

Rapid Isolation and Detection of *Escherichia coli* O157:H7 in Fresh Produce

ABSTRACT

This study describes a rapid method for the isolation and detection of *Escherichia coli* O157:H7 present at low-levels (0.04-0.4 cfu/g) in fresh salad produce.

Following a short enrichment (5 h at 42 °C) samples were processed using Pathatrix™, a novel re-circulating immuno-magnetic capture system, linked to Real Time PCR and/or selective agar plating.

Processing of post-enrichment sample aliquots (10 ml diluted 1:25) and the use of pre-blocked paramagnetic anti-*E. coli* O157:H7 beads was found to enhance target pathogen recovery on selective agar plates in cases where a high microbial load of non-target microflora was present. This is of particular relevance to the testing of bean sprouts.

This method has the potential to provide a result within 7 hours. The speed of result offers significant benefits for outbreak investigations and allows the rapid screening of salad produce for *E. coli* O157:H7 contamination prior to product entering the distribution chain.

INTRODUCTION

Fresh salad produce is commonly consumed in the raw state without heat processing or killing steps which would reduce or eliminate bacterial pathogens that may be present. Consequentially fresh salad, including spinach and lettuce, has been implicated as the vehicle in a significant number of *Escherichia coli* serotype O157:H7 outbreaks (Brackett-CFSAN Letter, 2005; Himathongkham *et al.*, 2006). Most recently, spinach contaminated with *E. coli* O157:H7 from cattle feces (FDA, 2006) was identified as the most likely source of widespread foodborne illness in the USA. This outbreak caused illness in at least 199 people across 26 states and resulted in 3 deaths (MMWR, 2006).

E. coli O157:H7 has become notorious as a foodborne pathogen due to its low infective dose and pathogenicity (Arthur *et al.*, 2005). A rapid screening method able to accurately and effectively detect the presence of *E. coli* O157:H7 in ready-to-eat salad could reduce the incidence of contaminated produce reaching the consumer. The availability of such a method could also benefit regulatory and health agencies, both for *E. coli* O157:H7 surveillance surveys and during outbreak investigations.





Naturally high levels of background microflora, often found associated with fresh salad produce, has proven a significant challenge for many rapid *E. coli* O157:H7 detection methods; this is especially the case when this pathogen is present at very low levels (<10 cfu/25 g) (Himathongkham *et al.*, 2006). Industry standards require that both conventional and rapid test methods must be capable of detecting initial levels of *E. coli* O157:H7 at as few as 1 cfu in 25 g of the food product. In order for a rapid test to be a significant improvement over conventional methodologies it must not only offer this level of sensitivity in a shorter time frame but also provide specificity, reliability and practicality to the end user.

This study describes the development of a rapid isolation and detection methodology for low-level *E. coli* O157:H7 contamination in fresh salad produce including bean sprouts and spinach. The method utilizes two commercially available technologies, Pathatrix™ (Matrix MicroScience Inc, Golden, CO) and Real Time PCR R.A.P.I.D.® LT (Idaho Technology Inc, Salt Lake City, UT).

Pathatrix™ (Matrix MicroScience Inc) is a unique, large volume, re-circulating immuno-magnetic capture system that selectively concentrates low levels of target microbial pathogens from complex food matrices. The system has been used to isolate specific bacterial pathogens from a range of foods including; ground beef, chocolate crumb, cocoa mass, cantaloupe rinses, produce rinses, tomato rinses, potato salad, powdered infant formulae, mashed potatoes and egg beaters among many others.

The use of paramagnetic beads coated with antibodies facilitates rapid, specific isolation and detection of target pathogens including *E. coli* O157:H7, *Yersinia pestis*, *Shigella sonnei*, *Enterobacter sakazakii*, *Salmonella* spp. and *Listeria* spp. (Wu *et al.*, 2004; Arthur *et al.*, 2005; Hanes *et al.*, 2005, 2006; Himathongkham *et al.*, 2006; Lau *et al.*, 2006; Madson *et al.*, 2006; Mullane *et al.*, 2006; Fu *et al.*, 2006; Warren *et al.*, 2006; Yuk *et al.*, 2006).

Stevens and Jaykus (2004) described several desirable attributes for an effective sample preparation technique and, with its ability to selectively concentrate target bacteria from food samples, Pathatrix™ fulfills many of these criteria, including AOAC Validation status for several of the most common foodborne pathogens.

The use of molecular methods of detection such as PCR are becoming more widely used in the food industry and have been particularly well used in the detection of *E. coli* O157:H7 in raw ground beef (Arthur *et al.*, 2005) both directly and in combination with immuno-magnetic separation methods.

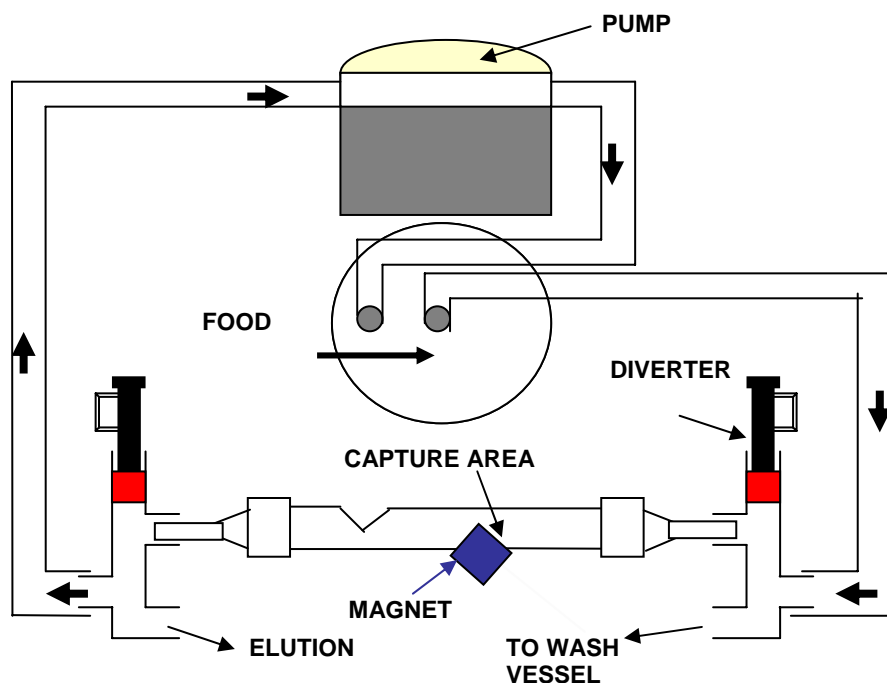
In this paper the authors investigate the development of a method that combines the Pathatrix™ immuno-magnetic separation technology with the R.A.P.I.D.® LT real time PCR to create an assay with a time to result in <7 h.

MATERIALS AND METHODS

Pathatrix™

Pathatrix™ is a unique re-circulating, immuno-magnetic capture system that concentrates low levels of target microbial pathogens from complex food matrices (FIG. 1.). The entire sample (total volume of 250 ml) is recirculated over the antibody coated paramagnetic beads held on the capture phase, for a total of 30 min. Through the use of the diverter valves the washing and elution steps are contained within the system, without the need for disconnections. This allows rapid, specific isolation and detection of target pathogens, such as *Salmonella* spp, *Listeria* spp and *E. coli* O157:H7. The Pathatrix *E. coli* O157:H7, *Salmonella* spp, *Listeria* spp. and Pathatrix Pooling protocols are AOAC validated methods

FIG.1. THE SCHEMA OF PATHATRIX



R.A.P.I.D.® LT

The R.A.P.I.D. LT is a real-time PCR instrument capable of analyzing samples for the presence of targeted nucleic acid sequences. Built upon LightCycler® technology, this instrument combines rapid air thermocycling and a real-time fluorimeter to reliably identify test samples in less than 25 minutes. The reagents are provided in freeze-dried format and can be stored at room temperature for convenience.

Spinach leaves and bean sprouts. Samples were obtained from two supermarket sources.



Inoculation Preparation. *Escherichia coli* O157:H7 NCTC12900/ATCC® 700728 was incubated overnight in Buffered Peptone Water (BPW Oxoid Ltd, Basingstoke, UK) at 37° C. Serial dilutions were performed on turbid suspensions of log phase cells in PBS. Direct plate counts in duplicate of the appropriate serial dilutions on TSA and CT-SMAC (Cefixime-Tellurite Sorbitol MacConkey agar, Oxoid Ltd, Basingstoke, UK) plates were used to confirm the final inoculum level of 0.04-0.4 cfu/g of product.

Sample inoculation. Samples of bean sprouts, pre-packed spinach and mixed lettuce were inoculated in accordance with the recommended procedure for AOAC method validations. Samples were weighed into a sterile container and inoculated with *E. coli* O157:H7 at low level (0.04-0.4 cfu/g). The inoculated samples were then refrigerated overnight at 2-8 °C. Further samples were weighed into another sterile container. These samples remained un-inoculated and were also refrigerated overnight at 2-8°C.

Sample Enrichment. 25g of inoculated or un-inoculated samples were weighed into sterile stomacher bags and a 1:10 dilution carried out using pre-warmed (42 °C) Buffered Peptone Water (BPW). Samples were hand mixed and incubated statically at 42 °C for 5 hours. Following the enrichment period, 10 ml aliquots were removed from each of the samples and 240 ml fresh BPW was added creating a 250 ml sample for analysis using Pathatrix™.

Bead blocking. Standard 50 µl doses of paramagnetic anti *E. coli* O157:H7 beads were pre-blocked (prior to Pathatrix run) using either 1% or 2% Skim Milk Solution (SMS). For each analysis a 50 µl dose of beads was suspended in 100 µl of sterile SMS and gently mixed for 1 hour on an end-over-end rotator at 35 rpm. 150 µl of this bead/milk solution (50 µl standard dose equivalent) was used for each Pathatrix™ analysis.

Pathatrix Analysis. Samples were analyzed on Pathatrix™ using the standard 30 minute cycle at 37° C. Either standard or SMS blocked beads were used to capture any target *E. coli* O157:H7 present. The final wash step was carried out using 100 ml of sterile PBS.. Bead suspensions recovered after the 30 minute Pathatrix™ cycle were placed on the magnetic rack to concentrate the beads from the PBS. The liquid was removed and the bead pellet was re-suspended in 100 µl sterile PBS.

Real Time PCR. A 25 µl aliquot was removed from the final 100 µl bead suspension and transferred into a bead beating tube containing 0.1 mm glass beads (IDAHO Technology Inc.) Mechanical lysis of the target cells to release DNA for PCR was carried out by performing a bead beating step on a Vortex Genie with Turbo Mix™ (Scientific Industries Inc, Bohemia, NY) for 5 min. The bead beating tubes were placed against a magnetic rack to concentrate the paramagnetic beads. Bead free supernatant (40 µl) was transferred into reagent tubes (IDAHO Technology Inc.) and then processed according to the R.A.P.I.D® LT Real-Time PCR protocol provided by Idaho Technology Inc.

Recovery of *E. coli* O157:H7 on CT-SMAC agar plates. The remaining 75 µl of beads were streak plated onto CT-SMAC agar plates, inverted and incubated overnight at 37°C for the isolation of *E. coli* O157:H7 colonies.

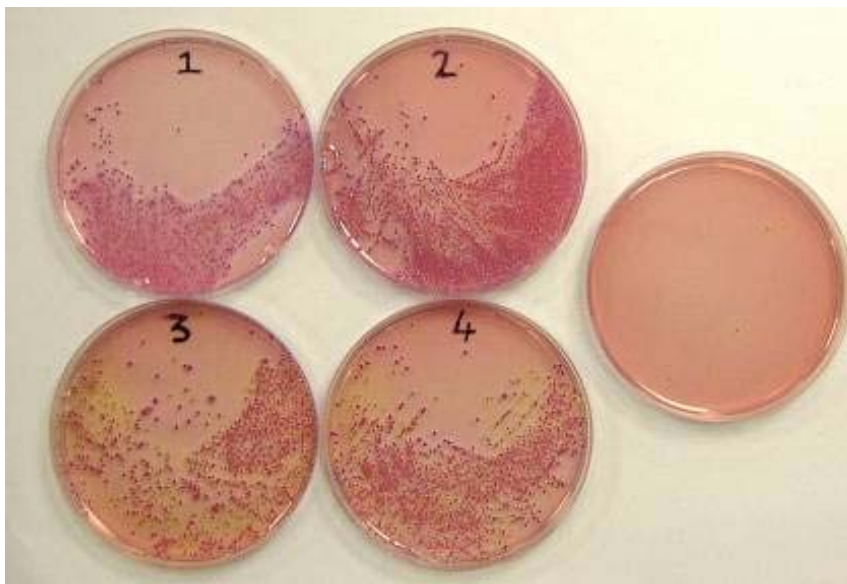


Confirmation Procedure. Colonies isolated during this study displaying typical morphology and color on CT-SMAC (straw/neutral grey with darker smoky centers) were selected for further examination. Confirmation of *E. coli* O157:H7 colonies isolated on CT-SMAC was performed using a Latex agglutination kit (Oxoid O157, Oxoid Ltd, Basingstoke, UK) and PCR.

RESULTS AND DISCUSSION

The CT-SMAC plates from the Bean Sprout samples are shown below in Figure 2 and indicate that recovery of isolated *E.coli* O157:H7 colonies is possible using the protocol outlined above.

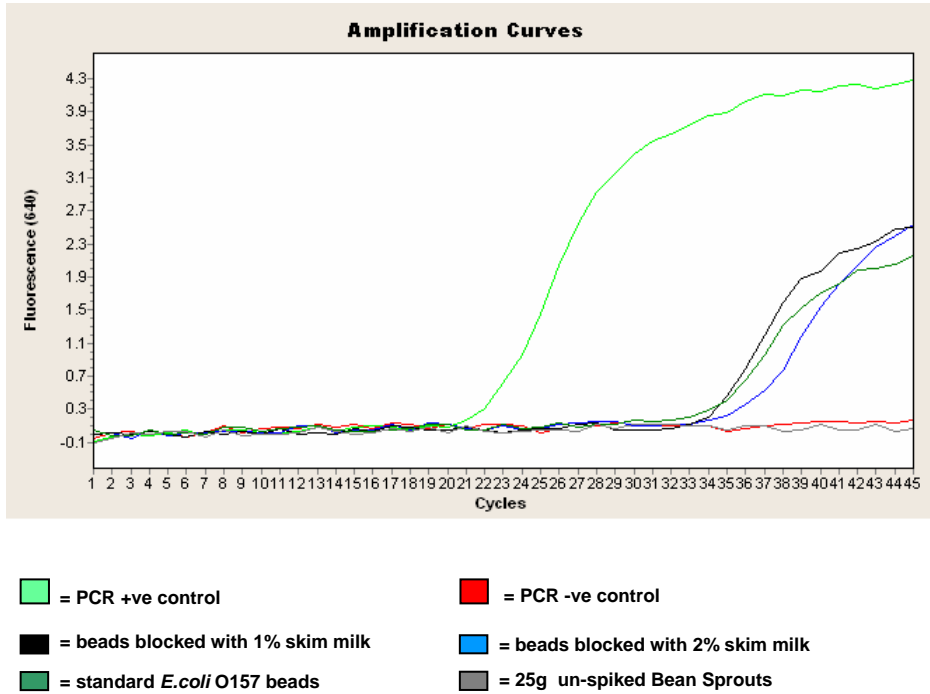
FIG. 2. PATHATRIX SAME DAY PROTOCOL: CT-SMAC PLATES FOR BEAN SPROUTS



The corresponding R.A.P.I.D. LT PCR data is shown in Figure 3



FIG. 3. BEAN SPROUT SAMPLE ANALYSIS: PATHATRIX + PCR



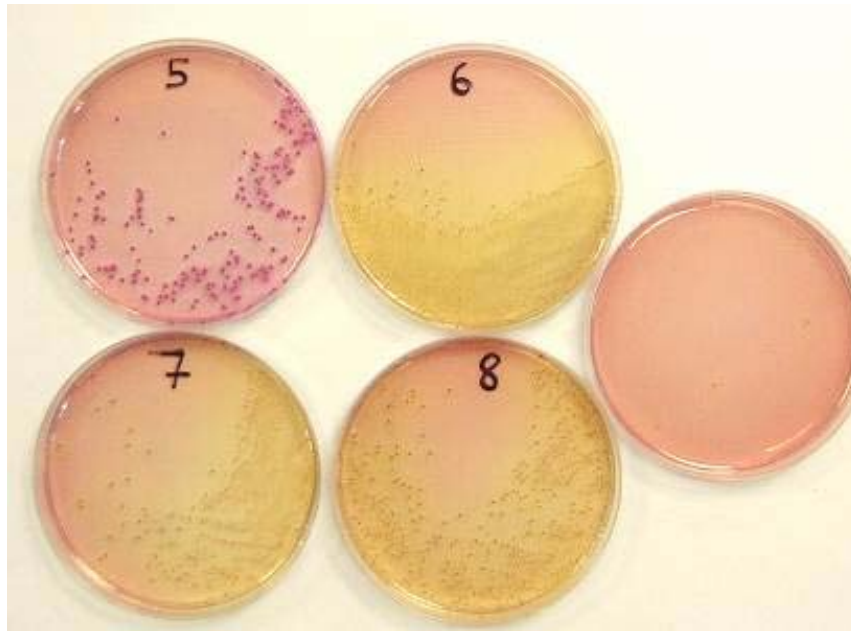
Samples were enriched in BPW for 5 hrs at 42°C. Samples were inoculated with 0.04-0.4 cfu/ g *E. coli* O157:H7

The data shows that whilst the detection of *E.coli* O157:H7 from Bean Sprouts with “standard” beads alone using CT-SMAC is virtually impossible, the PCR has detected them easily suggesting that they are clearly being captured and merely obscured by background microflora on the agar plates. In addition, it is clear that the beads blocked in SMS (1% or 2%) generate noticeably cleaner plates on CT-SMAC with well-isolated *E. coli* O157:H7 colonies making confirmation easier to achieve.

The CT-SMAC agar plates from the Spinach samples are shown below in Figure 4 and they indicate that *E.coli* O157:H7 can be readily isolated using “standard” and SMS blocked beads.



FIG. 4. PATHATRIX SAME DAY PROTOCOL: CT-SMAC PLATES FOR SPINACH

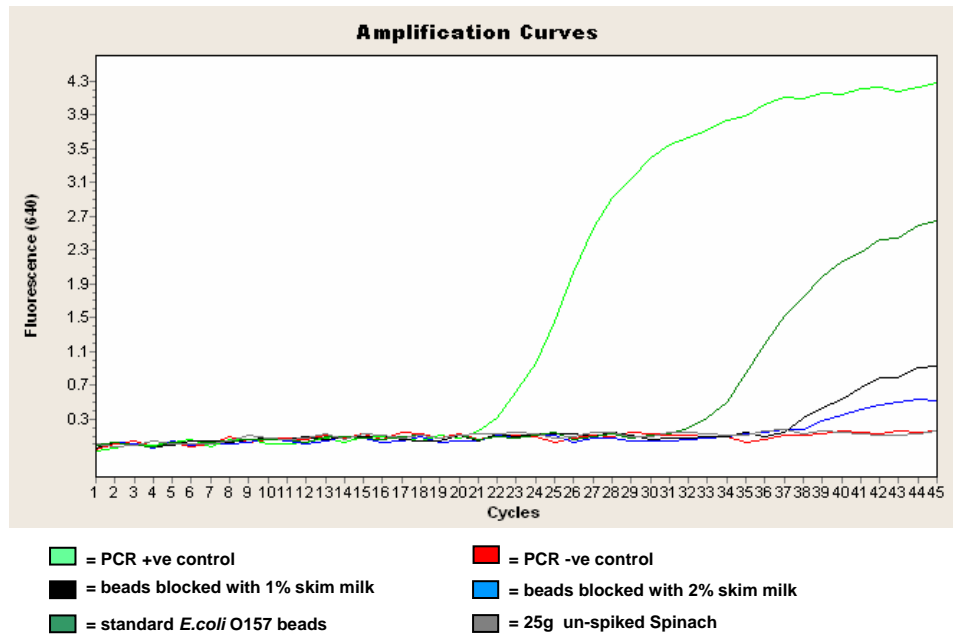


The data shows that the detection of *E.coli* O157:H7 in Spinach from “standard” beads alone using CT-SMAC is as good or even marginally better than the recovery achieved from the SMS (1% or 2%). This indicates less interference from background microflora than was observed with Bean Sprouts (see Figure 1)

The corresponding R.A.P.I.D.[®] LT PCR data is shown in Figure 5.



FIG.5. SPINACH SAMPLE ANALYSIS: PATHATRIX + PCR



Samples were enriched in BPW for 5 hrs at 42°C. Samples were inoculated with 0.04-0.4 cfu/g *E. coli* O157:H7

The data indicates that detection of *E.coli* O157:H7 in Spinach can be achieved with or without SMS blocking of the beads and that the SMS blocked beads appear to give a slightly lower recovery *i.e.* appear to have PCR crossover points later than the standard beads. This observation is consistent with the numbers of colonies recovered on the plates (see figure 3).

The data indicates that the combination of Pathatrix™ linked to real time PCR can be used to detect low levels of *E. coli* O157:H7 (0.04-0.4 cfu/g) in fresh salad produce including bean sprouts and spinach. Detection can be achieved in approximately 6.5 hours from point of sampling.

Whilst the data for spinach and some mixed salads (data not shown) indicates that the blocking of the beads with the SMS is not necessary, the authors suggest that pre-blocking the beads with 1% SMS should still be used to assist with the isolation of *E.coli* O157:H7 on CT-SMAC agar plates and in particular for the analysis of Bean Sprouts.

The authors believe that this represents a significant breakthrough in the rapid detection of *E.coli* O157:H7 in produce samples and could facilitate the possibility for growers and producers to operate a “test” and “hold” program for *E.coli* O157:H7.

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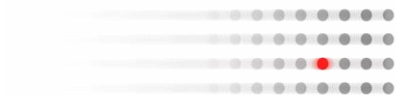
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