 ml of TSB in stomacher blender. The homogenates were incubated at 30°C for 16-18 h. Such material that was enriched during overnight incubation was both the subject of the analysis using the Real-time PCR and culture methods.

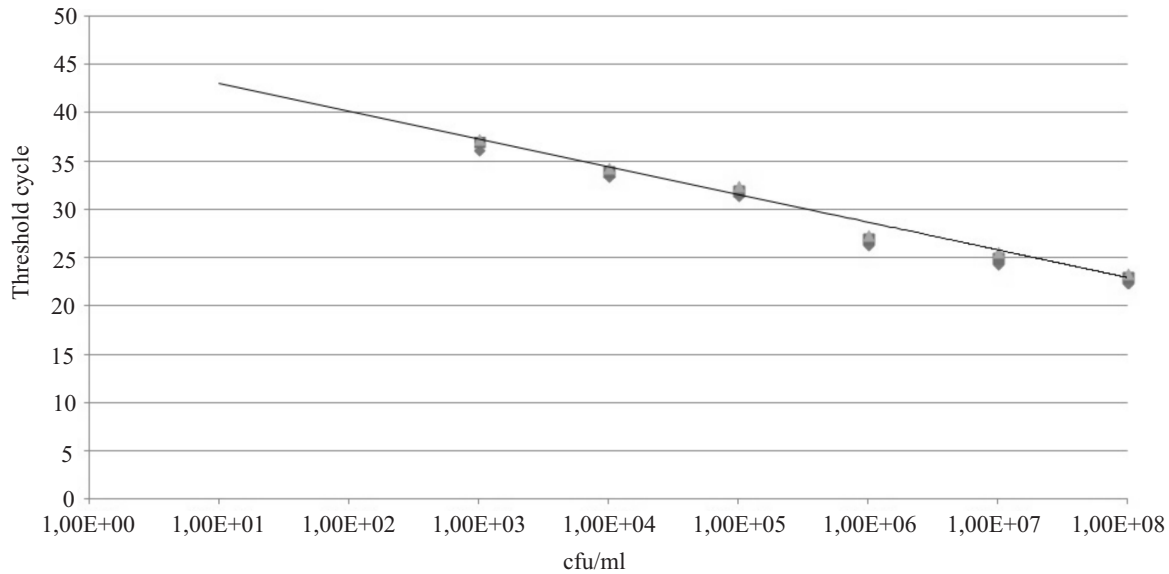

Fig. 1. Standard curve for 10-fold serial dilution series of locus _tag CH49_3099-positive *Y. enterocolitica* strain (10^3 – 10^8 cells per ml) in triplicate plotted as the threshold cycle (Ct) on the Y-axis.

Table 5. Detection of *Y. enterocolitica* 8081 in artificially contaminated minced pork meat samples after overnight enrichment by probe-based PCR and culture methods.

Inoculation level (cfu/g)	PCR-positive samples		Number of culture positive samples
	Number of positive samples	Threshold cycle Ct	
1-10	4/4	37-38	3/4
10^2	4/4	31-32	4/4
10^3 – 10^4	4/4	20-21	4/4

Table 6. Detection of *Y. enterocolitica* 8081 in artificially contaminated minced pork meat samples after overnight enrichment by culture methods.

Inoculation level (cfu/g)	A number of *Y. enterocolitica* 8081 cells in meat after overnight enrichment (cfu/g)
1-10	2.1×10^3
10^2	1.7×10^5
10^3 – 10^4	2.4×10^7 – 1.3×10^9

Table 7. Detection of pathogenic *Y. enterocolitica* in 150 naturally contaminated pig tonsils samples after overnight enrichment using the real-time PCR and culture methods.

Method	Number of positive samples (%)			Number of negative samples (%)
	Ct[a] ≤30	Ct ≤ 35	Ct ≤ 40	Ct > 41
The real-time PCR method	63 (42%)	109 (73%)	128 (85%)	18 (12%)
Culture method	34 (23%)	30 (45%)	48 (32%)	97 (65%)

Ct[a] – threshold cycle.

Table 8. Detection of pathogenic *Y. enterocolitica* in 80 naturally contaminated raw pork samples after overnight enrichment using the real-time PCR and culture methods.

Sample	Number of samples	Number of PCR positive samples (%)	Number of culture positive samples (%)
Raw pork meat	80	7 (9%)	6 (8%)

Results

The specificity of the designed probe-based real-time PCR assay for detection of locus _tag CH49_3099-positive *Y. enterocolitica* was checked. The set of a probe and primers developed for the needs of this study occurred to be specific for all potential pathogenic strains of *Y. enterocolitica* (Table 4).

Yersinia enterocolitica strains (a concentration of 104 bacteria per PCR) which belonged to pathogenic bioserotypes gave a positive reaction with Ct values in range from 20 to 21, *Y. enterocolitica* strains (a concentration of 10^2 bacteria per PCR) which belonged to pathogenic bioserotypes gave a positive reaction with Ct values in range from 31 to 32, *Y. enterocolitica* strains (a concentration of 10^1 bacteria per PCR) which belonged to pathogenic bioserotypes gave a positive reaction with Ct values in range from 37 to 38, whereas non-pathogenic *Y. enterocolitica*, *Yersinia* spp. including *Y. kristensenii*, *Y. intermedia*, *Y. pestis, Y. pseudotuberculosis* and *Y. rohdei* gave a negative result.

The lowest detection limit of this PCR protocol designated for the detection of the locus _tag CH49_3099 gene in pure cultures of *Y. enterocolitica* was 10^3 – 10^4 cfu/ml (Fig. 1), because DNA was isolated from 100 µl of pure culture, and only 2 µl was used as template. However, the detection limit of the PCR reaction amounted to 1-10 cfu per PCR. It was possible to detect such a low number of *Y. enterocolitica* cells as the DNA was prepared from material achieved after overnight enrichment. After overnight inoculation, the PCR method enabled to detect 1-10 cfu per g of minced meat (Table 5). The numbers of *Y. enterocolitica* 8081 cells in artificially contaminated minced pork meat samples after overnight enrichment by culture methods are presented in Table 6.

Discussion

The inoculation step prior to the Real-time PCR is required in order to increase the sensitivity especially in naturally contaminated samples. What is more, the inoculation step diminishes the risk of false-positive PCR results due to the ability to detect dead cells (Stenkova et al. 2008). However, it is commonly known that there is no need to carry out overnight enrichment of initial bacterial culture to successfully perform the PCR. The direct isolation of DNA from bacterial culture omitting the overnight enrichment enables to detect *Y. enterocolitica* using the PCR method starting from 1-10 cfu/g. Such research will be carried out in further papers focusing on the detection of *Y. enterocolitica* omitting the enrichment step.

Molecular methods find their applications in monitoring viral, bacterial, and protozoan pathogens, and in tracking pathogen- and source-specific markers in the environment. Molecular techniques, specifically polymerase chain reaction-based methods, deliver sensitive, rapid, and quantitative analytical tools enabling to study such pathogens, including newly emerging strains. However, they also possess some disadvantages. The molecular techniques available today and those under development need further refinement in order to be standardized and applicable to a diversity of matrices. The pros and cons of molecular techniques for the detection and quantification of pathogens should be the subject of further investigations as there is a huge variety of different food matrices and some bacterial strains contained in food matrices should undergo the enrichment step in order to be detectable by the PCR.

Naturally contaminated pig tonsils were subjected to the analysis to make comparison of the PCR method with culture method. Pig tonsils were taken for the analysis because they are thought to be very often contaminated with pathogenic strains of *Y. enterocolitica*. The detection level of locus _tag CH49_3099-positive *Y. enterocolitica* was indicated to be clearly higher when the real-time PCR method was applied in comparison to culture methods and this detection level amounted to even lower Ct values than 30 (Table 5). Pathogenic *Y. enterocolitica* was isolated from 128 samples (85%) using the real-time PCR method and from 48 samples (32%) using the culture method (Table 7). The detection rates of pathogenic strains of *Y. enterocolitica* in raw pork samples were significantly very low using both PCR and culture methods (Table 8). The main reason for the appearance of a very high number of negative results can be a very low number of pathogenic strains in the examined samples (Fredriksson-Ahomaa et al. 2003, Lambertz et al. 2007). The study involved the template which amounted to 2 µl which might be too low. The next alternative is to increase the amount of the template to 5 µl in order to increase the sensitivity of the assay and such amount of the template should be added to 45 µl of the master mix. Another reason for the appearance of negative results can be very high numbers of accompanying background microflora present in food matrix. The non-selective inoculation step applied in this study enables to detect increasing numbers of all bacteria and such phenomenon leads to the decrease in the sensitivity of the protocol in the situation when the accompanying microflora is present in too high number (Vhzlerovh et al. 2006). The presence of too high amount of extraneous background DNA possesses a very unwanted influence on the amplification efficiency. It can be caused by different in-

teractions taking place between DNA coming from bacteria and the PCR reagents. To avoid such a phenomenon, an incubation in selective media not influencing the inhibition of the PCR is suggested to be applied. Such medium should contain the selective components which would successfully inhibit the growth of accompanying background flora. What is more, it should not contain any inhibitory components which might to some extend influence the fluorescence based detection. The low detection rate with the application of the real-time PCR can also be caused by receiving the false-negative results coming from inhibitors present in pork (Lambertz et al. 2008). The DNA isolation protocol applied in this study was fast and easy to perform. However, it may not be the most efficient to eliminate any inhibitors which might be present in pork. The necessity is to include an internal positive control in this reaction in order to have a possibility of monitoring so called false-negative results.

In conclusion, the Real-time PCR method for rapid detection and identification of pathogenic *Y. enterocolitica* using the Real-Time PCR Detection System (Agilent Technologies, USA) has been described. The developed rapid and accurate real-time PCR method to detect pathogenic *Y. enterocolitica* in pork appeared to be highly sensitive and specific. The prevalence of pathogenic *Y. enterocolitica* strains present in naturally contaminated pig tonsils and raw pork meat determined with the Real-time PCR method was relatively higher in comparison to that estimated with the classical culture method. The application of molecular techniques such as the Real-time PCR gives a possibility of the detection of these pathogenic bacteria very rapidly and with higher specificity, sensitivity and reliability than with the use of classical culture methods.

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