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Detection of Shiga toxin-producing *Escherichia coli* (STEC) in ground beef and bean sprouts: Evaluation of culture enrichment conditions

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ABSTRACT

The main purpose of this work was to evaluate culture enrichment conditions, with particular regard to those reported in ISO/TS 13136:2012, for STEC detection in food. The culture media evaluated included mTSB with novobiocin 0–16 mg/l (mTSB + $N_{0–16}$) or acriflavin 12 mg/l (mTSB + A_{12}); BPW; mBPWp with acriflavin 10 mg/l, cefsulodin 10 mg/l, vancomycin 8 mg/l (mBPWp + ACV); and mBPWp with cefsulodin 10 mg/l, vancomycin 8 mg/l (mBPWp + CV). They were used for the growth of STEC O157, O26, O103, O111, O145 and O104 in pure cultures or in artificially contaminated food matrices (ground beef, mung bean sprouts). STEC detection was accomplished using commercially available multiplex real-time PCR assays targeting *stx*1-*stx*2 and *eae*, and serogroup-associated genes. More rapid multiplication of STEC in pure cultures occurred in mBPWp + CV, while an inhibitory effect of novobiocin and acriflavin was observed for some STEC serogroups in media with these selective agents. mBPWp + CV allowed the detection of all serogroups in bean sprouts when inoculated at levels as low as 1 CFU/25 g. A reduced novobiocin concentration of 2 mg/l in mTSB was required for STEC detection in ground beef samples. A temperature of 42 °C for the entire duration of the enrichment or 44 °C after an initial phase of 6 h at 37 °C was important to limit the multiplication of non-target bacteria. Results of this study suggest that media and protocols should be adapted to the food being analyzed, since protocols provided in official reference methods may produce insufficient sensitivity.

1. Introduction

Shiga toxin producing *Escherichia coli* (STEC) strains are defined as *E. coli* possessing genes encoding for Shiga toxins, which are important virulence factors in the pathogenesis of disease. It has been estimated that STEC cause 2,801,000 acute illnesses annually worldwide, leading to 3890 cases of HUS and 230 deaths (Majowicz et al., 2014). The most commonly reported serogroup was O157, although its relative proportion compared to other serogroups has declined (EFSA & ECDC, 2016). STEC O157, O26, O103, O111, and O145, the so called 'top five', along with the O104 epidemic strain responsible of the German outbreak in 2011, are considered those epidemiologically associated with the largest number of cases and the most severe disease (ECDC, 2013; EFSA BIOHAZ Panel, 2013).

Surveillance programs in many countries have traditionally targeted STEC O157; however, food analysis methods that detect any STEC, regardless of the serogroup, are now increasingly needed. In recent years, molecular methods such as the PCR and mainly real-time PCR-based protocols, have been shown to have high specificity and sensitivity, can reduce the analysis time, and have had a significant impact for food safety (Jenkins et al., 2015).

Recently, the Food Safety and Inspection Service of the United States Department of Agriculture (FSIS USDA) has expanded the zero-tolerance policy for *E. coli* O157 in raw beef products to include six non-O157 serogroups and have incorporated real-time PCR assays into their detection protocol (Fratamico et al., 2011; USDA, 2012), while the International Organization for Standardization (ISO) has also reported a real-time PCR-based method for detecting the 'top five' STEC (ISO, 2012). These methods are based on real-time PCR screening of sample enrichment cultures for the presence of the *stx* and *eae* genes, followed by serogroup identification and the characterization of the isolated strains.

Although PCR-based methods yield good results with testing of DNA isolated directly from the food enrichments (Amagliani et al., 2004;

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Omiccioli, Amagliani, Brandi, Bruce, & Magnani, 2009), a culture-enrichment step cannot be neglected. The enrichment phase has the twofold advantage of selectively increasing target bacteria concentration, thus enhancing assay sensitivity, and ensuring that positive results are obtained from viable cells. Therefore, the success of DNA-based pathogen detection and identification still heavily relies on sample preparation and culture enrichment. Indeed, the selection of an appropriate enrichment medium and incubation temperatures is very important. Some recent reports have demonstrated the effectiveness of different culture conditions for STEC growth and subsequent detection by PCR-based and/or plating protocols (Fratamico et al., 2014; Kanki, Seto, Harada, Yonogi, & Kumeda, 2011; Margot, Tasara, Zwietering, Joosten, & Stephan, 2016; Margot, Zwietering, Joosten, O'Mahony, & Stephan, 2015; Singh & Mustapha, 2015; Stromberg, Lewis, Marx, & Moxley, 2015; Verhaegen et al., 2016). However, to our knowledge, a systematic approach for the comparison of ISO and Food and Drug Administration Bacteriological Analytical Manual (FDA/BAM) media for the growth of the top STEC serogroups, followed by real-time PCR detection, has not been described. For this reason, the aim of our work was the comparison of STEC ("top five" plus O104) growth, both in pure cultures and in artificially contaminated food enrichments, in media recognized by official methods (ISO/TS 13136:2012 and FDA/BAM) and proposed by other authors (Weagant, Jinneman, Yoshitomi, Zapata, & Fedio, 2011), with the objective of proposing protocols compliant with sensitivity levels provided by EU Regulations.

2. Materials and methods

2.1. Real-time PCR methods

Two commercial real-time PCR kits (Diatheva, Fano, Italy) compliant with the ISO/TS 13136:2012 were used in the current study. The STEC FLUO Detection kit – Real-Time PCR is a screening test that first detects the combination of *stx*1/*stx*2 and *eae* virulence genes, in the presence of an Internal Amplification Control (IAC); the STEC Serotypes FLUO kit, here provided in the form of a prototype designed according to ISO/TS 13136:2012 specifications for primer and probe sequences, is a three panel-assay for serogroup identification (two serogroups in each assay plus the IAC according to the following combinations: O157-O111; O26-O103; O145-O104).

All real-time PCR reactions were conducted on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) with the following thermal cycling protocol: denaturation at 95 °C 10 min and 50 cycles at 95 °C 15 s and 60 °C 1 min.

2.2. Inclusivity and exclusivity testing

The specificity of the STEC FLUO Detection kit – Real-Time PCR was initially assessed with a panel of 18 bacterial species different from *E. coli*, 5 *E. coli* lacking *stx* and *eae* genes (exclusivity), 7 *stx*-negative and *eae*-positive *E. coli* and 24 STEC strains positive for *stx* and *eae* (inclusivity). A list of the strains used in this study is provided in Table 1. In addition, strains kindly provided by the European Reference Laboratory for Shiga-toxin producing *E. coli* (VTEC EURL, http://www.iss.it/vtec/), possessing all *stx*1$_{a,\ b,\ d}$ and *stx*2$_{a–g}$ genetic variants, were tested (control strains D2435, D2587, D3435, D3509, D3522, D3546, D3602, D3648). Moreover, *eae* gene subtyping was carried out in 20 *eae*-positive strains according to Madic et al. (2010), to determine *eae* gene variants carried by the strains (Table 1). Finally, STEC strains carrying the *stx*1 and *stx*2 gene variants were used to compare the inclusivity of the commercial kit with the real-time PCR protocol of the ISO/TS 13136:2012 (Annex E, Primers and probes for the PCR assays).

Table 1
Strains used for specificity testing using the STEC FLUO kit and for artificial inoculation.

Bacterial species	Strain genetic features*			STEC FLUO kit results	
	*stx*1	*stx*2	*eae*	*stx*	*eae*
Enterococcus faecalis UU 4421[a]	–	–	–	–	–
Escherichia coli ATCC 25922	–	–	–	–	–
E. coli UU1[a]	–	–	–	–	–
E. coli UU2[a]	–	–	–	–	–
E. coli UU3[a]	–	–	–	–	–
Listeria innocua ATCC 33090	–	–	–	–	–
L. monocytogenes ATCC 9525	–	–	–	–	–
Klebsiella oxytoca ATCC 8724	–	–	–	–	–
K. pneumoniae ATCC 13883	–	–	–	–	–
Serratia marcescens ATCC 14756	–	–	–	–	–
Aeromonas hydrophila ATCC 7966	–	–	–	–	–
Yersinia pseudotuberculosis[a]	–	–	–	–	–
Salmonella enteritidis UU7[a]	–	–	–	–	–
Salmonella Newport UU2[a]	–	–	–	–	–
Citrobacter freundii ATCC 8090	–	–	–	–	–
Proteus vulgaris[a]	–	–	–	–	–
Shigella flexneri ATCC 12022	–	–	–	–	–
Enterobacter cloacae ATCC 13047	–	–	–	–	–
E. aerogenes ATCC 13048	–	–	–	–	–
Pseudomonas aeruginosa ATCC 10145	–	–	–	–	–
Staphylococcus aureus ATCC 6538	–	–	–	–	–
S. aureus ATCC 25923	–	–	–	–	–
E. coli D2435 O48[b]	*stx*1a	*stx*2a	–	+	–
E. coli D2587 O174[b]	–	*stx*2b, *stx*2c	–	+	–
E. coli D3435 O73[b]	–	*stx*2d	–	+	–
E. coli D3509 O2[b]	–	*stx*2g	–	+	–
E. coli D3522 O8[b]	*stx*1d	–	–	+	–
E. coli D3546 O128[b]	–	*stx*2f	β1	+	+
E. coli D3602 O174[b]	*stx*1c	*stx*2b	–	+	–
E. coli D3648 O139[b]	–	*stx*2e	–	+	–
E. coli ED495 O113[b]	–	*stx*2c, *stx*2d	–	+	–
E. coli ED513 O128[b]	–	*stx*2b	–	+	–
E. coli ED546 O159[b]	–	*stx*2e	–	+	–
E. coli ED585 O111[b]	*stx*1a	–	θ	+	+
E. coli ED600 O26[b]	*stx*1a	–	β1	+	+
E. coli ED603 O121[b]	–	*stx*2a	ε	+	+
E. coli ED643 O26[b]	*stx*1a	–	β1	+	+
E. coli ED645 O145[b]	–	*stx*2a	γ1	+	+
E. coli ED654 O26[b]	–	*stx*2a	β1	+	+
E. coli EF292 O145[b]	–	–	γ1	–	+
E. coli EF299 O145[b]	–	–	γ1	–	+
E. coli EF333 O26[b]	–	–	β1	–	+
E. coli EF334 O26[b]	–	–	β1	–	+
E. coli EF335 O26[b]	–	–	β1	–	+
E. coli EF337 O26[b]	–	–	β1	–	+
E. coli UU O26[a]	*stx*1	*stx*2	β1	+	+
E. coli UU O103[a]	–	*stx*2	θ	+	+
E. coli UU O145[a]	*stx*1	–	γ1	+	+
E. coli UU O111[a]	–	–	β1	–	+
E. coli 1952 O157[a]	–	*stx*2	γ1	+	+
E. coli ATCC 35150 O157	*stx*1	*stx*2	γ1	+	+
E. coli F146 O157[a]	–	*stx*2	γ1	+	+
E. coli SSI 82110 O104[c]	–	–	–	–	–
E. coli 2011-3493 O104[d]	–	*stx*2a	–	+	–
E. coli O104:H4[e]	–	*stx*2a	–	+	–

Strain origin: a, strains from our in-house collection; b, kindly provided by the EURL VTEC; c, purchased from Statens Serum Institut (Copenhagen, Denmark); d, USDA collection, EAggEC epidemic strain responsible of the German outbreak 2011; e, DNA of the same EAggEC2011 epidemic strain kindly provided by Prof. Helge Karch (University of Münster, Germany).

* Gene variants, if known, are reported.

2.3. Effect of various culture conditions on STEC growth in pure cultures

STEC strains of six serogroups (ISO/TS 13136:2012 target serogroups O157, O111, O26, O103, O145, and including O104, which is