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Automated Detection and Characterisation of Foodborne Pathogens

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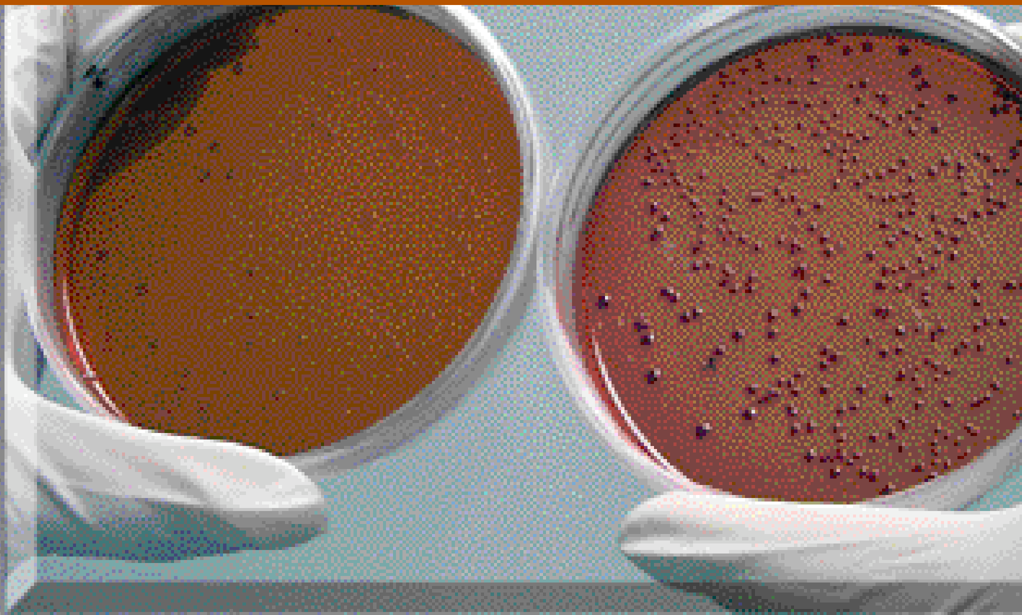

Ashtown Food Research Centre

RESEARCH & TRAINING FOR THE FOOD INDUSTRY

RESEARCH REPORT NO 89

AUTOMATED DETECTION AND CHARACTERISATION OF FOODBORNE PATHOGENS

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SUMMARY

This study focused on the development of molecular tools for the rapid detection and characterisation of food-borne pathogens including Verocytotoxigenic *Escherichia coli* (VTEC) (serotypes O157, O26 and O111) and *Salmonella* spp. The study involved the development of enrichment systems and the identification of unique genetic targets in these pathogens which could be amplified and detected by Real Time Polymerase Chain Reaction (PCR).

Enrichment and plating procedures were developed and optimised for the recovery of emergent pathogens *E. coli* O26 and O111 from beef. The optimum enrichment broth for *E. coli* O26 was Tryptone Soya Broth with cefixime, vancomycin and potassium tellurite at an incubation temperature of 41.5°C. Similar enrichment conditions were optimal for *E. coli* O111 with the omission of potassium tellurite. The optimum agar for recovery of *E. coli* O26 was MacConkey Agar (lactose replaced by rhamnose) with cefixime and potassium tellurite while optimum recovery of *E. coli* O111 was on Chromocult Agar supplemented with cefixime, cefsulodin and vancomycin. The optimised enrichment conditions were then applied in combination with a real-time PCR method targeting VTEC virulence genes *(VT1 and VT2)* and species-specific genes (O157 *per* gene, O26 *fliC-fliA* genes and O111 *wzy* gene). This yielded rapid (24 h) and sensitive methods for the detection of these pathogens in beef. The application of the new developed methods demonstrated the presence of *E. coli* O26 and O111 in retail minced beef samples. Two molecular sub-typing methods were used for comparison of VTEC bacteria at a genomic level including pulsed field gel electrophoresis (PFGE) and repetitive extragenic palindromic (Rep-PCR) fingerprinting. Results showed that Rep-PCR had a good correlation with PFGE but the latter had a higher discriminatory power in differentiating between VTEC isolates.

A real-time PCR method was developed for *Salmonella* targeting the 16S rRNA gene and was shown to be a rapid (24 h) and sensitive method for the

detection of this pathogen in naturally-contaminated meat samples. The methods developed have potential to be of considerable benefit in epidemiological investigations for these pathogens in the food chain.

INTRODUCTION

Traditional cultural methods for the isolation and identification of foodborne pathogens are time-consuming and labour intensive. This situation causes particular difficulties in relation to routine food testing, especially in circumstances when customers and statutory authorities require “evidence of the absence” of the pathogen. Therefore, there is an increasing need in the food industry to access rapid and sensitive detection methods.

Molecular detection methods based on the polymerase chain reaction (PCR) are increasingly accepted as alternatives to conventional cultural procedures for the detection of bacterial contamination in food. Conventional PCR assays require amplification of a target gene in a thermocycler, separation of PCR products by gel electrophoresis followed by visualisation and analysis of the resultant electrophoretic patterns. However, this process can take a number of hours. More recently, real-time PCR, an automated PCR assay, which uses fluorescence to detect the presence/absence of a particular gene in real-time, is being used as a rapid, sensitive and specific molecular diagnostic technique for the testing and identification of food pathogens. The advantages of real-time PCR technology include speed (approximately 30-40 min per 40 PCR cycles) and the ability to continuously monitor the progress of the PCR.

In this study, real-time PCR-based methods were applied to the detection of two important groups of foodborne pathogens *i.e.* verocytoxigenic *E. coli* (VTEC) and *Salmonella*. While *E. coli* O157:H7 is the most notable of the VTEC strains in terms of public health significance, other serotypes, including *E. coli* O111 and O26, are increasingly linked to human illness. However, no specific or routine methods are available for the detection of these two

emergent strains in foods and no data have been available on the role that food plays in their transmission. This study aimed to develop specific methods for the detection of O26, O111 and O157 and to then apply these methods to provide some initial data on these emergent VTEC organisms in minced beef.

Salmonella is the second most common cause of bacterial foodborne illness and with EU Microbiological criteria for fresh meat requiring fresh meat and poultry to be tested for *Salmonella*, there is a considerable demand by the meat industry for a rapid test for this pathogen. This study aimed to apply real-time PCR technology to the development of a rapid test for *Salmonella* spp and to carry out initial studies on its correlation with the standard cultural technique.

CULTURAL DETECTION OF *E. COLI* O26 AND O111

Verocytotoxin-producing *E. coli* include more than 100 serotypes, the most notorious from a public health perspective being *E.coli* O157:H7. However, serotypes O26 and O111 are now also increasingly implicated in human infection. As yet, no routine methods for these strains have been developed and the aim of this study was to optimise enrichment and recovery methods for these two serotypes from minced beef. *E. coli* O111 and O26 were inoculated into minced beef, placed in various enrichment broths with different selective agents and incubated at 41.5 or 37°C. The pathogens were then extracted by immunomagnetic separation using beads coated with antibodies specific for *E. coli* O111 or O26 (Dynal) and plated onto different selective plating media to allow development of colonies and detection/enumeration of the pathogen. Growth of the pathogens was recorded over time to identify optimum conditions for recovery of the pathogens. The optimum enrichment conditions for *E. coli* O26 were observed in samples enriched at 41.5° C in Tryptone Soya Broth supplemented with cefixime (50µg l^{-1}), vancomycin (40mg l^{-1}) and potassium tellurite (2.5mg l^{-1}). Similar enrichment conditions were optimal for *E. coli* O111 with the omission of potassium tellurite. Optimum recovery of *E. coli* O111 was on

Chromocult Agar supplemented with cefixime (50 mg ml^{-1}), cefsulodin (5mg l^{-1}) and vancomycin (8mg l^{-1}) while the optimum agar for recovery of *E. coli* O26 and most effective suppression of contaminants was MacConkey Agar [lactose replaced by rhamnose (20g l^{-1})] supplemented with cefixime (50 mg ml^{-1}) and potassium tellurite (2.5 mg l^{-1}).

REAL TIME PCR DETECTION OF *E. COLI* O157/O26 AND O111

The aim of this study was to develop a rapid detection method for *E. coli* O157, O26 and O111 from minced meat by real-time PCR using the Lightcycler Instrument (Roche). Initially, primers specific for the verotoxin genes *(vt1 and vt2)* and serotype-specific primers against conserved regions of genes coding for the outer membrane proteins of *E. coli* O157, O26 and O111 (O157 *per* gene; O26 *fliC-fliA* genes and O111 *wzy* gene) were designed. These primers were adapted for use with the light cycler using either Syber green dye (species-specific genes) or hybridisation fluorescent probe format (VT genes) (Table 1).

Minced beef was inoculated with *E. coli* O157, O26 and O111 at a level of 10-20 CFU g^{-1} and enriched for 6 h in either *E. coli* broth + novobiocin 200 mg ml^{-1} (*E. coli* O157); Tryptone Soya Broth + Cefixime 50 mg l^{-1}, Vancomycin 40 mg l^{-1} and Potassium tellurite 2.5 mg l^{-1} (*E. coli* O26) or Tryptone Soya Broth + Cefixime 50mg l^{-1} and Vancomycin 40mg l^{-1} (*E. coli* O111). Bacterial cells were extracted from enriched broths using either an immunomagnetic separation technique or direct extraction in which 1 ml of enriched culture was removed. In both cases, DNA was extracted using a DNA extraction kit (DNAeasy kit - Qiagen). The presence of VTEC was confirmed using real-time PCR (hybridisation probes) and then species-specific genes. *E. coli* O111, *E. coli* O26 and *E. coli* O111 were detected using a Syber green format approach. The presence of *E. coli* O157, O26 and O111 in meat was determined within 24 h using this real-time PCR technology (Figure 1).

Table 1: Primers used in detection of VT or serotype-specific genes (O157, O26 and O111)

Primer/Probe	T (°C)	Target Gene	Sequence
VT1F	55.4	*vt1*	ATAAATCGCCATTCGTTGACTAC
VT1R	57.0	*vt1*	AGAACGCCCACTGAGATCATC
VT1 FL	63.5	*vt1*	CGTAACATCGCTCTTGCCACAGAC
VT1 LC	63.0	*vt1*	CGTCAGTGAGGTTCCACTATGCGA
VT2 S	55.3	*vt1*	GGCACTGTCTGAAACTGCTC
VT2 R	55.4	*vt2*	TCGCCAGTTATCTGACATTCTG
VT2 FL	62.4	*vt2*	CCCCGAWACTCCGGAAGCAC
VT2 LC	63.7	*Vt2*	TTGCTGATTCKCCCCCAGTTCAGW
O157F	56.9	*per*	TCTGCGCTGCTATAGGATTAGC
O157A	56.0	*per*	CTTGTTTCGATGAGTTTATCTGCA
O111S	55.5	*wzy*	CTTTTTTTGAACCTACAGCAAGTAA
O111R	54.5	*wzy*	GATAAACCAATGCTCCTATCACAC
O26FlipCF	61.8	*fliC*	GCAGCGGATGGCAATGGGAAT
O26 FliAR	65.3	*fliA*	TCCACGCTCGCGGGCAGTC

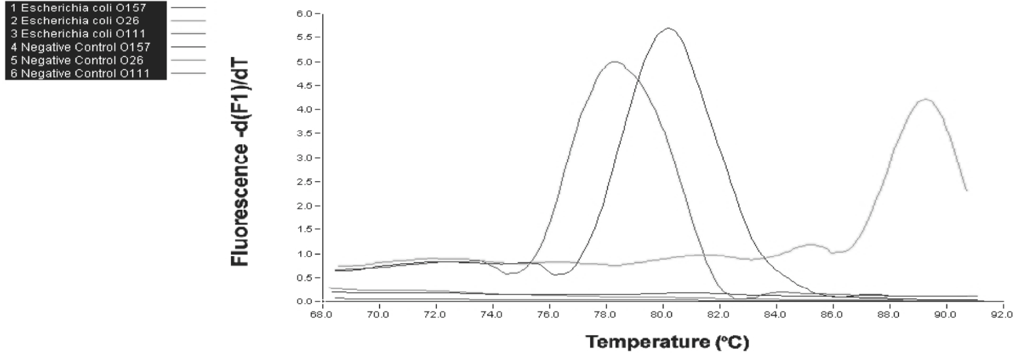

Figure 1: SYBR Green Species-Specific Real-time PCR assay for VTEC (O157, 026 and 0111)

APPLICATION OF DEVELOPED METHOD FOR VTEC 026 AND O111

This study compared the developed immunomagnetic separation (IMS)/cultural method and real-time PCR method to detect *E. coli* O26 and/or O111 in minced beef. A total of 65 samples was examined, 40 of which were frozen beef samples previously established as containing *E. coli* O157, and 25 of which were samples of fresh minced beef purchased from butcher shops in the Dublin area. After selective enrichment, all samples were (a) subjected to IMS, plated on differential media and identified as *E. coli* O26 or O111 using biochemical and immunological methods, or (b) subjected to DNA extraction and real-time PCR analysis using primers and probes against *E. coli* O111 and O26 species-specific genes and verotoxin genes. Overall, from the 65 minced beef samples collected, 3 were positive for *E. coli* O26 by real-time PCR with only one of these samples positive for *E. coli* O26 by the cultural method. One sample was positive for *E. coli* O111 by both real-time PCR and the cultural method. In conclusion, the developed real-time PCR was more sensitive than the developed IMS/cultural method for the detection

of *E. coli* O26 and O111 in minced meat. This study highlights the importance of mince beef in the transmission of non-O157 VTEC. Further research is necessary to establish the public health significance of these emergent pathogens.

Table 2: Samples positive for *E. coli* O26 or *E. coli* O111 by cultural and/or real-time PCR method

Sample set and number	Number Positive	Cultural method		Real-time PCR method			
		O26	O111	O26	O111	VT1 gene	VT2gene
Group A	3	+	-	+	-	+	+
		-	-	+	-	+	+
		-	-	+	-	+	-
Group B	1	-	+	-	+	+	+

Group A: 40 Frozen Retail Mince Beef Samples: 1A-40A
Group B: 25 Fresh Retail Mince Beef Samples: 1B-25B

SUB-TYPING OF VTEC (*E. COLI* O157/O26 AND O111) BY PFGE AND REP PCR

The comparison of bacterial strains at genetic level (sub-typing) is now an important part of epidemiological studies. This is carried out as part of outbreak investigations to establish if there is a link between bacterial strains isolated from different patients and to link strains from clinical specimens to the source of infection *i.e.* food, environment etc. It is also a useful tool to establish the routes by which strains are transmitted through the food chain and can provide the scientific basis for identifying where control measures are best applied.

In the past, laboratories depended heavily on conventional epidemiological typing systems based on phenotypic traits, such as cellular and colonial morphology, biochemical and physiological features. More recently, the advent of high resolution molecular methods which allow rapid and effective "molecular fingerprinting" of pathogens has led to a widespread application of such techniques as pulse field gel electrophoresis (PFGE) and repetitive extragenic palindromic-polymerase chain reaction (Rep-PCR). Such methods offer considerable scope for the differentiation of pathogens as they allow characterisation of those areas of the pathogen genome which contribute to genetic heterogenicity among closely-related strains.

In this study, two sub-typing methods PFGE and Rep-PCR were compared for the characterisation and differentiation of *E. coli* O157, O26 and O111 isolates from veterinary, clinical and food sources. Both methods grouped *E. coli* O157 isolates as a single cluster which could be reliably distinguished from non-O157 *E. coli* isolates. Simpson's index of diversity of Rep-PCR was 0.91 as compared to 0.95 for PFGE; this indicates that Rep-PCR has a lower discriminatory power than PFGE. Small differences between the capacities of PFGE and Rep-PCR to differentiate between *E. coli* O157 strains and non-O157 isolates were observed and it was evident that the two techniques can complement each other. Data obtained from this study could be used to establish a VTEC database library for epidemiological studies.

REAL-TIME PCR DETECTION OF *SALMONELLA* SPP.

The aim of this study was to develop a rapid detection method for *Salmonella* spp. by real-time PCR using the Lightcycler Instrument (Roche). A set of primers specific for a 16S rRNA gene in *Salmonella* was designed; these primers were adapted for use with the light cycler using a Syber green dye (Table 3).

The real-time PCR method developed in this study was then compared with a conventional International Standard Organisation (ISO) method to detect

Table 3: Primers used in detection of *Salmonella*

Primer	T (°C)	Target gene	Sequence	Product size
16SF	51.6	16S rRNA	ACGGTAACAGGAAG(AC)AG	402bp
16SR	45.5	16S rRNA	TATTAACCACAACACCT	

the presence of *Salmonella* in various food sources. This investigation also served to provide a snapshot of *Salmonella* in Irish retail beef, chicken, pork and turkey. After selective enrichment, all meat samples (n = 100) were (a) plated on differential media and identified as *Salmonella* using immunological methods or (b) subjected to DNA extraction and real-time PCR analysis using primers against a *Salmonella* species-specific gene (16S rRNA). Overall, from the 100 retail samples purchased in the Dublin region, 5 were found positive for *Salmonella* using the cultural method and the 16S primers real-time PCR assay (Table 4). Results indicate that the specificity and sensitivity of the real-time PCR method developed using the 16S primers were comparable to the currently used cultural diagnostic method and have potential for use in routine food testing for *Salmonella*.

Table 4: Summary of the number of products sampled and the number of products positive for *Salmonella* in this study.

Product type	Total number of samples	PCR Method (positive samples)	Cultural Method (positive samples)
		16S rRNA primer	
Beef	26	0	0
Chicken	38	2	2
Turkey	10	2	2
Pork	26	1	1
TOTAL	100	5	5

CONCLUSIONS AND RECOMMENDATIONS TO INDUSTRY

Rapid real-time PCR methods were developed for the detection of Verocytotoxigenic *Escherichia coli* (VTEC) (*Escherichia coli* O157, O26 and O111) and *Salmonella* in food. Such developments are important since current methods for the isolation and identification of these organisms from contaminated food are time consuming and labour intensive. The study developed routine and rapid methods for the recovery of emerging pathogens *Escherichia coli* O26 and O111 from food which are important emergent but to-date relatively uninvestigated pathogens. Using the methods developed in this study, non-O157 VTEC (*E. coli* O26 and O111) were demonstrated to be present in some samples of retail minced beef.

Two sub-typing methods, PFGE and Rep-PCR, were compared for genetic characterisation of VTEC. Speed and accuracy of these two techniques were assessed and they were evaluated as to their suitability for epidemiological investigation of *Escherichia coli* O26, O111 and O157. The data obtained from this study will contribute to an epidemiologically useful VTEC database library.

The method developed for *Salmonella* shows considerable potential as a rapid method for detecting this pathogen in fresh meats. This is particularly important in light of EU "Microbiological criteria for foodstuffs" which requires testing of fresh meat carcasses for *Salmonella*. This testing may be carried out by a cultural method (ISO 6579), which takes 3-4 days for positive clearance of samples, or by an equivalent rapid method (EU 2003/470). This requirement for testing for *Salmonella* means that industry needs an alternative rapid method as the delays incurred in waiting for tests results can otherwise involve a large product holding area and delayed product release. Further studies will need to be conducted to determine the equivalence of the developed methods with standard cultural method as outlined in EN/ISO: 16140.

PUBLICATIONS

Catarame, T.M.G., O'Hanlon, K.A., Duffy, G., Sheridan, J.J., Blair, I.S. and McDowell, D.A. (2003). Optimisation of enrichment and plating procedures for the recovery of *E. coli* O111 and O26 from Minced Beef. *Journal of Applied Microbiology*, **95,** 949-957.

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