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Inhibition of polymerase chain reaction for the detection of *Escherichia coli* O157:H7 and *Salmonella enterica* on walnut kernels

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ABSTRACT

The aim of this study was to determine whether *Escherichia coli* O157:H7 can be reliably detected and isolated from walnut kernels using standard methods of analysis. The limit of detection approached 1 cell per analytical unit (25 g) for *E. coli* O157:H7 on walnut kernels enriched in modified tryptic soy broth with 20 μg/ml novobiocin and plating onto selective agar media. The presence of PCR inhibitors in walnut kernels was indicated by the failure to detect *E. coli* O157:H7 from culture positive enrichment broths analysed by PCR, with two separate polymerase and reagent compositions (Dupont BAX *E. coli* O157:H7 MP system, Promega GoTaq Green for *stx*) and three methods of template preparation (DuPont BAX, Qiagen DNeasy, Bio-Rad InstaGene). PCR inhibition was overcome by 1:100 dilution in TE buffer of the DNeasy or InstaGene template. PCR inhibition was not relieved by dilution of the BAX template. Similar results were observed for walnut kernels inoculated with *Salmonella enterica* and analysed for *invA*, indicating that PCR inhibition is not specific to the organism or primer/template. These results indicate that analysis of walnut kernels for pathogens should be with culture based methods or use protocols for DNA template preparation modified to remove or dilute inhibitors and the need for internal amplification controls in PCR methods.

1. Introduction

The reliability and sensitivity of both culture and non-culture (Rossen et al., 1992) methods for the microbiological analysis of foods have been shown to be affected by the food matrix. In 2011 the Canadian Food Inspection Agency (CFIA) conducted two recalls of raw shelled walnut kernels (CFIA, 2011a; CFIA, 2011b) due to an identified risk of consumer exposure to *Escherichia coli* O157:H7. The methods of analysis used in these investigations, culture method MFLP-80 (Warburton and Christensen, 2008) and BAX® System PCR Assay *E. coli* O157:H7 MP (Health Canada, 2003) are well established for the detection of *E. coli* O157 in a wide range of foods, but the performance had not been previously evaluated for contaminated walnuts or any other tree nut.

Walnuts and other tree nuts are generally considered products with a very low risk of contamination with bacterial pathogens. A study conducted in the United Kingdom reported that, of 2886 samples (25 g analytical units) of various nut kernels, 0.1% were positive for *Salmonella* and 0.8% had detectable levels of *E. coli* (Little et al., 2010). Of the 2886 nut samples analysed in the study, 441 samples were walnuts. *E. coli* was detected on 3 of the walnut samples (Little et al., 2010). An Australian study reported no *Salmonella*, *E. coli* or *Staphylococci* in 564 samples from ready-to-eat retail packs of nuts, though 3 were positive for *Listeria monocytogenes* at levels below 10 CFU/g (Eglezos, 2010). However, the occurrence of outbreaks associated with these products indicates that contamination with an infectious dose of bacterial pathogens can occur (Keady et al., 2004; Miller et al., 2012). Both wet and dry contamination routes for walnuts with enteric pathogens have been proposed, but the primary routes of contamination have not been identified (Blessington et al., 2013).

In the study described here, we determined the limit of detection (LOD) for uninjured, viable *E. coli* O157:H7 cells on walnut kernels using MFLP-80. We also investigated the possibility of the food matrix interfering with the PCR screening of enrichment broths. Interference of PCR was confirmed and experiments were conducted to determine a solution to this problem.

2. Materials and methods

2.1. Walnuts

All walnut samples were taken from a single 15 kg case of shelled walnuts halves originating from California. The walnuts

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were stored at room temperature (20–25 °C) until analysis. Aerobic colony counts and total *E. coli* and coliform counts of the walnut kernels were performed on triplicate 25 g walnut samples. Samples were diluted 1:10 in 0.1% peptone water and stomached at 200 rpm for 4 min. 0.1 ml of the walnut solution was plated on to Plate Count Agar (Oxoid, Basingstoke, UK), incubated at 24 °C for 48 h (Health Canada, 2001) and 3M Petrifilm *E. coli*/Coliform Count Plates (3M, St Paul, MN) incubated at 35 °C for 48 h (Warburton, 2001).

2.2. Bacterial strains

E. coli O157:H7 strains 11-1024 and 11-1865, were kindly provided by Dr. Celine Nadon (Public Health Agency of Canada, National Microbiology Laboratory, Winnipeg, MB). Both were clinical isolates from an outbreak in which walnuts were identified as the potential source. The *Salmonella enterica* subsp. *enterica* strain used was ATCC 13076. All cultures were maintained frozen at −80 °C. Cultures were prepared by streaking onto Brain Heart Infusion (BHI) agar (Difco Granulated Agar and Difco Brain Heart Infusion, Becton Dickinson and Co., Sparks, MD) and incubating for 16–24 h at 37 °C.

2.3. Inoculation of walnuts with E. coli O157:H7

A single colony of the strain to be used was inoculated to 50 ml of BHI broth. Following incubation for 16–24 h at 37 °C, cells were harvested by centrifugation at 8000 × *g* for 10 min. The supernatant was removed and cells re-suspended in phosphate buffered saline pH 7.0 (PBS). The cell suspension was then adjusted by dilution to an optical density of 0.10 at 600 nm in 1 cm path length methacrylate cuvette (Fisherbrand, Fisher Scientific, Ottawa, ON). This gave a cell density of approximately 7.5 log CFU/ml.

Inoculated walnut samples were prepared in stomacher bags by the addition of 100 μl of either pooled or individual cell suspensions of *E. coli* O157:H7 11-1024 and 11-1865, the walnuts were then manually mixed in the stomacher bag and stored at 4 °C for two hours prior to analysis.

2.4. Enumeration of E. coli O157:H7

E. coli O157:H7 was enumerated using a most probable number of organisms (MPN) method. Walnut kernels were suspended 1:10 (50 g in 450 ml) in modified tryptic soy broth (mTSB) (30 g/l Bacto tryptic soy broth, Becton, Dickinson and Co, 1.5 g/l Difco bile salts No. 3 Becton, Dickinson and Co, 1.5 g/l K_2HPO_4 Sigma–Aldrich, St Louis, MO. pH 7.4) and blended in a Stomacher 400 circulator (Seward, Worthing, UK) for 4 min at 200 rpm. A 10 tube three-fold dilution MPN was set up by dividing the suspended walnut sample into ten tubes of 4.5 g, ten tubes of 0.45 g and ten tubes of 0.05 g. All samples were then incubated for 1 h at 42 °C before the addition of novobiocin (Sigma–Aldrich) to a final concentration of 20 μg/ml. The tubes were then incubated for 16–20 h at 42 °C. Growth of *E. coli* O157 in MPN tubes was determined by streaking 10 μl of broth media onto Sorbitol MacConkey agar (Difco, Becton Dickinson and Co.) with cefixime and tellurite supplement (CT-SMAC) (Oxoid) and modified hemorrhagic colitis agar with cefsulodin and tellurite (mHC-CT) plates (Bacto Tryptose 20.0 g/L Becton, Dickinson and Co, Difco Bile Salts no.3 1.12 g/L Becton, Dickinson and Co, sodium chloride 5.0 g/L Sigma–Aldrich, sorbitol 20.0 g/L Sigma–Aldrich, 4-Methylumbelliferyl-ß-D-glucuronide 0.10 g/L Sigma–Aldrich, 1.6% Bromocresol purple 0.94 ml/L Sigma–Aldrich, Difco agar 15.0 g/L Becton, Dickinson and Co, cefsulodin 10 mg/L Sigma–Aldrich, potassium tellurite 2.5 mg/L Sigma–Aldrich). The CT-SMAC and mHC-CT plates were incubated overnight at 37 and 42 °C, respectively. The O157 serology of sorbitol negative colonies was confirmed by latex agglutination with the O157 DrySpot (Oxoid).

Tubes from which *E. coli* O157:H7 was isolated were scored as positive and the MPN/g was calculated according to the method of Blodgett (2010) using the Excel spreadsheet provided for download (available at http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm).

2.5. Limit of detection of culture method MFLP-80

The LOD of MFLP-80 (Warburton and Christensen, 2008) was determined using the method recommended in the Compendium of Microbiological Methods (Health Canada, 2011), with enumeration of the inoculated *E. coli* O157 by MPN as described above.

A cell suspension of *E. coli* O157:H7 strains 11-1024 and 11-1865 was prepared and two-fold serial dilutions of the cell suspension were used to inoculate 25 g portions of walnuts at six different concentrations above and below 1 cell/25 g. For each dilution six parallel 25 g sub-samples were prepared for analysis with MFLP-80 and an additional two 25 g samples of the highest dilution were prepared and composited for enumeration of *E. coli* O157 by MPN. A diagram of the experimental design is presented in Fig. 1.

MFLP-80 was performed on each sub-sample with isolation of *E. coli* O157 attempted for all enrichment broths by immunomagnetic separation (IMS) using anti-*E. coli* O157 DynaBeads (Invitrogen, Oslo, Norway) (Warburton, 2006) and plating onto mHC-CT and CT-SMAC agar. Enrichment broth was also streaked directly onto mHC-CT and CT-SMAC plates. The O157 serology of sorbitol negative colonies was confirmed by latex agglutination (O157 DrySpot, Oxoid). The LOD was determined by duplicate experiments.

2.6. Screening of enrichment broths by PCR for E. coli O157:H7

For experiments to investigate the reliability of PCR screening of walnuts for *E. coli* O157 a 65 g sample size was adopted following notification that this sample size had been used in recent CFIA investigation. Samples of walnuts (65 g) were inoculated with *E. coli* O157 (strain 11-1865) in a range of 3–1500 CFU per 65 g and analysed by MFLP-80. Samples were incubated at 42 °C overnight. Following incubation, an aliquot of enrichment broth was taken and stored at −20 °C for PCR analysis. Enrichment broth samples were analysed using the BAX *E. coli* O157:H7 MP system (DuPont Qualicon, Wilmington, DE) as described in MFLP-30 (Health Canada, 2003). DNA template for PCR analysis was also prepared from enrichment broth using 1 ml of enrichment broth with the InstaGene matrix (Bio-Rad, Hercules, CA) or DNeasy Blood and Tissue kit (Qiagen, Germantown, MD). The template generated was screened for the presence of the genes for verotoxin 1 and verotoxin 2 with primers stxU-F 5′ ATACAGAGGAGGAATTTCGT 3′ and stxU-R 5′ TGATGATGAGCAATTCAGTAT 3′ which targets a conserved 212–215 bp region in the A-subunit of verotoxin. Primers and thermocycling conditions were used according to the method of Paton and Paton (2003). The PCR mix per sample was composed of 12.5 μl of GoTaq Green Master Mix (Promega, Madison, WI), 0.25 μl of 100 μM stxU-F, 0.25 μl of 100 μM stxU-R, 9.5 μl of water and 2.5 μl template. Amplicon production was detected by gel electrophoresis, with PCR product and a DNA ladder (Fisher 50 bp mini DNA ladder) run for 6 min at 275 V on a 2.2% FlashGel™ (Lonza, Allendale, NJ) and visualised by UV transillumination. Positive control samples were included for each of the three trials and consisted of enrichment broth inoculated at 3, 6, or 15 CFU without the presence of walnuts. A negative control was also included which contained a 65 g walnut sample that was not inoculated. An additional negative control was prepared for the BAX analysis which did not contain any walnuts, and contained only the mTSB enrichment broth.