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EXTENDED ABSTRACT

Improvement of biomolecular methods for the identification and typing of *Escherichia coli* O157:H7 isolated from raw meat

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Abstract The aim of the study was to evaluate the sensitivity of two m-PCR methods for the quantitative determination of *E. coli* O157:H7 in foodstuffs. Genomic serotyping was carried out on bacterial cultures, and the necessary time was optimized to increase the resolution of the method. Subsequently, artificial contamination trials using meat were conducted to assess method accuracy in foodstuffs and pursue the genetic typing of pathogens. Measurement thresholds were shown to range between 10^5 and 10^6 CFU/mL, but were reduced by four logarithmic cycles in 80% of samples. Relative to the meat contamination trials, serotypes were identified after 24 hours, corresponding to 10 CFU/mL inoculum, with higher rates seen when m-TSB was used for enrichment. Inoculated samples were found to contain three virulence factors (*hly*A, *eae*A, and *stx1*).

Keywords *E. coli* VTEC · Genomic serotyping · Multiplex PCR · Virulence factors

Abbreviations

STEC	*E. coli* shiga-like toxin
VTEC	*E. coli* verocytotoxin
HUS	hemolytic uremic syndrome
CFU	colony forming units
EHEC	*E. coli* enterohemorragic

Introduction

In the last decades, *Escherichia coli* strains producing Shiga-like toxin (STEC) or verocytotoxin (VTEC) have taken on an important epidemiological role as emerging foodborne pathogens. Within the group of *E. coli*, the *E. coli* enterohemorragic (EHEC)

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microorganisms have a primary role. This subgroup includes strains able to produce a hemolysin that induces hemolytic uremic syndrome (HUS) (Levine 1987), a serious enterohemorrhagic disease of the human urinary system. Historically, serotype O157 has been the most investigated, even considering that other serotypes have been involved in several epidemic situations (Caprioli et al. 2001; Beutin 2006). Between 1988 and 2004, an overall number of 344 cases of VTEC infection were reported in Italy.

The surveillance system ENTER-NET has reported 19 cases of EHEC in 2006, identifying the following as the most common *E. coli* serogroups: O26 (36.8%), O103 (21.1%), O157 (21.1%), and O145 (15.8%) (ENTER-NET Italia 2005; Galetta et al. 2008). In 2003, the Scientific Veterinary Committee Public Health (SCVPH) highlighted the necessity of improving the sensitivity of detection methods for pathogens that produce verocytotoxin during food-monitoring processes. This improvement will also allow comparable data to be obtained from different member states (SCVPH, 2003).

Well-known isolation and identification procedures in the food industry should yield results in a very short time to avoid sanitary risks and, at the same time, have high sensitivity given the low infectious doses of these pathogens (Willshaw et al. 2001). In this context, biomolecular methods may represent a valuable option or may integrate traditional culture methods that normally require a long time to be performed (Fratamico et al. 1995).

Materials and methods

Considering all of the above, the sensitivity of two multiplex polymerase chain reaction (m-PCR) techniques were tested for the quantitative determination of *E. coli* O157:H7 directly from foodstuff matrices. Initially, sensitivity was evaluated without interfering factors from the matrix, and genomic serotyping was carried out from bacterial cultures. Subsequently, the time necessary to lower the limit of detection was determined. Afterwards, meat contamination tests were carried out to determine efficiency under conditions of low contamination, which are still relevant for the transmission of infection. Finally, genomic typing of the pathogens was performed. The study of virulence factors for single strains importantly assigned a different role to the strains at the onset of toxic infectious episodes. These biomolecular investigations regarding sensitivity (10 tests) were performed using an *E. coli* O157:H7 strain isolated from bovine feces, possessing the *rfb*E_{O157} and *fli*C_{H7} genes. These genes encode, respectively, the O157 somatic antigen and the H7 flagellar antigen. Moreover, the pathogen has virulence sequences *stx*1, *stx*2 (including its variants), *hly*A, and *eae*A, which are linked to the synthesis of verocytotoxins, an enterohemolysin, and an intimin. For this reason, dilutions up to 10^{-8} were prepared in Tryptone Soya Broth (TSB, Oxoid) taken from inoculated broth media with an average titer of 2×10^{9} CFU/mL. A quantity of 500 μL was taken from every dilution for DNA extraction through a 6% Cheelex resin (BioRad). Serotyping identification was carried out using an m-PCR method, according to Wang G et al. (2002) protocol, followed by electrophoresis on an agarose gel 2%.

To directly apply this technique on foodstuff matrices, it became necessary to use a pre-enrichment phase to reduce false-negative results from contamination levels lower than the limit of detection of the method. Therefore, either the first aliquot, which was negative, or the last aliquot, which was positively serotyped, were subjected to confirmation using a pre-enrichment phase at 37°C in TSB for a short (6 h) and long (24 h) time period. A 500 μL aliquot was taken from each sample at both incubation times to carry out genomic extraction and amplification using a similar protocol to the one previously described above.

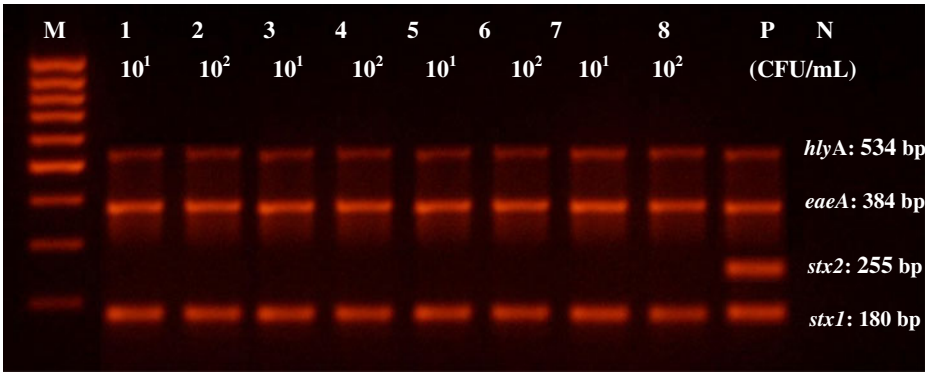

Fig. 1 Electrophoresis of *hly*A (534 bp), *eae*A (384 bp), *stx2* (255 bp), and *stx1* (180 bp) amplicons carried out by artificial contamination trials on meat matrices after a 24-h incubation. Lanes: M, Mass Ruler^{TM} DNA Ladder Low Range, (Fermentas); 1-2, test no. 3 in m-TSB; 3-4, test no. 3 in EC broth; 5-6, test no. 4 in m-TSB; 7-8, test no. 4 in EC broth; P, wild-type strain (VTEC *stx1, stx2*, *hly*A, and *eae*A); N, negative control

In meat matrix contamination tests, the verocytotoxic strain used in the first phase with a concentration between 10^1 to 10^2 CFU/mL was studied.

The homogenate was prepared using modified TSB (Oxoid) and EC Broth (Oxoid) added to novobiocin and incubated at 37°C. At respectively 0, 6, and 24 h, three aliquots were taken from each sample for the genomic identification of the serotype to avoid or reduce false-negative results. All positive, extracted samples were then subjected to m-PCR to determine the pathogenic profile (Paton and Paton 1998).

Results

The results on method sensitivity underlined a rather high detection threshold; in fact, four out of 10 tests yielded positive results for *rfb*E_{O157} and *fli*C_{H7} genes up to 10^5 CFU/mL titer, while the others were positive with sensitivity of 10^6 CFU/mL. As expected, inoculated broth media was positive with longer incubation times, even for very low concentrations (10^1-10^2 CFU/mL). Forty percent of tests had already reached this same sensitivity after just 6 h; method sensitivity was enhanced by 5 logs in 60% of tests after a 24 h incubation (Fig. 1, Table 1).

Regarding the contamination tests on meat matrices, all aliquots were negative for both inocula, after either 0 or 6 h incubation times. After a 24 h incubation, 80% of m-TSB-inoculated media and 60% of EC-broth media were positive, even ones with the lowest

Table 1 Sensitivity on TSB without incubation, after short pre-enrichment (T_6), and enhanced period incubation (T_{24})

Time (hours)	Detection limit (CFU/mL)									
	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1	10^0
T_0	+	+	+	+	–	–	–	–	–	–
T_6	/	/	/	+	+	+	+	+	–	–
T_{24}	/	/	/	+	+	+	+	+	+	–

bacterial titer (10^1 CFU/mL). The samples that were positive by genomic serotyping also possessed three virulence factors (*hly*A, *eae*A, and *stx*1), while no *stx*2 verocytotoxin amplicons were found.

Discussion

The limit of detection of genomic serotyping was rather high even after 6 h of incubation. Both $rfbE_{O157}$ and $fliC_{H7}$ amplicons were found at very low contamination rates (10^1 CFU/mL). The limit of detection was reduced of 4 logs in 80% of cases during short pre-enriching times as reported by others (Vimont et al. 2006). Regarding meat contamination, our results confirmed what has been observed previously. After 24 h of incubation and even at the lowest inoculation rates, a higher frequency of serotypes was found using m-TSB for enrichment than using EC-broth for isolation of the microorganism. This evidence is even more interesting considering that the inoculated media broth is diluted 10 fold during sampling. The s*tx*2 fragment, which is specific for a contaminating strain, was not found in the homogenates even though Paton's technique involves the application of primers linked to its conserved gene sequences. It could therefore be supposed that the matrix represents an interfering or inhibitory factor to sequence amplification. Information concerning strain specificity and virulence in the food sample could be obtained in a one day with optimization of analysis using m-PCR methods.

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