Rapid and sensitive detection of pathogenic *Yersinia enterocolitica* strains in food using selective enrichment and real-time PCR

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Summary

Yersinia enterocolitica is an important human enteroinvasive pathogen with a global distribution. Food contamination is associated with pigs considered a major reservoir of *Y. enterocolitica*. The aim of this study was to develop and evaluate a rapid and sensitive real-time PCR-based method for the detection of pathogenic *Y. enterocolitica* in food. A TaqMan probe-based real-time PCR detection system targeting a sequence of chromosomally located *ail* (attachment and invasive locus) gene was designed. The real-time PCR system was evaluated as 100% selective for pathogenic *Y. enterocolitica*, which was determined using 20 *Y. enterocolitica* strains, 16 non-enterocolitica *Yersinia* spp. and 30 other *Enterobacteriaceae* strains. The PCR detection limit was 1×10^2 CFU.ml^{-1}. Single selective enrichment based on the evaluation of various enrichment procedures was optimized. Out of 20 food samples analysed, four were positive by the PCR-based method and only one by the standard method. When 10 food matrices were artificially contaminated at a level of 10^0 CFU, ten and six sets of samples were positive by respective methods. The developed method may be used for the detection of pathogenic *Y. enterocolitica* as a specific and reliable alternative to the currently used microbiological method, furthermore providing a considerable reduction in the required analysis time.

Keywords

pathogenic *Yersinia enterocolitica*; real-time PCR; detection; food

Yersinia enterocolitica is a food-borne pathogen causing yersiniosis, which can range from a self-limiting gastroenteritis to a potentially fatal septicemia [1]. The primary route of infection of humans is most probably through ingestion of contaminated food. Healthy pigs have been identified as a major reservoir of the human pathogenic *Y. enterocolitica* strains and pork is therefore likely to be the most important vehicle for its transmission to humans [2].

Species *Y. enterocolitica* is divided into six biotypes: 1A, 1B, 2, 3, 4 and 5 [3]. Five biotypes (except for 1A) are considered pathogenic for humans. The strains belonging to biogroup 1A are regarded as avirulent, although they may be opportunistic pathogens [4, 5]. The serotype most frequently implicated in human infection is O:3 (almost all strains belonging to biogroup 4), then serotypes O:8 (biogroup 1B) and O:9, O:5,27 (biogroup 2 or 3) [6, 7]. Virulence of *Y. enterocolitica* isolates depends on the expression of several chromosomal and plasmid-encoded genes, and on a complex interplay between the secreted virulence factors [8]. First chromosomal factor required for the infection, *ail* (attachment invasion factor) gene, is a stable virulence marker limited to only pathogenic strains of *Y. enterocolitica*. The virulence plasmid pYV is not a good DNA target for the detection of pathogenic *Y. enterocolitica*, because of the possibility to obtain false negative results due to a potential loss of the plasmid during subculturing.

The currently available standard method for the detection of presumptive pathogenic *Yersinia enterocolitica*, EN ISO 10273 [9], is labour-intensive, time-consuming and does not provide the required selectivity and detection limit for reliable results. PCR-based methods are known to be a powerful tool for highly specific and sensitive identification of pathogenic bacteria in foods and are considered

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reliable alternatives to conventional methods [10]. The second generation of PCR methodologies, i.e. real-time PCR, is able to overcome several limitations of conventional PCR. The dominant target for the PCR-based detection of pathogenic *Y. enterocolitica* strains is *ail* gene, which has been used either in conventional PCR [11–13] or in real-time PCR [14–16].

In this study, a TaqMan real-time PCR-based detection targeted to *ail* gene is described. Being found rapid and sensitive, *Y. enterocolitica* PCR detection suitable for routine application has been applied to natural and artificially contaminated foods and evaluated by comparing real-time PCR with conventional detection. Different enrichment media and conditions have been used and compared to improve the sensitivity of the detection, in particular in the presence of high levels of the competing microflora.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Yersinia spp. used in the study (listed in Tab. 1) were obtained from Czech Collection of Microorganisms (CCM, Brno, Czech Republic), Spanish Type Culture Collection (CECT, Valencia, Spain), kindly provided by Prof. Naydenski (Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria), or isolated and identified in our laboratory (VÚP Food Research Institute, Bratislava, Slovakia). The strains from *Enterobacteriaceae* family other than *Yersinia* spp. were obtained from official collections. All strains were maintained at –18 °C in 60% (v/v) glycerol solution or freeze-dried for long-period storage. Working cultures were prepared in Brain Heart (BH) broth (Merck, Darmstadt, Germany) by overnight incubation at 37 °C with shaking. Decimal dilutions were prepared in 0.85% NaCl and cell concentrations were determined by the plate-count method.

Identification of *Yersinia enterocolitica* strains

Strains of *Yersinia* spp. were analysed by using published conventional PCR for selected chromosomal markers (*ail* and *ystB*), virulence plasmid pYV (*yadA*) and species-specific markers (Ye16S rDNA) described in Tab. 2. Growth and characteristic colony morphology of *Y. enterocolitica* strains was followed on a selective agar with cefsulodin, irgasan and novobiocin (CIN agar, Merck).

Preparation of DNA and 5'-nuclease PCR

DNA was prepared from overnight cultures using lysis by boiling, as previously described for other pathogenic bacteria [17]. As a positive control for PCR, DNA was extracted from *Y. enterocolitica* CCM 5671 using QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany). Each PCR reaction contained 300 nmol·l^{-1} of each of primers ailrtF and ailrtR, 200 nmol·l^{-1} of the TaqMan probe ailrtP (Qiagen Operon, Cologne, Germany), 500 μmol·l^{-1} of each dNTP (Applied Biosystems, Foster City, California, USA), 1.5 U of HotStar Taq DNA polymerase (Qiagen), 1× concentrated PCR buffer, 4.5 mmol·l^{-1} magnesium chloride, TaqMan exogenous internal positive control labelled with the dye VIC (Applied Biosystems), 2.5 μl of the DNA sample and water to make the total volume up to 25 μl. Real-time PCR was performed in a PTC-200 thermal cycler coupled to a Chromo 4 continuous fluorescence detector (MJ Research, Waltham, Massachusetts, USA) using a thermal programme consisting of the initial denaturation of 15 min at 95 °C, and 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Three negative and two positive controls were included in each experiment.

Tab. 1. Sequences of oligonucleotide used in the study.

Designation	Sequence and labelling	Product size	Reference
ailF/ailrtF	5´-TTAATGTGTACGCTGCGAGTG -3´	425 bp	[15]
ailR	5´-GGAGTATTCATATGAAGCGTC -3´		
ystBF	5´-TTGGACACCGCACAGCTTAT -3´	263 bp	this study
ystBR	5´-ACAGGCAGGATTGCAACATA -3´		
Ye16SF	5´-AATACCGCATAACGTCTTCG-3´	329 bp	[11]
Ye16SR	5´-CTTCTTCTGCGAGTAACGTC -3´		
yadAF	5´-AGATTCGGCAGTTACTTATGG-3´	308 bp	[15]
yadAR	5´-ATTGCGCGACATTCACT -3´		
ailrtR	5´-CAGGTTAAAACCTTTAGGGTTCA -3´	107 bp	this study
ailrtP	5´- FAM-TTAATGTGTACGCTGCGAGTG -TAMRA-3´		

Tab. 2. *Yersinia* spp. identification using PCR targeting different virulence markers.

No.	Strain and designation	PCR result				
		Ye16S (330 bp)	ail (425 bp)	ailrt (107 bp)	ystB (262 bp)	yadA (308 bp)
1	*Y. enterocolitica* VUP 25	+	–	–	+	–
2	*Y. enterocolitica* VUP 26	+	–	–	+	–
3	*Y. enterocolitica* VUP 27	+	–	–	+	–
4	*Y. enterocolitica* VUP 28	+	–	–	+	–
5	*Y. enterocolitica* VUP 29	+	–	–	+	–
6	*Y. enterocolitica* VUP 30	+	+	+	–	–
7	*Y. enterocolitica* VUP 31	+	+	+	–	–
8	*Y. enterocolitica* VUP 33	+	+	+	–	–
9	*Y. enterocolitica* VUP 54	+	+	+	–	–
10	*Y. enterocolitica* VUP 55	+	+	+	–	–
11	*Y. enterocolitica* VUP 61	+	+	+	–	–
12	*Y. enterocolitica* VUP 62	+	+	+	–	–
13	*Y. enterocolitica* VUP 64	+	–	–	+	–
14	*Y. enterocolitica* VUP 65	+	+	+	–	–
15	*Y. enterocolitica* VUP 155	+	+	+	–	–
16	*Y. enterocolitica* VUP 167	+	–	–	+	–
17	*Y. enterocolitica* CCM 5671	+	+	+	–	–
18	*Y. enterocolitica* CECT 4054	+	+	+	–	–
19	*Y. enterocolitica* CECT 4055	+	+	+	–	–
20	*Y. enterocolitica* CECT 559	+	+	+	–	–
21	*Y. bercovieri* CCM 4206	–	–	–	–	–
22	*Y. bercovieri* IP 22935	–	–	–	–	–
23	*Y. bercovieri* IP 22914	–	–	–	–	–
24	*Y. frederiksenii* CCM 3555	–	–	–	–	–
25	*Y. frederiksenii* IP 22775	–	–	–	–	–
26	*Y. intermedia* CCM 3558	–	–	–	–	–
27	*Y. intermedia* IP 22702	–	–	–	–	–
28	*Y. intermedia* IP 22803	–	–	–	–	–
29	*Y. kristensenii* CCM 3559	–	–	–	–	–
30	*Y. kristensenii* CCM 3561	–	–	–	–	–
31	*Y. kristensenii* IP 22468	(+)*	–	–	–	–
32	*Y. kristensenii* IP 22914	(+)	–	–	–	–
33	*Y. mollaretti* CCM 4208	–	–	–	–	–
34	*Y. pseudotuberculosis* CCM 5666	–	–	–	–	–
35	*Y. pseudotuberculosis* IP 32979	–	–	–	–	–
36	*Y. pseudotuberculosis* IP 32981	(+)	–	–	–	–
	Y. enterocolitica Y79**	+	+	+	–	+

* – weak PCR product, ** – DNA reference material obtained from Institute for Reference Materials and Measurements, Geel, Belgium.

Determination of PCR detection limit

DNA from the overnight culture of *Y. enterocolitica* CCM 5671 was isolated using QiaAmp DNA Mini Kit (Qiagen) and used to determine the DNA-based detection limit. Concentration of total extracted DNA was determined using Quant-iT PicoGreen Assay (Invitrogen, Gaithersburg, Maryland, USA) with fluorescence measured in a Tecan Saphire2 plate reader (Tecan, Salzburg, Austria). Two parallel PCR analyses of diluted DNA solutions were performed. For the determination of the practical PCR detection limit and PCR detection probability, two independent sets of three to ten parallel PCR analyses of suitable dilutions (ranging from 10^6 to 10^0 CFU·ml^{-1}) of *Y. enterocolitica* CCM 5671 overnight culture were performed.

Evaluation of enrichment procedures

Two selective broths and related enrichment procedures were used according to EN ISO 10273: enrichment in Peptone Sorbitol Bile (PSB) broth (Fluka, Buchs, Switzerland) at 25 °C for 2 to

3 days, and enrichment in Irgasan Ticarcillin Potassium Chlorate (ITC) broth (Fluka) at 25 °C for 48 h. Culture in a new Yersinia Selective Enrichment Broth (YSEB) according to Ossmer (Merck) at 30 °C for 24 h was also tested for comparison. For evaluation of different enrichment procedures, model *Enterobacteriaceae* microflora at levels of 10^8, 10^6 a 10^4 CFU per 100 ml of medium was used as the background. Real background microflora was prepared by homogenization of a raw meat sample in a bag with a filter insert. Total bacterial count of 6×10^8 CFU·ml^{-1} and *Enterobacteriaceae* count of 2×10^6 CFU·ml^{-1} were determined in this homogenate. *Y. enterocolitica* growth in individual enrichment cultures was followed by real-time PCR and the results were expressed in terms of *Y. enterocolitica* recovery.

Food samples and artificial contamination

Food samples were obtained from retail market in Slovakia. All food samples were analysed for the presence of *Y. enterocolitica* using a microbiological method according to EN ISO 10273 [9] and using the developed real-time PCR-based method. For artificial contamination, *Y. enterocolitica* CCM 5671 were inoculated at two initial concentrations of 10^0 and 10^1 CFU per 10 g sample and 90 ml of medium.

Reference method

The method according to EN ISO 10273 (Microbiology of food and animal feeding stuffs – Horizontal method for the detection of presumptive pathogenic *Yersinia enterocolitica*) [9] was used, simultaneously utilizing two selective enrichment media with incubations up to 5 days, followed by identification of characteristic *Y. enterocolitica* colonies on two selective agar media, and their subsequent confirmation using biochemical and serological tests.

RESULTS AND DISCUSSION

Evaluation of virulence markers application

Results of duplex PCR targeting *ail* gene for "more pathogenic" *Y. enterocolitica* strains (biotypes 1B, 2 to 5) and *ystB* gene for "less pathogenic" *Y. enterocolitica* strains (biotype 1A) clearly distinguished these two groups (Tab. 2, Fig. 1). Out of 20 analysed *Y. enterocolitica* strains, 13 were positive for *ail* gene marker and 7 for *ystB* gene marker. Marker of *yadA* gene detecting pYV plasmid was not identified for any *Y. enterocolitica* strain, the detection ability of PCR being verified using DNA reference material from Institute for Reference Materials and Measurements (Geel, Belgium). Species-specific marker targeting 16 S rDNA gave positive results for all *Y. enterocoolitica* analysed, but also false positive results for some other *Yersinia* spp. (Tab. 2). Our results confirmed successful application of *ail* gene, a chromosomally located marker. The *yadA* gene located on the virulence plasmid was considered unsuitable as a target for the detection because of plasmid instability during the laboratory treatment of strains.

Design of oligonucleotides and selectivity of real-time PCR

Based on published *Y. enterocolitica ail* gene sequence, a new reverse primer and a probe were designed using Primer Express software (Applied Biosystems) to amplify a 107 bp product; the forward primer previously designed for conventional PCR [15] was used. No non-*Y. enterocolitica* homological DNA sequences were found in the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA). Selectivity of the developed real-time PCR system was evaluated using 63 *Yersinia* spp. and other *Enterobacteriaceae* strains demonstrating a 100% inclusivity and 100% exclusivity.

PCR detection limit

The DNA-based detection limit, determined using dilutions of DNA isolated from *Y. entero-*

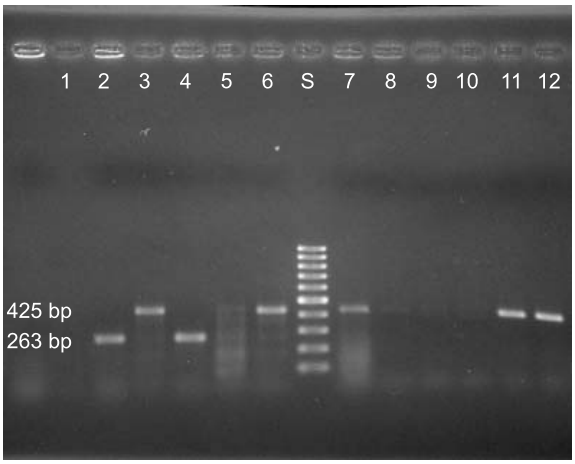

Fig. 1. Example of a duplex PCR targeting *ail* gene (425 bp) and *ystB* gene (263 bp).
1 – *Y. pseudotuberculosis* CCM 5666, 2 – *Y. enterocolitica* VUP25, 3 – *Y. enterocolitica* CCM 5671, 4 – *Y. enterocolitica* VUP27, 5 – *Y. intermedia* CCM 3558, 6 – *Y. enterocolitica* VUP31, 7 – *Y. enterocolitica* VUP62, 8 – *Citrobacter freundii* CCM 4475, 9 – *Serratia marcescens* CCM 303, 10 – *Y. frederiksenii* CCM 3555, 11 – *Y. enterocolitica* VUP65, 12 – *Y. enterocolitica* VUP155, S – molecular standard $n \times 100$ bp.

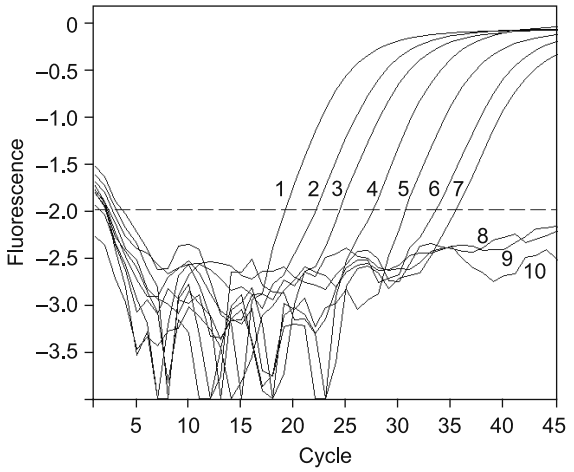

Fig. 2. Record of real-time PCR using *Y. enterocolitica* CCM 5671 culture dilutions.
1 – 6×10^7 CFU·ml^{-1}, 2 – 6×10^6 CFU·ml^{-1}, 3 – 6×10^5 CFU·ml^{-1}, 4 – 6×10^4 CFU·ml^{-1}, 5 – 6×10^3 CFU·ml^{-1}, 6 – 6×10^2 CFU·ml^{-1}, 7 – 1.2×10^2 CFU·ml^{-1}, 8 – 6×10^1 CFU·ml^{-1}, 9 – 1.2×10^1 CFU·ml^{-1}, 10 – 6×10^0 CFU·ml^{-1}.

colitica CCM 5671, was approximately 10 fg per PCR reaction (data not shown). This is equivalent to two genome copies, calculated on the basis of *Y. enterocolitica* genome size of 4.62 Mbp ≈ 5 fg, (BLAST database, AM286415, National Center for Biotechnology Information). The practical PCR detection limit was estimated using decimal dilutions of the overnight culture and DNA samples prepared by cell lysis (Fig. 2). The detection probability of the cell suspension was 100% at a concentration of 1.2×10^2 CFU·ml^{-1} and at higher concentrations, and 75% at a concentration of 6.0×10^1 CFU·ml^{-1} (data not shown). Comparable or higher detection limits were obtained by other researchers [14, 15]. Thisted-Lambertz et al. [16] showed in their study, that the probability of detecting the cell count level of 10 *Y. enterocolitica* cells per ml was 47% and that of cell count level of 100 *Y. enterocolitica* cells per ml was 97%. These results mean that a concentration of target *Y. enterocolitica* greater than 1.2×10^2 CFU·ml^{-1} must be reached by enrichment to ensure the reliable detection of *Y. enterocolitica* in the food sample using the developed real-time PCR method.

Evaluation of enrichment procedures

Y. enterocolitica CCM 5671 recovery was evaluated, using real-time PCR detection during the enrichment in three different selective media at two levels of artificial contamination and enrichment conditions according to manufacturer's instructions, and using model and real background microflora. PCR provided more sensitive detection in the case of high concentration of model *Enterobacteriaceae* background microflora, but at the extreme load of 10^8 CFU·ml^{-1}, the detection of 2×10^0 CFU of *Y. enterocolitica* per sample completely failed using both methods. On the other hand, using real microflora background (probably containing stressed cells) at a total bacterial count of 6×10^8 CFU·ml^{-1} with *Enterobacteriaceae* count of 2×10^6 CFU·ml^{-1}, the detection was 100% successful. Results for the detection at contamination levels 10^0 CFU, 10^1 CFU and 10^2 CFU per 100 ml culture in combinations with background microflora of 10^4, 10^6 and 10^8 total bacterial count are presented in Tab. 3 and Tab. 4. The obtained results demonstrate that the best results of positive samples recovery and most rapid PCR detection of *Y. enterocolitica* were obtained using Yersinia Selective Enrichment Broth (YSEB). Summarized results of enrichment recovery for positive samples are expressed in percentage in Tab. 5. Applicability of this medium was verified by model detection of 2×10^0 CFU of pathogenic *Y. enterocolitica* strains of different serotypes (CECT 4054 serotype O:8, CECT 4055 serotype O:3, VUP62 serotype O:5) and the same performance of the medium was observed (data not shown). Similar results were described by Thisted-Lambertz et al. [16], who determined the accuracy of the method according to ISO 10273 to be only 40%, compared to 95% of the real-time PCR, as determined using food samples artificially inoculated with low levels of *Y. enterocolitica* (10 to 300 CFU per 25 g sample).

Detection of pathogenic *Y. enterocolitica* in food samples

Subsequently, the enrichment for 24 to 48 h in Yersinia enrichment selective broth at 30 °C and the detection by the developed real-time detection was compared to the detection method according to EN ISO 10273 [9] for the detection of presumptive pathogenic *Y. enterocolitica* by analysis of 20 food products. Four samples were found positive for *Y. enterocolitica* by the real-time PCR-based method and only one sample was found positive by the standard detection method (Tab. 6).

The efficiency of the developed real-time PCR-based detection method was further evaluated using ten food samples artificially contaminated with *Y. enterocolitica* CCM 5671 at levels of 2×10^1 CFU per 10 g and 2×10^0 CFU per 10 g, respectively. For all 10 samples, positive detection results were obtained by the real-time PCR-based method. On the other hand, one and four samples produced negative results using the standard method at levels of 10^1 CFU per 10 g and 10^0 CFU

Tab. 3. Evaluation of different selective enrichment procedures by *ail*-targeted real-time PCR and standard method, using three levels of *Y. enterocolitica* contamination and three levels of model background microflora (*Enterobacteriaceae* strains).

Y. enterocolitica CCM 5671 [CFU]	Bacground microflora [CFU]	24 h		48 h		72 h	
		rtPCR	EN ISO	rtPCR	EN ISO	rtPCR	EN ISO
PSB medium, 25 °C, with shaking (EN ISO 10273)							
10^0	–	nd	nd	+	–	+	+
	10^4	nd	nd	+	–	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	–	–
10^1	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	–	–
10^2	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	+	–	+	+
ITC medium, 25 °C (EN ISO 10273)							
10^0	–	–	–	+	+	nd	nd
	10^4	–	–	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	–	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	–	–	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	–	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	–	–	+	+	nd	nd
YSEB medium, 30 °C (new)							
10^0	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	–	–	+	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	+	–	+	–	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	+	nd	nd

nd – not determined.

Tab. 4. Evaluation of different selective enrichment procedures by real-time PCR and standard method using three levels of *Y. enterocolitica* contamination and three levels of real background microflora (raw meat filtered homogenate).

Y. enterocolitica CCM 5671 [CFU]	Background microflora [CFU]	24 h		48 h		72 h	
		rtPCR	EN ISO	rtPCR	EN ISO	rtPCR	EN ISO
PSB medium, 25 °C, with shaking (EN ISO 10273)							
10^0	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	–	–
10^1	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	–	+	+
	10^8	nd	nd	–	–	+	–
10^2	–	nd	nd	+	+	+	+
	10^4	nd	nd	+	+	+	+
	10^6	nd	nd	+	+	+	+
	10^8	nd	nd	+	–	+	+
ITC medium, 25 °C (EN ISO 10273)							
10^0	–	–	–	+	+	nd	nd
	10^4	–	–	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	–	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	–	–	+	+	nd	nd
	10^8	–	–	+	+	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	–	+	+	nd	nd
	10^6	+	–	+	+	nd	nd
	10^8	–	–	+	+	nd	nd
YSEB medium, 30 °C (new)							
10^0	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	–	nd	nd
10^1	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	–	nd	nd
10^2	–	+	+	+	+	nd	nd
	10^4	+	+	+	+	nd	nd
	10^6	+	+	+	+	nd	nd
	10^8	+	–	+	+	nd	nd

nd – not determined.

Tab. 5. Detection ability of *Y. enterocolitica* CCM 5671 at contamination levels of 10^0, 10^1 and 10^2 CFU with background microflora using three enrichment procedures.

	24 h		48 h		72 h	
	rtPCR	EN ISO	rtPCR	EN ISO	rtPCR	EN ISO
PSB medium, 25 °C (with shaking)	nd	nd	83%	46%	88%	79%
ITC medium, 25 °C	42%	25%	88%	88%	nd	nd
YSEB medium, 30 °C	96%	63%	100%	83%	nd	nd

nd – not determined.

Tab. 6. Results of the detection of pathogenic *Y. enterocolitica* in natural food samples.

Sample	Number of samples	Number of positive results	
		rtPCR detection	EN ISO detection
raw meat	6	2	1
bowels	3	0	0
fish	3	0	0
frozen vegetable	4	0	0
fresh chopped vegetable	4	2	0
Total	20	4	1

Tab. 7. Results of the detection of pathogenic *Y. enterocolitica* in food samples artificially contaminated at two levels.

Sample	Number of samples	Number of positive results			
		rtPCR detection		EN ISO detection	
		10^0 CFU	10^1 CFU	10^0 CFU	10^1 CFU
raw meat	2	2	2	1	2
bowels	2	2	2	2	2
fish	2	2	2	1	2
frozen vegetable	2	2	2	2	2
fresh chopped vegetable	2	2	2	2	2
Total	10	10	10	6	9

per 10 g, respectively (Tab. 7). Insufficient detection ability of the standard method was observed also by other investigators [11, 14, 16]. Fredriksson-Ahomaa and Korkeala [18] concluded that the reported low rate of pathogenic *Y. enterocolitica* strains isolated from foods is most probably due to a limited sensitivity of the traditional culture detection methods, which provided the detection limits between 10^3 to 10^6 CFU or more per g of pork products.

CONCLUSSION

The currently established standard method for the detection of pathogenic *Y. enterocolitica* in food (EN ISO 10273) is based on enrichment simultaneously in two selective broths followed by microbiological and biochemical identification. This method is labour-intensive, time-consuming and analytically insufficient [16]. Generally, molecular-based methods have a well-grounded potential to overcome the insufficiences of identification procedures associated with biochemical characteristics. The aim of our study was to develop and optimize a highly sensitive and reliable method for the rapid and definitive detection of *Y. enterocolitica* pathogenic biotypes in food, in particular in food products containing high levels of competing microflora. The method would be coupled to selective enrichment and ensure the required detection limit. The developed real-time PCR-based method, which involves an optimized, reduced selective enrichment, facilitates the detection of pathogenic *Y. enterocolitica* strains on the next day after the food sample reception. The developed

real-time PCR system targeting *ail* gene may be complemented by PCR targeting *ystB* gene, which would result in the detection of all *Y. enterocolitica* biotypes.

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