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Designing a time-effective TaqMan probe-based real-time polymerase chain reaction protocol for the identification of *Yersinia enterocolitica* in raw pork meat

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Abstract

The aim of this study was to design a time-effective method comprising a short pre-enrichment step in a non-selective broth in combination with the TaqMan probe applied in the real-time polymerase chain reaction to detect *Yersinia enterocolitica* strains in raw pork meat. The method enabled to detect 1 colony forming unit per 25 mg of *Yersinia enterocolitica* in pork meat. The specificity and reliability of the method was not diminished by the company of microflora naturally present in meat. The method was found successful to detect pathogenic *Yersinia enterocolitica* strains in pork meat. It is advised to be used for assessing the microbial risk and for controlling the microbial quality of meat and meat products.

Pathogenicity, molecular biology, enrichment, detection

Meat and meat products possess very high nutritional value and are rich with compounds required for the growth of naturally present microflora. Meat is quite often contaminated with different pathogenic microorganisms. Such phenomenon imposes the necessity to guarantee the safety of meat and meat products in order to eliminate the risk of exposing the consumer to food-borne diseases caused by the consumption of contaminated foodstuffs. The development of successful analytical methods to detect the presence of pathogens in meat and meat products becomes crucial (Kocher et al. 1989; Herman 2001; Heptinstall and Rapley 2002). Such detection methods enable to confirm or exclude the presence of pathogenic bacteria in meat and meat products with reliable results (Koppel et al. 2011). They could be used for food hygiene purposes and may also predict the potential risk of the presence of pathogenic strains. Classical culture-based detection methods sometimes do not deliver accurate and reliable results; moreover, they are tedious and time-consuming. They do not find their application because they do not meet the requirements of the meat industry which produces on a massive scale, when the safety of large amounts of meat and meat samples must be determined relatively fast (Girish et al. 2007; Kesmen et al. 2007).

Nowadays, molecular methods based on the sequencing of nucleic acids involving the polymerase chain reaction (PCR) and the real-time polymerase chain reaction (real-time PCR) are used as they are reliable, specific, sensitive and time saving protocols for both qualitative and quantitative detection of various pathogenic microorganisms in meat and meat products (Tanabe et al. 2007; Jonker et al. 2008). Special attention should be paid to the real-time PCR method which is gaining popularity in different analytical applications in food processing, making it possible to detect various pathogenic bacteria and monitor the gene expression of bacteria caused by industrial procedures (Dooley et al. 2004). Real-time PCR is the molecular method which enables the simultaneous amplification and detection of specific deoxyribonucleic acid (DNA) sequences. The analysis is carried out in a thermocycler. This method is rapid, specific, sensitive and reliable. It is less risky in terms of carryover contamination in comparison to the conventional PCR method (Walker

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et al. 2003; Mane et al. 2009). A large number of chemistries have been applied for the detection of targeted DNA during real-time PCR analysis (Kesmen et al. 2007). They were widely defined as "specific" or "non-specific" for the amplified sequence. In the present study, a TaqMan probe was used, being a kind of "specific" detection chemistry, in which a short sequence of oligonucleotides is specified and labelled with two fluorescent dyes which specifically attach to the targeted DNA and release a fluorescence signal with simultaneous DNA amplification (Higgins et al. 1992). The sequence of nucleotides in the TaqMan probe is compatible with the sequence of the targeted DNA in the genome of identified bacteria which makes it possible to detect this bacteria without the need for any further confirmatory assays (Monteil-Sosa et al. 2000; Hird et al. 2005; Rodriguez et al. 2005).

To guarantee the successfulness and sensitivity of the designed real-time PCR assay for the detection of a very small number of cells of *Yersinia enterocolitica* (*Y. enterocolitica*) in raw meat, a 20 h-long pre-enrichment stage in a nutritious non-selective medium was necessary for the multiplication of Yersinia cells to a number high enough to be detected by the PCR analysis (Saez et al. 2004; Tanabe et al. 2007; Martín et al. 2009). There have been some successful attempts to shorten this long pre-enrichment step to 8 h to detect and quantitatively specify the number of pathogenic cells in pig carcasses. However, this step was still too time-consuming. For the purpose of the present study, a time-effective and highly specific TaqMan probe-based real-time polymerase chain reaction protocol was designed involving a brief pre-enrichment step lasting for 4 h and enabling to detect a very low number of pathogenic Yersinia cells in raw pork meat (Rodríguez et al. 2003; Sawyer et al. 2003; Walker et al. 2003).

Materials and Methods

Bacterial strains and growth conditions

A mixture of 12 *Yersinia enterocolitica* strains (collection of Ghent University, Belgium) was used for the purpose of this study. Each strain culture was prepared by taking a single colony which was grown on tryptone soya agar (TSA) (Oxoid, UK) and placing it into 10 ml of tryptone soya broth (TSB) (Oxoid, UK). It was incubated at 30 °C for 16–24 h. An overnight culture was carried out individually for each strain, then it was mixed equally together, and serially diluted to different cell concentrations in TSB. There were also some other microorganisms which were prepared in the same way and cultured in the conditions specified above. The only difference was that they were incubated at 37 °C for 16–24 h. These microorganisms involved *Escherichia coli* ATCC 25922, *Shigella flexneri* ATCC 12022, *Enterobacter cloacae* ATCC 13047, *Klebsiella pneumoniae* ATCC 35657, and *Pseudomonas aeruginosa* ATCC 27853.

Evaluation of a viable number of cells of *Yersinia enterocolitica* directly from naturally contaminated raw pork meat

Viable cells of *Y. enterocolitica* strains in raw pork meat were evaluated by the standard plate count technique. Samples of raw pork meat were taken aseptically, placed into sterile plastic bags and stored under chilled conditions for the purpose of our study. The samples of meat were checked for the presence of pathogenic *Y. enterocolitica* by direct plating and different enrichment protocols according to the International Organization for Standardization (ISO) method. Meat samples were aseptically cut into small pieces, and 10 g of meat were put into a 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). Then, 0.1 ml of such homogenate was placed on cefsulodin-irgasan-novobiocin (CIN) agar plates (Yersinia Selective Agar Base and Yersinia Selective Supplement, Oxoid, UK) by a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). Then, the CIN agar plates were incubated at 30 °C for 24 h and investigated for characteristic Yersinia colonies using a stereo microscope with Henry illumination (Olympus, Germany).

Suspected colonies were taken by a sterile loop and incubated in trypton soy broth (PCA, Oxoid, UK) at 30 °C for 4 h and for 20 h. Then the culture grown in trypton soy broth was taken for the real-time PCR analysis. Suspected colonies of *Y. enterocolitica* were also inoculated on Plate Count Agar at 30 °C for 24 h and then taken for biochemical testing.

Pre-enrichment step

Twenty-five mg of raw pork meat samples free from pathogenic cells of *Y. enterocolitica* were inoculated with a mixture of 12 *Y. enterocolitica* strains listed in Table 1. The inoculation level was 1 ml of bacterial mixture

containing 10^2 cfu. The samples were inoculated in 225 ml of pre-warmed TSB and buffered peptone water (BPW) (Oxoid, UK), incubated at 30 °C for 4 h with simultaneous shaking at 200 rpm. Samples were examined in duplicate. Ten ml of pre-enriched culture were subjected to DNA extraction. Raw pork samples which were used for inoculation were previously tested negative with a PCR method.

Table 1. List of *Yersinia enterocolitica* strains used in the study.

Species	Original strain no.	Biotype	Serotype	Virulence gene/virulence plasmid	Collection
Y. enterocolitica	YE1	4	O:3	ail[1], ystA[2]	Ghent University, Belgium
Y. enterocolitica	YE3	4	O:3	ail, ystA	Ghent University, Belgium
Y. enterocolitica	YE5	4	O:3	ail, ystA	Ghent University, Belgium
Y. enterocolitica	YE7	4	O:3	ail, ystA	Ghent University, Belgium
Y. enterocolitica	YE165	3	O:3	ail, ystA	Ghent University, Belgium
Y. enterocolitica	KNG22703	2	O:9	pYV[3]	Ghent University, Belgium
Y. enterocolitica	2516-87	2	O:9	pYV	Ghent University, Belgium
Y. enterocolitica	8081	1B	O:8	ail, pYV	Ghent University, Belgium
Y. enterocolitica	WA	1B	O:8	pYV	Ghent University, Belgium
Y. enterocolitica	W22703	2	O:9	pYV	Ghent University, Belgium
Y. enterocolitica subsp. *palearctica*	105.5R(r)	3	O:9	pYV	Ghent University, Belgium
Y. enterocolitica subsp. *palearctica*	Y11	4	O:3	ail, ystA	Ghent University, Belgium

ail[1] (it encodes the attachment invasion locus protein), ystA[2] (it encodes the production of enterotoxins Yst (Yersinia stable toxins)) – these target genes are found in the chromosome, they are virulence factors found in pathogenic *Y. enterocolotica* strains; pYV[3] (Yersinia virulence plasmid) – it is the virulence plasmid which encodes a type III secretion system (*ysc* and *lcr* genes) essential for delivery of additional plasmid-borne anti-host factors collectively referred to as Yops (Yersinia outer proteins). It is a virulence factor found in pathogenic *Y. enterocolotica* strains.

Biochemical testing

Urea Agar according to Christensen

Bacterial material from the PCA was taken with a loop and incubated on Urea Agar according to Christensen (Oxoid, UK) at 30 °C for 24 h. Pink-violet or red-pink colour indicated a positive urease reaction. An orange-yellow colour indicated a negative urease reaction.

Kligler Iron Agar

Bacterial material from the PCA was taken with a loop, put on the slant surface of Kligler Iron Agar (Oxoid, UK) and incubated at 30 °C for 24 h. *Yersinia enterocolitica* is glucose positive and lactose negative, and does not form H_2S or gas.

Bile Esculine Agar

Bacterial material from the PCA was taken with a loop, put on the surface of Petri dishes of Bile Esculine Agar (Oxoid, UK) and incubated at 30 °C for 24 h. A black halo around the colonies indicated a positive reaction. Pathogenic *Y. enterocolitica* were negative.

DNA extraction

A 10-ml portion of 4h-bacterial culture in TSB and BPW was centrifuged at 11.700 × *g* for 10 min, washed in 5 ml of 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), suspended in 1 ml of 0.01 M sodium phosphate buffer in 20% sucrose (pH 7.0) with lysozyme (2.5 mg/ml) and incubated 45 min at 37 °C. A 9-ml portion of lysis buffer (10 mM Tris-hydro-chloride [pH 8.0], 1 mM ethylenediaminetetraacetic acid (EDTA), 500 µg of pronase B per ml, 1% sodium dodecyl sulphate) were then added. After additional 30 min at 37 °C, the samples were deproteinized by extraction with phenol and chloroform, and nucleic acids were precipitated with ethanol. The samples were suspended in 10 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA and stored at 4 °C. The concentration of DNA (ng/µl) was measured by the spectrophotometer (Evolution 220, Thermoscientific, USA).

The mass (M) of 1 genomic molecule was counted on the base of the equation $M = n \times (1.01 \times 10^{-21}\ g \cdot bp^{-1})$; for Yersinia 4.616×10^{6}bp. The quantified DNA (number of genomic copies/µl) was serially diluted in water and underwent the real-time PCR according to the conditions specified below.

Real-time PCR reactions

DNA extracted from *Y. enterocolitica* was subjected to a TaqMan probe-based real-time PCR assay targeting a DNA sequence within the CH49_3099 locus gene specific for pathogenic strains of *Y. enterocolitica*. The assay included a forward primer, a reverse primer, a target TaqMan probe (FAM-MGB-NFQ labelled), the internal amplification control (IAC) template, and IAC probe.

The primer/probe set targeting locus _tag CH49_3099 gene was designed on the base of 50-nucleotide sequence GACGATACCTTGGTATAGCAATCTATTTAGCACTGATGTGTCGGTTCCGG specific for *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 (Table 2). The primer/probe set was designed using Primer Express Software v3.0 (Applied Biosystems, Foster City, 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'-GACGATACCTTGGTATAGC-3'; reverse primer 5'- ATAGCTGATGACTTTAT-3'; probe 5'-FAM- CCGGAACCGACACATCAGTGCTAAATAGAT-3'-MGB-NFQ. The amplicon size was 66 bases. The oligonucleotides were synthesised and purchased from Eurofins Genomics (Germany).

The reaction total volume was 20 µl. Real-time PCR analysis was performed using the thermocycler of Stratagene Mx3005P (Agilent Technologies, USA). The PCR mixture contained 5 µl DNA template, 12.5 µl of TaqMan Universal Master Mix (Syngen Biotech, United Kingdom), 600 nM of primers R and F respectively, and 200 nM of FAM-MGB-NFQ labelled probe. A non-template control (NTC) contained 5 µl of water instead of DNA and was included in each run. The real-time PCR cycling parameters were the following: 1 cycle of initial denaturation of DNA at 95 °C for 10 min followed by 40 cycles of amplification with denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. The real-time PCR reaction and amplification step were carried out using a DNA amplification curves which were the subject of analysis. The calculation of the threshold cycle (C_T) value was carried out using Stratagene Mx3005P software version 2.1 (Agilent Technologies, USA). The C_T value was described as the real-time PCR cycle, at which the generated fluorescence increased exponentially and exceeded its background level.

Statistical analysis

Each experiment was repeated at least three times and data were analysed using analysis of variance (ANOVA) and Duncan multiple range test ($P < 0.05$).

Results

Sensitivity of the TaqMan probe-based real-time PCR protocol for the detection of *Yersinia enterocolitica* species in raw pork meat without pre-enrichment

A detection limit of the TaqMan probe-based, real-time PCR protocol which made it possible to directly assess different concentrations in the range from 1 to 10^8 cfu·25 mg^{-1} of *Y. enterocolitica* in raw pork meat without the pre-enrichment step was the subject of investigation. It was observed that higher C_T values were found when there was a decrease in the total number of viable cells of *Y. enterocolitica.* Such phenomenon was attributed to the decrease in the amount of DNA coming from cells (Fig. 1). The real-time PCR analysis enabled to detect viable cells

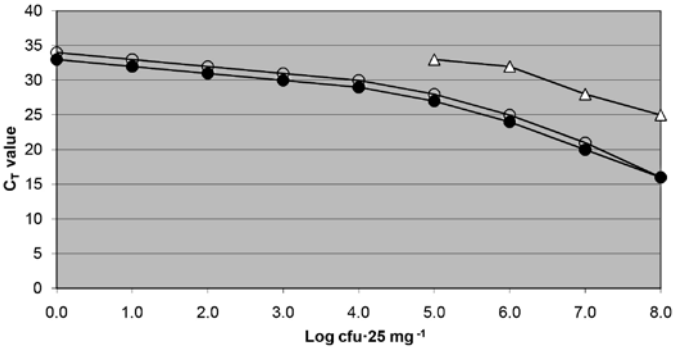

Fig. 1. Detection of various concentrations of *Yersinia enterocolitica* strains in 25 mg of raw pork meat samples by the real-time PCR without pre-enrichment (▲) and after brief pre-enrichment for 4 h in BPW (○), and after brief pre-enrichment for 4 h in TSB (●). C_T values are means of at least 3 separate determinations.

at a level of 10^5–10^8 cfu·25 mg^{-1} of *Y. enterocolitica* in raw pork meat without the pre-enrichment step, and the C_T values were in a range from 33 to 25 (Fig. 1). It was not possible to detect viable cells of *Y. enterocolitica* in a number lower than 10^5 cfu·25 mg^{-1}. So the detection limit of the protocol designed for the need of this study was a value of 10^5 cfu·25 mg^{-1} for viable cells.

Table 2. Partial locus _tag CH49_3099 gene sequence alignment of 6 strains of Yersinia with the database sequence indicating sequence differences.

	Region of gene from 3300786 to 3300855									
	Forward primer 5'-3'			Probe 3'-5'			Reverse primer 3'-5'			
Y. enterocolitica (CP009846.1)	C	GACGATACCTTGGTATAGC	A	ATCTATTTAGCACT GATGTGTCGGTTCCGG		CA	ATAAAGTCATCAGCTAT			
Y. kristensenii (CP009997.1)	T	CGCGCGTTCGGTGTTGGAG	A	AACTGGGCAACAGTGTCGAGGTGGCGATGA		AT	GGCCACGATGCGCTGGC			
Y. intermedia (CP009801.1)	T	CACTCGTTTGGCACAATTC	A	TACAGGAAAGGCCATGCAAGGCCAAGACTG		TG	GTTTGCAACATAATTAA			
Y. pestis (AE009952.1)	G	CACCCGCCAATAGCAGTAA	A	CCCAATAACAGCAG CTCTGCAAACAAGATA		AT	CAACCAATAGAGGGCGA			
Y. pseudotuberculosis (CP009786.1)	C	GGTGCTGGTTTCATGGCTG	C	CCCAATAACAGCAGCTCTGCAAACAAGATA		AT	CTATTGATTTGTACATG			
Y. rohdei (CP009787.1)	C	TAAAGACAACCAACTGGCA	G	CT TT CCAACATTCCGGT TT CTGGC AACCTT		GT	GATACTCTCCGCGACAA			

Application of the 4 h pre-enrichment step in BPW and TSB

In order to evaluate the sensitivity of the TaqMan probe-based real-time PCR protocol towards the assessment of the number of viable cells of *Yersinia enterocolitica* in raw pork meat, a brief pre-enrichment step lasting for 4 h before real-time PCR analysis was applied. Its aim was to establish the detection limit of the protocol which meant the lowest number of detectable viable cells. The pre-enrichment steps involved in this protocol were carried out in two culture media, TSB and BPW. They were applied with the conjunction of the real-time PCR to assess the number of viable cells of *Y. enterocolitica* in the range of 1–10^8 cfu·25 mg^{-1}. The involvement of pre-enrichment steps in the real-time PCR protocol enabled to detect a number of pathogenic cells in the range from 1 cfu·25 mg^{-1} which constitutes the detection limit (Fig. 1). It was found that slightly higher C_T values were observed in the case of application of the 4-h pre-enrichment step in BPW in comparison to the 4-h pre-enrichment in TSB. However, the differences were very small ($P < 0.05$). It means that both media are suitable to be used in combination with the real-time PCR analysis. The present study proves that both culture media can be successfully used as a pre-enrichment step to multiply the number of *Y. enterocolitica* cells to be detected during the real-time PCR analysis.

Discussion

Yersinia species very often naturally contaminate meat and meat products. As they are often

accompanied by other members of the *Enterobacteriaceae,* the designed 4-h pre-enrichment/real-time PCR protocol was checked for the ability to detect different concentrations of *Y. enterocolitica* in mixed cultures in raw pork meat (López-Andreo et al. 2006; Laube et al. 2007). The accompanied bacteria were represented by *E. coli, S. flexneri, E. cloacae, K. pneumoniae,* and *P. aeruginosa.* To check the competition among these species, mixed cultures were applied to deliver a large number of cells at a level of 10^7–10^8 cfu·25 mg^{-1}. The accompanied bacteria did not influence the method sensitivity and *Y. enterocolitica* was detectable at a level even as low as 1 cfu·25 mg^{-1} of raw pork meat with the application of either TSB or BPW as a pre-enrichment media.

The results of the present investigation prove that the 4 h pre-enrichment step either in TSB or BPW in the combination with the real-time PCR method is a very time-effective and highly specific TaqMan probe-based real-time polymerase chain reaction protocol for the detection of *Y. enterocolitica* in raw pork meat (Sawyer et al. 2003). The analysis lasts for approximately 5.5 h in comparison to at least 3 days when the traditional culture-based method is applied. The shortening of the pre-enrichment time to 4 h was crucial and contributed to making the analysis more rapid in comparison with cultivating the strains overnight. Application of the 4-h pre-enrichment step in the present research enables to provide the results within the same working day. Moreover, the detection limit for the designed 4-h pre-enrichment/real-time PCR protocol amounted to 1 cfu·25 mg^{-1}, which proved a very high sensitivity of the probe/primers set for the identification of *Y. enterocolitica* species in raw pork meat (Jonker et al. 2008; Kesmen et al. 2009; Koppel et al. 2011). The very low detection limit of Yersinia strains can be also explained by the fact that in meat there is a very high availability of nutrients, and lack of growth inhibitors in pre-enriched meat samples which might encourage the multiplication of Yersinia to achieve the levels that are detectable within only 4 h (Kesmen et al. 2007). Moreover, the designed protocol was found to be successful in the detection of as few as 1 cfu·25 mg^{-1} of Yersinia in meat samples not only thanks to the specific sequences of nucleotides in the set of probe/primers but also thanks to the application of the pre-enrichment step in BPW and TSB pre-warmed to 30 °C, and shaking cultures during the pre-enrichment time.

In conclusion, the results of the study proved that the designed TaqMan probe-based real-time polymerase chain reaction protocol for the identification of *Y. enterocolitica* in raw pork meat is very time-effective and highly specific because within approximately 5.5 h it is able to detect as few as 1 cfu·25 mg^{-1} of *Y. enterocolitica* in raw pork meat. It enables the detection of Yersinia in a quantitative form in meat and meat products. It constitutes a very useful tool in the assessment of microbial contamination and in the hazard analysis critical control points plans.

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