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OPTIMIZATION OF REAL-TIME PCR PROTOCOL FOR DETECTION OF PATHOGENIC *YERSINIA ENTEROCOLITICA* STRAINS

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Summary

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Detection and isolation of pathogenic *Yersinia enterocolitica* strains from foods of animal origin is difficult and inefficient in many cases. During the last decade, many classical microbiological and immunochemical methods are successfully complemented with molecular based techniques. In the current study, an optimized protocol for Real-Time PCR and an efficient system for detection of *ail* gene in *Yersinia enterocolitica* is presented. The selected primer pair MP1/MP2 with the MPFR as a positive control and TaqMan® probe were successfully applied for detection and quantification of *Yersinia enterocolitica* WA 314 strain of bio/serotype 1b/O:8. The established protocol could be implemented for detection and quantitation of pathogenic *Yersinia enterocolitica* strains in clinical samples like meat, milk, faeces, etc.

Key words: *ail* gene, optimization, real-time PCR, *Yersinia enterocolitica*

INTRODUCTION

Pathogenic strains of the *Yersinia enterocolitica* species have a significant impact on the epidemiological dynamics of infections associated with consumption of contaminated foods and the related negative social and healthcare problems (Bottone, 1999). Despite the evident role of foods of animal origin in the transmission of yersiniae, isolation of pathogenic strains in many cases is laborious and inefficient (De Boer, 1995).

Adequate detection of *Y. enterocolitica* in pork meat is important for consumer's health, because pigs are a recognized reservoir and factor in the transmission of pathogenic strains and pork meat is a basic component in the food industry (Pavlov, 1989; Fredriksson-Ahomaa & Korkeala, 2003).

The pathogenic potential of *Y. enterocolitica* is determined by complex interaction of chromosomal and plasmid genes (Parkhill *et al.*, 2001). The presence of a 70 kb plasmid (pYV, plasmid *Yersinia* virulence) and specific chromosomal determinants (genes *ail*, *yst,* etc.) are necessary for the full expression of pathogenic potential. Loss of plasmid is a phenomenon, which is frequently observed in laboratory practise, due to conditions of cultivation and storage. The psychrophilic nature of pathogenic *Yersinia* strains deter-

mines their ability for growing and multiplication in low-temperature regimes, used for storage of food products. That is why chilled and frozen foods are potential reservoirs and sources of infection, factors in transmission of pathogenic yersiniae and source of food-borne disorders and related complications.

During the last decade classical microbiological and immunochemical schemes for detection of food-borne pathogens are successfully supplemented with DNA-based methods. Besides direct hybridization techniques, the majority of these methods include *in vitro* amplification. Some of these techniques like NASBA, 3SR, LCR still have limited application in food microbiology. PCR is the most developed method up to now based on *in vitro* amplification and has a potential for rapid and selective detection of microorganisms.

During the last years there is a significant progress in development of Real-Time PCR aiming at the quantitation of bacterial load in different complex samples. Its principle is based on detection of a fluorescent signal, which is proportional to the number of amplicons in the tested sample (Higuchi *et al.*, 1992; 1993; Lee *et al.*, 1993; Livak *et al.*, 1995).

There are two major groups of methods for the detection of the fluorescent signal during PCR-sequence specific probes (TaqMan®, Molecular Beacons, Scorpions) (Heid *et al.*, 1996; Vet *et al.*, 1999; Mhlanga & Malmberg, 2001; Saha *et al.*, 2001; Solinas *et al.*, 2001; Abravaya *et al.*, 2003; Tan *et al.*, 2004) and DNA-binding agents (SYBR-green I, EtBr). The latter allows a relatively low-cost detection (Wittwer *et al.,* 1997; Morrison *et al.*, 1998; Donohoe *et al.*, 2000; Siraj *et al.*, 2002; van der Velden *et al.*, 2003).

The main goal of the recent study is the optimization of a Real-Time PCR protocol based on the *ail* gene, in order to quantify *Yersinia enterocolitica*. This will contribute to the fast and quantitative detection of pathogenic yersiniae in complex samples like meat, milk, faeces, etc.

MATERIALS AND METHODS

Bacterial strain and preparation of genomic DNA standard

Yersinia enterocolitica strain WA 314, bio/serotype 1b/O:8 (kindly provided by prof. Wolf-Wotz, Umea, Sweden) was maintained as a stock culture with 20% DMSO at −70 °C.

Bacterial cells were incubated on Mac Conkey agar (Difco Laboratories, Detroit, Michigan, USA) at 25 °C for 24 hours and DNA isolation was carried out by a standard protocol (Ausubel *et al.*, 1995). The concentration of obtained DNA fraction was determined spectrophotometrically on SmartSpec3000 (BioRad, USA) at 260 nm. The quality was tested by standard gel electrophoresis (1.5% agarose, 30 min, 100 V). The molecular weight (MW) of double strand (ds) DNA was calculated according to the formula:

MW ds DNA=number of base pairs×649.

Decimal dilutions from 2.0×10^{10} copies/μL to 2.0×10^{7} copies/μL were prepared in TE buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA). Working scheme included 8 dilutions in HPLC water (2.0×10^{6} copies/μL to 0.2 copies/μL).

Primer pairs for detection of ail gene (Acc. No M29945, EMBL database)

The sequences of the primers, probes and ssDNA fragment used in this study are given in Table 1.

All primer sequences were designed by Primer Express® software (Applied

Table 1. Sequences of primers, probes and ssDNA fragment and their location on the *Y. enterocolitica ail* gene

Name	Sequences	Location and direction (based on EMBL/Genbank Acc. No. M29945)
MA1	5′-GCC-TGT-TTA-TCA-ATT-GCG-TCT-GT-3′	521–543 >
MA2	5′-GCT-TTG-CGC-ATA-ACC-AAT-AGA-A-3′	< 574–595
MA3	5′-GGT-TAA-GGC-ATC-TGT-ATT-TGA-TGA-ATC-3′	846–873 >
MA4	5′-TGG-AAG-TGG-GTT-GAA-TTG-CA-3′	< 915–934
MP1	5′-GCC-TGT-TTA-TCA-ATT-GCG-TCT-GT-3′	521–543 >
MP2	5′-TGG-CTT-TGC-GCA-TAA-CCA-A-3′	< 579–594
TaqMan® probe	5′-TGT-ACG-CTG-CGA-GTG-AAA-GTA-GTA-TTT-3′*	549–584
ssDNA fragment (MPFR)	5‘–TT-GCC-TGT-TTA-TCA-ATT-GCG-TCT-GT-TAA-TGT-GTA-CGC-TGC-GAG-TGA-AAG-TAG-TAT-TTC-TA-TTG-GTT-ATG-CGC-AAA-GCC-A-TT–3‘	521–594

* On the 5’ and 3’ ends, a carboxyfluorescein as reporter dye and a black whole quencher are respectively present.

Biosystems, USA) and ordered by Eurogentec S.A. (Belgium).

SYBR Green-I method for Real-Time PCR detection of the ail gene

The protocol was tested using 1×SYBR® Green I PCR Master Mix (Applied Biosystems, USA) with addition of 600 nM from each primer and 5 μL template DNA. The final volume of the reaction was 25 μL.

TaqMan® method for Real-Time PCR detection of ail gene

The TaqMan® PCR Master Mix (Applied Biosystems, USA) was applied, with addition of 600 nM from each primer and 5 μL template DNA. The final volume of reaction was 25 μL.

All reactions were carried out by using the ABI 7000 (Applied Biosystems, USA) detection system. The amplification programme included incubation at 95 °C for 10 min, followed by 40 cycles (denaturation at 95 °C – 15 s; annealing and elongation at 60 °C for 1 min).

RESULTS AND DISCUSSION

The experimental scheme included the initial test of amplification efficiency with MA1/MA2 and MA3/MA4 primer pairs. Both primer sets were tested with the SYBR Green method. With the MA1/MA2 primer pair, non-specific reaction products were encountered after analysis of the dissociation curves obtained during denaturation of Real-Time PCR amplification products. Furthermore, with both primer pairs, positive fluorescent signals from negative (blank) controls were frequently observed. For these reasons, MA1/MA2 and MA3/MA4 primer pairs were not restrained for further tests. At this point of the experimental scheme the primer pair MP1/MP2 (521–543>/<579–594) was included.

The obtained results allowed quantification in the interval of 1.0×10^7 to 1.0×10^3 copies genomic DNA. The standard curve based on these dilutions of DNA showed a linear relationship be-

tween log input DNA and the threshold cycle (Ct) (Fig. 1). The regression equation was $y = -3.272 \log(x) + 41.54$ with a square regression coefficient (R^2) of 0.997 and an amplification efficiency of 102.3%.

All blanc reactions were negative using the MP1/MP2 primer pair. The lack of fluorescent signal from blanc controls confirmed the absence of aspecific reactions and contamination of the PCR reaction mix. The amplification of a single DNA fragment of the expected length was demonstrated by the analysis of the dissociation curves, obtained by denaturation of the amplification products.

In parallel, a Real-Time PCR assay for detection of the *ail* gene was carried out with TaqMan® probe. As a positive control, a single stranded DNA fragment (MPFR) was synthesized based on the sequence information of the *ail* gene (Acc. No M29945, EMBL database).

The obtained results illustrated the linear relation between the initial DNA amount and the detected fluorescent signal and allowed determination of target DNA in the interval of 1.0×10^6 to 1.0×10^1 copies/reaction. The regression equation was

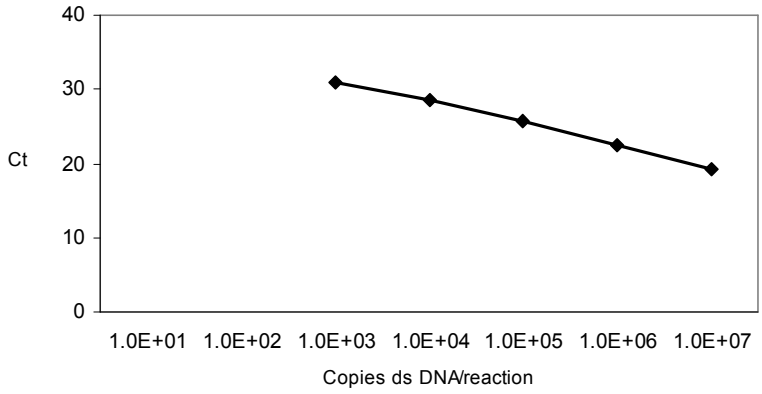

Fig. 1. Standard curve with MP1/ MP2 primer pair.

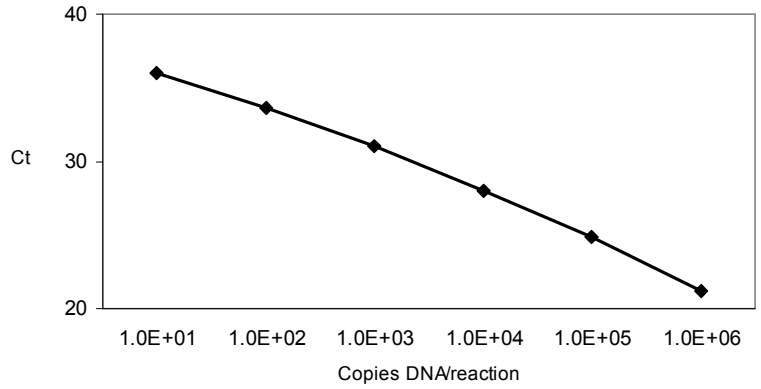

Fig. 2. Standard curve with MP1/ MP2 primer pair and TaqMan® probe.

$y = -2.783 \log(x) + 39.082$ with a square regression coefficient (R^2) of 0.9783 (Fig. 2).

The optimized protocol of real-time PCR including SYBR Green I as well as the TaqMan® probe revealed a definitive capacity for detection of the chromosomally encoded *ail* gene in pathogenic *Yersinia enterocolitica* strains. The selected primer pair MP1/MP2, with positive control MPFR and TaqMan® probe could be successfully applied for detection and development of system for quantity assessment of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* strains in food (meat, milk, etc.) and clinical samples (blood, faeces, etc.). This will contribute to the effective monitoring and evaluation of bacterial contamination of food products.

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