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ORIGINAL ARTICLE

Development of a real-time PCR assay with an internal amplification control for the screening of Shiga toxin-producing *Escherichia coli* in foods

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food analysis, internal amplification control, real-time PCR, Shiga toxin, Shiga toxin-producing *Escherichia coli*, *stx*.

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Abstract

Aims: To develop and evaluate a real-time PCR assay incorporating an internal amplification control (IAC) suitable for the screening of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) in foods.

Methods and Results: A competitive IAC was constructed and included in an *stx*-specific real-time PCR assay. Coupled to 18-h enrichment and automated DNA extraction, the assay could reliably detect the presence of STEC in minced meats inoculated at 10 CFU per 25 g. Its performance was evaluated on 415 minced beef and 112 raw milk cheese samples and compared with that of a PCR-ELISA method. Fifty-three minced meats and 31 cheeses were found *stx*-positive, giving 98·3% and 93·75% concordance, respectively, with the PCR-ELISA reference method.

Conclusions: A highly sensitive *stx*-specific real-time PCR method including an IAC was developed, facilitating monitoring of false-negative results due to PCR inhibitors.

Significance and Impact of the Study: Combined with automated DNA extraction, the *stx*-IAC real-time PCR assay represents a suitable method for rapid screening of STEC in foods.

Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC), also called verotoxin-producing *E. coli*, are a diverse group of food-borne pathogens. They cause a wide spectrum of human diseases, ranging from mild diarrhoea to haemorrhagic colitis and the life-threatening haemolytic-uraemic syndrome (Paton and Paton 1998). Their ability to cause serious illnesses in humans is related in part to their capacity to produce Stx1 and Stx2.

Several real-time PCR methods based on *stx1*- and *stx2*-specific fluorogenic probes have been proposed for STEC detection (Belanger *et al.* 2002; Ibekwe *et al.* 2002; Reischl *et al.* 2002; Jinneman *et al.* 2003; Nielsen and Andersen 2003; Sekse *et al.* 2005; Stefan *et al.* 2007). However, most of these assays are not applicable as diagnostic tools as they lack an internal amplification control (IAC) to detect false-negative results due to PCR-inhibitory compounds (Hoorfar *et al.* 2004a; Anonymous 2005).

In the present work, we developed a competitive IAC to improve a real-time PCR *stx*-typing assay designed previously (Perelle *et al.* 2004). The resulting PCR assay was evaluated on artificially and naturally contaminated food samples in comparison with a PCR-ELISA method used routinely in our laboratory (Fach *et al.* 2001).

Materials and methods

Bacterial strains

STEC strains B2F1 (*stx2d1*, *stx2d2*; Ito *et al.* 1990), PMK5 (*stx1*; Mariani-Kurkdjian *et al.* 1993) and 90·0327 (*stx1*, *stx2*; *E. coli* Reference Center) were grown at 37°C in

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tryptic soy broth supplemented with 0·6% yeast extract (TSBYE; AES Laboratoire, Combourg, France).

Primers and probes used in real-time PCR

The *stx*-specific primers (*stx*-fwd and *stx*-rev) and hybridization probes (*stx1*-probe and *stx2*-probe) have been described previously (Perelle *et al.* 2004), except that the *stx2*-probe was labelled with fluorescein and BHQ1. The IAC was amplified and detected using *stx*-fwd and *stx*-rev primers and a new specific IAC-probe [5′ (Red640)-CAAGGCGACAAGGTGCTGATGCCG-(BHQ2) 3′]. Oligonucleotides were purchased from Sigma-Aldrich (Evry, France).

Construction and preparation of the IAC

The IAC was designed so that its amplification product and those from the *stx1* and *stx2* targets had similar lengths (96, 131 and 128 bp, respectively). A recombinant 2·786-kb plasmid, designated pIAC-STEC, containing *stx*-fwd and *stx*-rev primer binding sites flanking a unique DNA fragment of the chloramphenicol acetyltransferase (*cat*) gene was constructed as follows. Two complementary 100-mer oligonucleotides (5′-AATTTTTGTTACTGTGACAGCTG AAGCTTTACGTGAATCGCCAG**CGGCATCAGCACCTT GTCGCCTTG**CGTATAGATGTTGATCTTACATTGAAC TGGGG-3′ and 5′-AATTCCCCAGTTCAATGTAAGATC AACATCTATACGCAAGGCGACAAGGTGCTGATGCCG CTGGCGATTCACGTAAAGCTTCAGCTGTCACAGTAA CAAA-3′; primers and IAC-probe binding site sequences indicated as underlined and bold letters, respectively) were hybridized and ligated to *Eco*RI-digested pUC19 (Invitrogen, Cergy-Pontoise, France). Following transformation into *E. coli* DH5α (Invitrogen) and purification, DNA concentration of pIAC-STEC was spectrometrically determined and its sequence confirmed (Cogenics, Meylan, France). The following equation was used to calculate the number of pIAC-STEC copies per microliter: $[6{\cdot}023 \times 10^{23} \times$ weight of pIAC-STEC $(g\ \mu l^{-1})]/[660\ g\ per\ mol \times 2786\ bp]$. The plasmid was freshly diluted to the working concentration of 64 copies per microlitre which corresponded to the lowest reproducible IAC concentration that amplified consistently and did not interfere with the amplification of the target sequence.

Preparation of template DNA

DNA was released from STEC cultures by heating using an InstaGene™ Matrix as described by the supplier (BioRad, Marnes-la-Coquette, France). Bacterial DNA was purified from food suspensions and eluted in 100 μl volume using the MagNA Pure LC DNA Isolation Kit III on the MagNA Pure system (Roche Diagnostics, Meylan, France) according to the manufacturer's recommendations.

PCR conditions for food analysis

PCR-ELISA was carried out by using 10 μl of template DNA, as described previously (Fach *et al.* 2001). Real-time PCRs were performed with the LightCycler® 1·2 instrument (Roche Diagnostics) as described previously (Perelle *et al.* 2004) with the following modifications. Three microlitre of DNA template were subjected to PCR (45 cycles of amplification) in a total reaction volume of 30 μl and in the presence of 200 nmol l^{-1} of the IAC-probe and 64 copies of pIAC-STEC. Each run systematically included four control reactions: one negative, one IAC and two positives containing *stx1* or *stx2* genomic DNA as template. For negative and positive controls, no IAC was included. Fluorescence was recorded at the end of the extension step in channel F1 (530 nm; *stx*) and F2/backF1 (640 nm; IAC).

Detection limit of the *stx* real-time PCR assay

STEC B2F1, PMK5 and 90·0327 were grown until a concentration of 10^9 CFU ml^{-1} was reached and 10-fold serially diluted in buffered peptone water. The number of CFU was determined by plating. DNA was extracted from 1-ml aliquots of dilutions 10^{-2} to 10^{-7} and 2 μl (1/100th) were analysed by real-time PCR in a total volume of 20 μl. Amplifications were performed in triplicate in two independent runs, with or without 64 IAC copies.

Artificially and naturally contaminated food samples, enrichment procedure and isolation of STEC

Artificially contaminated samples

Ten-fold serial dilutions of an *E. coli* B2F1 culture, grown at 37°C until it reached a concentration of 10^9 CFU ml^{-1}, were prepared. Aliquots of the dilutions were plated in duplicate on Tryptone Soy Agar plates (TSA; Oxoid, Dardilly, France) for direct enumerations of STEC cells that were introduced into minced beef. Samples (25 g) from a refrigerated minced beef batch were inoculated in triplicate with the *E. coli* B2F1 strain at three contamination levels (0, 10 and 100 CFU), diluted 10-fold (w/v) in modified TSB (mTSB; Oxoid) supplemented with novobiocin (Sigma-Aldrich) at 20 mg l^{-1}, and incubated at 37°C for 18 h.

Naturally contaminated samples

Four hundred and fifteen minced beef meats and 112 soft cheeses made from raw goat's milk (n = 51) or raw cow's

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milk (n = 61) were collected in France from 2005 to 2007 and analysed as described above except that 50-g portions of minced beef were tested, and acriflavin (Sigma-Aldrich) at 12 mg l^{-1} was used instead of novobiocin for cheeses analyses. STEC strains were isolated from *stx*-positive samples using a colony hybridization assay, as described previously (Fach *et al.* 2001).

Results

Sensitivity of the *stx*-IAC real-time PCR assay

The threshold sensitivity was experimentally assessed with DNA extracted from pure bacterial suspensions of an STEC B2F1 (*stx2d1*, *stx2d2*) strain ranging from 10^7 to 10^2 CFU ml^{-1}. A minimum of 100 CFU ml^{-1} corresponding to 200 *stx* copies per millilitre yielded a positive reaction (Table 1). When suspensions of STEC strains PMK5 (*stx1*) and 90·0327 (*stx1*, *stx2*) were tested, a detection limit of 100 CFU ml^{-1} was also determined, corresponding to 100 and 200 *stx* copies per millilitre, respectively (data not shown). The *stx* amplification plots obtained in the presence and absence of the IAC were similar, indicating that the IAC had no negative influence on target amplification and detection (data not shown).

Concerning the detection of the IAC, it occurred efficiently in the absence or presence of low amounts of STEC (i.e. $<10^5$ CFU ml^{-1}; Table 1). For higher STEC cells concentrations, nonproportional fluorescence signals with abnormal low Ct values were observed (Table 1). As the IAC probe sequence is homologous to a portion of the *cat* gene, it was also checked that a high concentration (10^9 copies per PCR) of the *cat* gene (carried on plasmids pLDR10 and pBAD33 for this assay) did not affect the detection of the IAC (data not shown), demonstrating that *cat* sequences which could naturally be present in food samples did not compete for the IAC probe.

The limit of detection of STEC in food was then examined using 25-g portions of refrigerated minced beef artificially contaminated and subjected to 18-h enrichment (Table 1). The noninoculated samples were all negative. Absence of PCR inhibition was confirmed by amplification of the IAC. All samples inoculated with 10 or 100 STEC CFU tested positive for *stx*, with target Ct values ranging from 22·73 to 24·80 cycles.

Table 1 Detection of STEC B2F1 in pure culture and in artificially contaminated minced beef samples

Sample type	Concentration (CFU ml^{-1})* or seeding level (CFU 25 g^{-1})†	*stx*-IAC real-time PCR‡	
		Ct (target)	Ct (IAC)§
Broth	10^7	21·52 ± 0·16	–
	10^6	25·69 ± 0·15	–
	10^5	29·16 ± 0·15	31·50 ± 0·40
	10^4	32·33 ± 0·31	33·64 ± 0·46
	10^3	33·99 ± 0·22	34·47 ± 0·42
	10^2	35·70 ± 0·15	34·48 ± 0·73
	0	–	34·61 ± 0·68
Minced beef	105	24·80 ± 0·21	–
	105	24·06 ± 0·06	–
	105	24·50 ± 0·30	–
	10	22·90 ± 0·06	–
	10	23·93 ± 0·06	–
	10	22·73 ± 0·16	–
	0	–	34·07 ± 1·11
	0	–	34·75 ± 1·13
	0	–	34·72 ± 0·87

STEC, Shiga toxin-producing *Escherichia coli*; IAC, internal amplification control; (–), negative PCR result.

*STEC concentration in broth.

†STEC seeding level in minced beef.

‡Ct values are mean ± SD for three determinations.

§For STEC levels higher than 10^4 CFU ml^{-1}, nonproportional IAC amplification curves were obtained with abnormal low IAC Ct values.

Detection of *stx* genes in naturally contaminated samples and isolation of STEC

A comparative study between the *stx*-IAC real-time PCR assay and a reference PCR-ELISA method was carried out on 415 minced beef and 112 raw-milk cheese samples suspected to be naturally contaminated. Both methods gave identical results for 408 out of the 415 minced meats (98·3% concordance) and for 105 out of the 112 unpasteurized cheeses (93·7% concordance) (Table 2). The target Ct values of the samples found *stx*-positive by real-time PCR ranged from 16·76 to 36·49 cycles for minced meats and from 16·82 to 38·93 cycles for cheeses (Table 2). Total of 7·5% and 16% of meat and cheese samples, respectively, showed high *stx* Ct values (>35; data not shown) corresponding to low STEC levels (i.e. $<10^3$ CFU ml^{-1}). Taking into account the 18-h enrichment step, it is uncertain whether those samples contained low amounts of stressed viable STEC or nonenrichable *stx* sequences. All matrices considered, eight (1·5%) out of the 527 samples were found to be PCR-inhibited and needed to be diluted for analysis (data not shown).

Colony hybridization was used to isolate STEC from the enriched food samples. STEC strains were obtained from 22 (42·3%) out of the 52 *stx*-positive minced meats and from three (10·3%) out of the 29 *stx*-positive cheeses (Table 2). No strains were isolated from the 14 samples that showed inconsistent results between real-time PCR and PCR-ELISA. It is worth noting that the mean target

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Table 2 Detection of *stx* genes in naturally contaminated food samples by *stx*-IAC real-time PCR and PCR-ELISA and STEC isolation by colony hybridization

Food type	PCR-ELISA* (OD_{450} target)	Real-time PCR* (Ct target)	No. of samples (%)	No. of samples with isolated STEC
Minced beef	+(0·114–4·000)	+(16·76–36·49)	52 (12·5)	22
	–	–	356 (85·8)	nd
	–	+(34·78)	1 (0·2)	0
	+(0·138–2·861)	–	6 (1·5)	0
Cheese	+(0·190–4·000)	+(16·82–38·93)	29 (25·9)	3
	–	–	76 (67·8)	nd
	–	+(30·14; 33·24)	2 (1·8)	0
	+(0·143–2·575)	–	5 (4·5)	0

STEC, Shiga toxin-producing *Escherichia coli*; IAC, internal amplification control; nd, not done.
*(–), negative result; (+), positive result; the range of OD_{450} and Ct values obtained for each sample category is indicated, except for categories containing one to two sample(s) for which all values are shown.

Ct value calculated for samples from which strains were isolated was significantly lower than the one found for the other *stx*-positive samples, i.e. 24·65 ± 4·47 compared with 30·57 ± 3·64 for the minced meats, and 21·55 ± 5·16 *vs* 32·57 ± 3·16 for the unpasteurized cheeses (data not shown).

Discussion

Until now, STEC screening in food products was performed at the French Food Safety Agency using a *stx*-specific PCR-ELISA detection system. This method, validated in 2001, has been shown to be specific and sensitive (Fach *et al.* 2001). Its results are obtained within 48 h. To save further time and labour, we developed a real-time PCR targeting the *stx* genes that includes a competitive IAC. The use of an IAC has been reported for two other *stx*-specific real-time PCR assays (Belanger *et al.* 2002; Stefan *et al.* 2007). In both cases, amplification of the IAC was performed using a different set of primers than for *stx* which may not accurately reflects the target amplification. By contrast, the IAC developed here is co-amplified with *stx* genes using a common set of primers, under the same conditions and in the same PCR tube, as recommended (Hoorfar *et al.* 2004b). Incorporation of a low number of IAC copies into the assay ensured competition advantages for *stx* target amplification and no negative effect of the IAC was seen on *stx* detection, even for low STEC concentrations. By contrast, the presence of high amounts of STEC affected the detection of the IAC, presumably because of nonoptimal amplification of the IAC during competitive PCR, as reported elsewhere (Malorny *et al.* 2004). Residual crosstalk between the two detection channels might also be suspected when high levels of fluorescence are emitted by the *stx* probes. This is however not problematic because the efficient production of an IAC fluorescence signal is only required when *stx* amplification has failed, i.e. in the absence of *stx* target. The *stx*-IAC real-time PCR was therefore proved to be highly sensitive and able to detect as few as 100 STEC CFU ml^{-1}, which is regarded as essential given the low infectious dose reported for STEC.

The *stx*-IAC real-time PCR assay was as effective as the PCR-ELISA test for detection of *stx* genes in contaminated foods. After enrichment, both methods were able to detect STEC in minced beef inoculated with as low as 10 CFU per 25 g. Both assays were also shown to perform equally well when applied on the same 527 naturally contaminated test portions, with 98·3% and 93·7% concordance according to the food products (minced meats and raw milk cheeses, respectively). Only 14 (2·7%) samples gave contradictory results. Differences in the volumes of the DNA samples subjected to PCR-ELISA and real-time PCR (10 μl *vs* 3 μl, respectively) might explain in part the discrepancies observed. The use of other real-time PCR instruments allowing the analysis of 10 μl DNA samples should contribute to increase the sensitivity of the assay. In addition, it cannot be excluded that some *stx* gene variants might be differentially amplified and/or detected by both methods. It was also noted that samples from which STEC isolates were recovered displayed a target Ct value significantly lower than that found for the other *stx*-positive samples. This reflects the presence of STEC at a higher density in the former enriched samples, which facilitated their isolation. The Ct value can therefore also be used for guidance to estimate the probability to recover a STEC strain from an enriched broth. Finally, it is worth mentioning that PCR results with Ct values above 35 should be interpreted with caution for the presence of viable STEC in the enrichment broths because a significant amount of nonenrichable *stx* templates (i.e. derived from bacteriophages or nonviable cells) might be

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present in the food samples and detected by PCR. More generally, when *stx*-positive PCR results are obtained, the presence of STEC in foods should be considered as presumptive unless confirmation by STEC isolation is achieved. It is however important to keep in mind that STEC isolation is still problematic, as illustrated here by STEC recovery rates of 10·3% and 42·3% from *stx*-positive cheese and meat samples, respectively, and as also reported by others (Pradel *et al.* 2000; Fach *et al.* 2001; Vernozy-Rozand *et al.* 2004, 2005).

In conclusion, the IAC-included real-time PCR assay described here represents a useful method for detection of *stx* genes and STEC screening in foods. Combination of high throughput automation and computer analysis allows to save time and labour and reduces the risks of carryover contamination in the laboratory compared with the previously employed PCR-ELISA method. With the 18-h enrichment step, results could be obtained within a total analytical time of 24 h.

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