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Development and assessment of a rapid method to detect *Escherichia coli* O26, O111 and O157 in retail minced beef.

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1 **Version 1**

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3 **Development and assessment of a rapid method to detect *Escherichia***
4 ***coli* O26, O111 and O157 in retail minced beef.**

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ABSTRACT

A molecular-based detection method was developed to detect *Escherichia coli* O26, O111 and O157 in minced (ground) beef samples. This method consists of an initial overnight enrichment in modified tryptone soya broth (mTSB) and novobiocin prior to DNA extraction and subsequent serogrouping using a triplex PCR. This method has a low limit of detection and results are available within 24 hours of receipt of samples. Once optimized, this rapid method was utilized to determine the prevalence of these *E. coli* serogroups in six hundred minced beef samples all of which were previously examined by immunomagnetic separation (IMS) and selective plating for *E. coli* O26 and O111. Using IMS, two *E. coli* O26 isolates were detected. No *E. coli* O111 were recovered. The multiplex PCR technique described here did not detect *E. coli* O111 nor O157 in any of the samples, however six minced beef samples were positive for *E. coli* O26 using our method, only two of these were previously detected by IMS and culture. Application of molecular methods are useful to support culture-based approaches thereby further contributing to risk reduction along the food chain.

Keywords: non-O157 *E. coli*; *E. coli* O157, O26 and O111; serogroup-specific PCR; rapid detection in food.

INTRODUCTION

Escherichia coli O157 was initially implicated in human disease in 1982 (Riley et al., 1983) and since then the reported incidence of verocytotoxigenic *Escherichia coli* (VTEC) related illness has increased worldwide. In addition to *Escherichia coli* (*E. coli*) O157, other serogroups of *E. coli* such as O111 and O26 have emerged as important etiological agents of gastrointestinal disease in humans (Tarr and Neill, 1996). There is evidence of these infections in Ireland including an outbreak of *E. coli* O26 which was reported in a crèche in 1999 (McMaster et al., 2001). However, the true incidence and clinical significance of non-O157 serogroups in Ireland still remains to be determined. Routine surveillance to detect these non-O157 *E. coli* in foods, animals and the environment need to be introduced so that public health measures can be implemented. Development of rapid diagnostics capable of detecting a wide range of *E. coli* serogroups associated with disease in humans is a priority.

In recent years, a great deal of work has been carried out to ascertain the most sensitive and specific methods for isolating and identifying *E. coli* O157. The current method of choice for the detection of *E. coli* O157 in foods is enrichment followed by concentration using Immunomagnetic separation (IMS), with subsequent plating onto the selective Cefixime-Tellurite Sorbitol MacConkey agar (CT-SMAC). This procedure was adapted to allow for the detection of *E. coli* O26 and O111 in foods with the use of serogroup-specific beads. However, a

major obstacle in detecting *E. coli* O26 and *E. coli* O111 by this method is the lack of suitable selective agars.

CT-SMAC is the selective agar of choice used to detect *E. coli* O157 from many food matrices as the majority of *E. coli* O157 are non-sorbitol fermenting. However sorbitol fermenting *E. coli* O157 strains capable of causing haemolytic uremic syndrome (HUS) have been reported (Bettleheim et al., 2002; Karch and Bielaszewska, 2001; Karch et al., 1993). While *E. coli* O26 and O111 have demonstrated abilities to ferment both sorbitol and lactose (Batson et al., 2002; Hiramatsu et al., 2002), non-sorbitol fermenting *E. coli* O26 and O111 have also been isolated (Batson et al., 2002; Hiramatsu et al., 2002; Louie et al., 1998). Batson et al. (2002) reported on the carbohydrate fermenting ability of forty-one isolates of *E. coli* O26 and twenty-two isolates of *E. coli* O111. These authors reported that all isolates could ferment lactose, 92.5% of the *E. coli* O26 isolates and only 64.7% of the *E. coli* O111 fermented sorbitol.

As sugar fermenting characteristics are not consistent, it is difficult to choose (or formulate) a selective agar for the detection of all of these serogroups in food matrices containing high numbers of background microflora. The situation has improved somewhat, with the introduction of Rhamnose MacConkey agar (Hiramatsu et al., 2002) as a selective media for *E. coli* O26 and with the development of other chromogenic agars. However, laboratories remain over-reliant on the fermentation characteristics of these micro-organisms which could

result in pathogenic isolates going undetected. Taking this into consideration along with the fact that culture methods are time-consuming, a PCR based method could be valuable in providing additional information to identify specific *E. coli* serogroups.

In this paper, we report on the development of a triplex PCR method to detect *E. coli* O26, O111 and O157 in a single tube reaction following enrichment. Our method was applied retrospectively to analyze 600 minced beef samples for the three serogroups. This food matrix was previously investigated by microbiological methods (Murphy et al., 2005), and in this case failed to identify a number of positive samples. Application of our method is discussed as a convenient tool to support risk reduction measures along the food chain.

MATERIALS & METHODS

Preparation of bacterial strains for inoculum.

A single colony of *E. coli* O26 (NCTC 8781) or *E. coli* O111 (NCTC 9111) or *E. coli* O157 (NCTC 12900), were inoculated into three separate falcon test-tubes containing 4 ml of nutrient broth (Oxoid, Basingstoke, UK) and incubated at 37°C for 4 h. Serial dilution of 4 h cultures were prepared in 9 ml Maximum Recovery Diluent (MRD; Oxoid) down to a dilution of 10⁻⁹. One hundred microliters of 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸ and 10⁻⁹ serial strain dilutions were plated directly onto nutrient agar (Oxoid) and incubated at 42°C for 16 h to determine the cell numbers in the initial inoculum. All inoculations were carried out in duplicate.

Inoculation of minced beef samples to determine limit of detection.

Minced beef was purchased at a local supermarket and stored at 4°C prior to inoculation. One ml of the 10⁻⁷, 10⁻⁸ and 10⁻⁹ serial dilutions of each strain were added to separate 25 g minced (ground) beef samples containing 225 ml of modified Tryptone Soya Broth (mTSB; Oxoid) with novobiocin (20 mg/L), (LabM Ltd., Lancashire, UK) and incubated at 42°C overnight (16 h). An uninoculated sample of minced beef was also included as a control.

DNA Extraction

DNA was extracted from the overnight (16 h) enrichments both immediately and after storage for approximately 7 days at -20°C, using the Dneasy tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions with minor

modifications. Briefly, 25 µl of each sample were added to eppendorf tubes containing 180 µl of the kit tissue lysis buffer (ATL). Twenty micro-liters of proteinase K (supplied in the kit) was added to each tube and mixed. All samples were heated for approximately 135 min at 55°C and were mixed occasionally during incubation to maintain homogeneity. Following incubation, samples were vortexed for 15 seconds and the Protocol for 'Isolation of total DNA from animal tissues', was followed from step 3, of the Dneasy tissue kit instructions (Qiagen).

Triplex-PCR mediated serogrouping

All spiked samples were examined for the presence of *E. coli* O26, O111 and O157 using the triplex PCR. This triplex reaction was designed by combining previously published primers by Paton and Paton (1998) for the *rfb* genes of *E. coli* O157 and O111 and primers published by Debroy et al. (2004) for *E. coli* O26 *wzx* gene. All primers were tested against several bacterial species other than *E. coli* and also against different serogroups of *E. coli* (Table 1).

Triplex-PCR

Samples (5 µl) of each purified template DNA [approx. 100 ng] were amplified in 50 µl reaction mixtures containing 5 µl of dNTP mixture (consisting of 3 mM each of dATP, dCTP, dGTP, and dTTP), 2 µl of forward and reverse primer mix (5 pmol/µl each primer—see Table 2), 5 µl of 10 x buffer (750 mM Tris-HCL, pH 8.8, 200 mM (NH₄)₂SO₄, 0.1% (v/v) Tween 20), 5 mM MgCl₂ and 2.5 U Red Hot *Taq* DNA Polymerase (Abgene, Surrey, U.K.). Thermal cycling consisted of 36 cycles,

each of 95°C for 30 sec, 60°C for 60 sec and 72°C for 60 sec, and a final extension at 72°C for 10 min. Each amplification reaction included a positive control and a no-DNA template control. Amplified DNA products were resolved by 2% (w/v) agarose gel electrophoresis in 1 x Tris-Borate-EDTA (TBE) buffer containing 0.5 mg/ml ethidium bromide at 100 V and the results visualized and photographed using the Gel Doc 2000 system (Bio-Rad, Hercules, CA, U.S.A.).

Purification and pooling of DNA templates.

In a previous study, six hundred retail minced beef samples (25 g each), were examined by IMS and culture for the presence of *E. coli* O26 and O111 (samples were not examined for *E. coli* O157 (Murphy et al., 2005)). Two ml aliquots of each beef sample were also frozen at -70°C after an overnight enrichment (for 16 h) in mTSB and novobiocin. Each frozen enrichment broth was thawed rapidly at 50°C (Ternent et al., 2004) and 1 ml aliquots were pooled into lots containing five representative meat samples. DNA was purified from each pool using the DNA extraction protocol previously outlined and the recovered DNA pools were then subjected to the triplex PCR. Individual samples from any positive pool were subsequently tested separately, to identify the positive sample(s). Further work to determine the virulence status of all positive samples was undertaken using real-time PCR for *vt1*, *vt2*, *eae* and *hlyA*.

192 *'Real-Time' PCR for detection of vt1, vt2, eae and hlyA-encoding genes*

193 Five micro-liters of purified template genomic DNA were added to the following
194 25 µl PCR mix containing 1 X SYBR-1 green PCR master mix (Bio-Rad
195 Laboratories) and 50 ng of each primer to target the *vt1*, *vt2* (Gannon et al.,
196 1992), *eae* (Beebakhee et al., 1992) and *hlyA* genes (Paton and Paton, 1998).
197 Samples were amplified in an I-Cycler (Bio-Rad, Hercules, CA, U.S.A), at 95°C
198 for 2 min followed by 30 cycles each of 45 sec at 95°C, 45 sec at 58°C, and 60
199 sec at 72°C and a final extension step of 72°C for 10 minutes. Amplification was
200 monitored by the accumulation of SYBR-1 Green as the amplification reaction
201 progressed and data captured was later analyzed to produce the corresponding
202 melt curve.

203
204 **RESULTS**

205 In this study, a triplex PCR was developed to detect *E. coli* O26, O111 and O157
206 in a single reaction. The specificity of the PCR was tested using a panel of
207 micro-organisms (Table 1). This triplex PCR method was used in conjunction
208 with the Dneasy animal tissue kit to detect *E. coli* O26, O111 and O157 from
209 minced (ground) beef, after an initial 16 h enrichment. The limit of detection for
210 the assay was determined to be 10 colony forming units (CFU) for *E. coli* O111,
211 15 CFU for *E. coli* O26, <10 CFU for *E. coli* O157 in 25 g of minced beef (Fig.
212 1a). The uninoculated minced beef sample was determined to be negative for *E.*
213 *coli* O157, O26 and O111 by this method. Results were available in less than 24
214 h. No difference in the limit of detection was observed between spiked broths

examined directly after enrichment and the enrichment broths that were frozen for a period before testing (data not shown). Enrichment time trials were also undertaken, that included times of 4 h, 6 h and overnight (16 h) enrichments. Data showed that an overnight enrichment (16 h) was required to obtain the low limits of detection described above (data not shown). These data correlated with a recent study by O'Hanlon et al. (2004) wherein these authors showed that a longer enrichment time allowed for the multiplication of slower growing serogroups, thereby reducing the possibility of false negative results being reported.

Compared to IMS and culture (Murphy et al., 2005) this molecular approach identified six *E. coli* O26 isolates, only two of which were previously detected. No *E. coli* O157 nor *E. coli* O111 were detected using this rapid method. The latter case correlates with the IMS and culture results where *E. coli* O111 was not detected (Murphy et al., 2005). All six *E. coli* O26 isolates were tested for the presence of the virulence factors *vt1*, *vt2*, *eae* and *hlyA* by real-time PCR. Table 3 showed that isolate-4 (Fig. 1b, 1c) contained both *vt2* and *hlyA* and isolate-6 contained the *eae* gene alone (Fig. 1c and Table 3). The remaining four isolates did not contain any virulence factors as determined by this method (Table 3).

DISCUSSION

A study, was recently carried out on the 'Prevalence and characterization of *Escherichia coli* O26 and O111 in retail minced beef in Ireland' (Murphy et al., 2005), which highlighted the need for an alternative method to detect these micro-organisms in food. IMS and culture is the technique routinely used worldwide for the detection and identification of *E. coli* O157 in food matrices. However, this technique is time-consuming and is sub-optimal when used for the detection of other *E. coli* serogroups such as *E. coli* O26 and O111, increasing the risk of reporting false negative results.

In our study, a triplex PCR was developed to detect the three *E. coli* serogroups in minced beef after an initial overnight enrichment step (16 h) followed by DNA purification. The presence of these micro-organisms in minced beef was determined within twenty-four hours, reducing the time required when compared to standard methods by up to four days. Speed of detection is a very important factor in outbreak scenarios and routine food surveillance. Our method could detect less than 15 CFU of all the serogroups tested, which is comparable to the limit of detection for the IMS and culture technique (Safarikova et al., 2001) and to data previously reported from studies using real-time PCR (O'Hanlon et al., 2004; Sharma et al., 2002). While, the molecular method described in this paper compliments the methodologies of O'Hanlon et al. (2004) and Sharma et al. (2002), a useful feature is its capability to detect all three serogroups in a single triplex reaction whether virulent or non-virulent. The additional real-time PCR

allows for further characterization of any positive samples for *vt1*, *vt2*, *eae* and *hlyA* genes.

Using the protocol described, serogrouping results are available within eight hours after enrichment therefore, if these broths were stored at 4°C employing suitable biosecurity measures, positive samples can be cultured, if required. A unique finding of our study relates to large longitudinal surveillance studies. In this case culture enrichments may be frozen and tested at a later stage in bulk or in pools, a point which would contribute positively to reducing the financial burden associated with these studies.

Analysis of the six hundred frozen enrichment broths demonstrated the ability of this method to detect target sequences in samples negative for IMS and culture. As these isolates were detected among high numbers of background micro-flora in enriched minced beef, it would be reasonable to assume that all these isolates were viable, an important point when applying PCR methods. As one of the *E. coli* O26 isolates detected using the rapid method contained *vt2* and *hlyA* genes and the other isolate contained the *eae* gene alone, these organisms could be of potential public health significance, should they be ingested by a vulnerable individual. This observation demonstrates the advantage of combining the approach described here along with conventional approaches, emphasizing the value of molecular methods.

Implementation of the method outlined in this study would be of value in food surveillance and public health laboratories. Results of analysis are available in less than twenty-four hours. It has a low limit of detection and it is less labour intensive compared to the more traditional methods applied. Our method could be extended to include a wider range of serogroups or other food-borne pathogens of public health significance.

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Figure Legends to Illustrations

Fig. 1a. Results of the limit of detection dilution assay for *Escherichia coli* (*E. coli*) O157, *E. coli* O111 and *E. coli* O26 in minced beef using this rapid technique. Lane M = molecular weight marker VIII (Roche); Lane 1 = *E. coli* O26 10^{-7} (15 CFU/ml); Lane 2 = *E. coli* O26 10^{-8} (0 CFU/ml); Lane 3 = *E. coli* O111 10^{-7} (10 CFU/ml); Lane 4 = *E. coli* O111 10^{-8} (0 CFU/ml); Lane 5 = *E. coli* O157 10^{-7} (40 CFU/ml); Lane 6 = *E. coli* O157 10^{-8} (4 CFU/ml); Lane 7 = *E. coli* O157 10^{-9} (0 CFU/ml); Lane 8 = Control (sample); Lane 9 = multiplex positive control consisting of equivalent template DNA from three independent *E. coli* serogroups [including O26, O111 and O157]; Lane 10 = No template PCR control.

A 2% (w/v) agarose gel in 0.5 X Tris-Borate-EDTA (TBE) buffer containing 0.5 mg/ml ethidium bromide.

Fig. 1b. Melt curve graph of SYBR-1 green real-time PCR assay for *vt2*. Melting temperature (T_m), 89°C. *E. coli* O157 positive control (NCTC 12900), (red peak); Isolate 4, (green peak). Other isolates and the negative control are shown as flat lines.

Fig. 1c. Melt curve graph of SYBR-1 green real-time PCR assay for *eae* and *hlyA* genes. Melting temperature (T_m) of 88°C for *eae* and 87°C for *hlyA*. *E. coli* O157 positive control, (pink peak, *hlyA*; green peak, *eae*); Isolate 4, (red peak, *hlyA*); Isolate 6, (blue peak, *eae*). Other isolates and the negative control are shown as flat lines.

Table 1. List of the micro-organisms used to test the specificity of the primers, with their corresponding reactions.

Micro-organism	Source	*O26 <i>wzx</i>	*O111 <i>rfb</i>	*O157 <i>rfb</i>
<i>Clostridia perfringens</i>	NCTC 8237	-	-	-
<i>Vibrio parahaemolyticus</i>	NCTC 10885	-	-	-
<i>Salmonella poona</i>	NCTC 4840	-	-	-
<i>Staphylococcus aureus</i>	NCTC 6571	-	-	-
<i>Listeria monocytogenes</i>	NCTC 11994	-	-	-
<i>Bacillus cereus</i>	NCTC 7464	-	-	-
<i>Pseudomonas aeruginosa</i>	NCTC 10662	-	-	-
<i>Citrobacter freundii</i>	NCTC 9750	-	-	-
<i>Enterobacter faecalis</i>	NCTC 775	-	-	-
<i>E. coli</i> O126	Clinical Isolate	-	-	-
<i>E. coli</i> O128	Clinical Isolate	-	-	-
<i>E. coli</i> O142	Clinical Isolate	-	-	-
<i>E. coli</i> O124	Clinical Isolate	-	-	-
<i>E. coli</i> O44	Clinical Isolate	-	-	-
<i>E. coli</i> O55	Clinical Isolate	-	-	-
<i>E. coli</i> O111	NCTC 9111	-	+	-
<i>E. coli</i> O26	NCTC 8781	+	-	-
<i>E. coli</i> O157	NCTC 12900	-	-	+

* target present (+), absent (-).

Table 2. Oligonucleotide primers used to identify *Escherichia coli* serogroups.

<u>Oligonucleotide primers</u>	<u>Gene</u>	<u>Sequence (5' to 3')</u>	<u>Size</u>	<u>% G+C</u>	<u>Amplicon (bp)</u>
O111-F	<i>rfb</i> <i>E</i> _{O111}	TAGAGAAATTATCAAGTTAGTTCC	[24-mer]	29	406
O111-R		ATAGTTATGAACATCTTGTTTAGC	[24-mer]	29	
O157-F	<i>rfb</i> <i>E</i> _{O157}	CGGACATCCATGTGATATGG	[20-mer]	50	259
O157-R		TTGCCTATGTACAGCTAATCC	[21-mer]	43	
O26-F	<i>wzx</i> <i>E</i> _{O26}	GCGCTGCAATTGCTTATGTA	[20-mer]	45	152
O26-R		TTCCCCGCAATTTATTCAG	[20-mer]	40	

Table 3. Characteristics of the *Escherichia coli* O26 isolates detected in raw minced beef utilizing the triplex PCR.

Isolate number	Package type	Detection Method		*Virulence Markers			
		IMS	New PCR Technique	vt1	vt2	eae	hlyA
1	Prepacked	Detected	Detected	-	-	-	-
2	Prepacked	Detected	Detected	-	-	-	-
3	Prepacked	Not Detected	Detected	-	-	-	-
4	Loose	Not Detected	Detected	-	+	-	+
5	Loose	Not Detected	Detected	-	-	-	-
6	Prepacked	Not Detected	Detected	-	-	+	-

* virulence target present (+), absent (-).

Figures

[a].

[b].

[c].