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Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) O104 from sprouts☆

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) strains belonging to serogroup O104 have been associated with sporadic cases of illness and have caused outbreaks associated with milk and sprouts. An outbreak that occurred in Europe in 2011 linked to fenugreek sprouts was caused by *E. coli* O104:H4 that had characteristics of an enteroaggregative *E. coli* (EAEC) but carried the gene that encoded for Shiga toxin 2. In this study, methods were developed for detection of this enteroaggregative STEC O104, as well as STEC O104 in sprouts. Multiplex PCR assays for enteroaggregative STEC O104:H4 targeted the stx_2, *aggR*, and wzx_{104} genes, and for STEC O104 targeted the $stx_{1\text{-}2}$, *ehxA*, and wzx_{104} genes. After incubating artificially contaminated sprouts at 4 °C for 48 h and overnight enrichment in modified buffered peptone water with pyruvate supplemented with three antibiotics (mBPWp), the pathogens were detected in all samples inoculated at a level of ca. 100 CFU/25 g. Several samples inoculated at lower concentrations of ca. 10 CFU/25 g were negative by the PCR assays, and this could have been due to cells not surviving or not being able to recover after the stress treatment at 4 °C for 48 h. For isolation of the pathogens, immunomagnetic separation (IMS) using magnetic beads coated with antibodies against O104 were employed, and this was followed by plating the beads onto mRBA and CHROMagar STEC O104 for isolation of *E. coli* O104:H4 and mRBA and CHROMagar STEC for isolation of *E. coli* O104:H7. Presumptive colonies were confirmed by agglutination using latex particles attached to antibodies against serogroup O104 and by the multiplex PCR assays. The methodologies described in this study for detection of enteroaggregative STEC O104:H4 and STEC O104 include the use of IMS and latex reagents for serogroup O104, and they enhance the ability to detect and isolate these pathogens from sprouts and potentially other foods, as well.

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1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are food-borne pathogens that cause gastrointestinal illness, hemorrhagic colitis and hemolytic uremic syndrome (HUS). *E. coli* O157:H7 is the STEC serotype most often implicated in outbreaks; however, there are numerous other STEC serotypes that have caused serious human illness and outbreaks. STEC strains that cause human illness were included as notifiable pathogens to the Nationally Notifiable Diseases Surveillance System in 2000. From 2000 to 2010, 7695 cases were reported; 5688 were associated with serogroup O157, and 83% of the other STEC were serogroups O26, O45, 103, O111, O121 and O145 (Gould et al., 2013). However, other serogroups, including O91, O113, O104 have also caused serious human illness (Bettelheim, 2007; Bielaszewska et al., 2011; Brooks et al., 2005; CDC, 1995; Gould et al., 2013).

Prior to 2011, STEC serogroup O104 was not considered as a major STEC; although it was associated with an outbreak involving 11 cases in the U.S., as well as many sporadic human cases in Germany, the United Kingdom, Korea, France, Finland, Norway, Denmark, Belgium, Sweden, and Austria, as well as other countries. Among all of these cases, 4 presented with HUS (ECDC and EFSA, 2011). The concern about this serogroup increased in May 2011 with the occurrence, primarily in Germany, of a large outbreak due to an *E. coli* O104:H4 strain that produced Shiga toxin. In this occasion, 3842 people presented with bloody diarrhea, 855 had HUS, and over 50 people died. Sprouts from fenugreek seeds were discovered to be the vehicle of infection in this outbreak (Robert Koch Institute, 2011). The genome sequence of the outbreak strain revealed that it carried virulence genes associated with both STEC (stx_2, *iha*, lpf_{O26}, lpf_{O113}) and enteroaggregative *E. coli* (EAEC) (*aggA*, *aggR*, *set1*, *pic*, *aap*) (Bielaszewska et al., 2011). Further studies confirmed that *E. coli* O104:H4 is an EAEC that had increased

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pathogenicity due to lateral transfer of the gene encoding for Shiga toxin 2 (stx_2) and antibiotic-resistance factors (Rasko et al., 2011; Rohde et al., 2011). Thus, this strain can be considered to be a member of both STEC and EAEC. STEC O104 strains, similar to STEC O91 and O113 that have caused outbreaks and cases of human illness, do not carry the *eae* gene (encodes for intimin); however, they generally carry the STEC enterohemolysin gene (*ehxA*) (Feng et al., 2001; Rump et al., 2012).

In recent years, the consumption of fresh produce, including sprouts, along with fresh and ready-to-eat food, has increased, and these foods have been associated with many outbreaks and cases of human illness (Berger et al., 2010; Taormina et al., 1999). Detection of pathogens in certain foods, including sprouts, is particularly difficult because of the relatively high background flora and the presence of coliforms that may interfere with detection and isolation of the target bacteria (Weagant and Bound, 2001). Weagant and Bound (2001) successfully used modified buffered peptone water with pyruvate (mBPWp) supplemented with acriflavin, cefsulodin, and vancomycin (ACV) for enrichment of *E. coli* O157:H7 in sprouts, and this medium is also described for STEC detection in the Food and Drug Administration Bacteriological Analytical Manual (Feng et al., 2010). Additionally, it has been shown that mBPWp supplemented with ACV was effective for detection of two strains of STEC O104 in sprout samples contaminated with approximately 1 CFU/g (Jinneman et al., 2012).

The use of immunomagnetic separation (IMS) improves the ability to isolate pathogens from food enrichments because the beads attached to target bacteria-specific antibodies bind, concentrate, and separate the bacteria from food particles and background microflora. In the present study IMS beads and latex particles attached to O104-specific antibodies were useful for isolation and identification of *E. coli* O104 strains from artificially inoculated sprout enrichments. In addition, two real-time multiplex PCR assays that included an internal positive control were optimized to detect two combinations of virulence genes, and were used for detection of the two *E. coli* O104 strains from the enrichments, as well as for confirmation of the latex agglutination-positive colonies. In the assay used for enteroaggregative STEC O104:H4 responsible of the German outbreak, the stx_2, *aggR*, and wzx_{104} genes are amplified, to determine the presence of the gene encoding for Shiga toxin 2, the presence of a plasmid encoding the AAF fimbriae, and the serogroup O104-specific *wzy* gene. The second assay was designed for STEC O104 non-EAEC and detects the presence of $stx_{1\text{-}2}$, *ehxA* (enterohemolysin), and wzx_{104}.

2. Materials and methods

2.1. Bacterial strains

Two serogroup STEC O104 strains were used for sprout inoculations in this study. Enteroaggregative STEC O104:H4 2011C-3493 was obtained from the Centers for Disease Control and Prevention and was isolated from a case of HUS in a U.S. traveler linked to the German outbreak in 2011 associated with sprouts. STEC O104:H7 RM9387 was obtained from Robert Mandrell at the USDA, Agricultural Research Service, Western Regional Research Center in Albany California and was isolated from cattle. The strains were maintained at −80 °C in tryptic soy broth (TSB) (Becton, Dickinson, Sparks, MD) supplemented with 20% glycerol.

2.2. Preparation of inoculum

The *E. coli* strains were grown from the frozen stock cultures in TSB overnight at 37 °C, and then the cultures were streaked onto tryptic soy agar (TSA) (Becton Dickinson) plates, incubated overnight at 37 °C and stored at 4 °C. A well separated colony from the plate was transferred with a sterile loop into 10 ml of TSB and incubated at 37 °C for 18 h. The culture was decimally diluted in sterile 0.1% peptone water (Becton, Dickinson), and the sprouts were inoculated with two different cell concentrations, a low level at approximately 10 CFU/25 g and a higher concentration at approximately 100 CFU/25 g. The number of bacteria in the inoculum was confirmed by plating onto TSA. An uninoculated 25-g sprout sample was included in each experiment.

2.3. Inoculation and enrichment of sprouts

From packages of dill and alfalfa sprouts purchased at a local market, 25-g aliquots were aseptically removed, placed into Stomacher® Strainer Bags (Seward Laboratory Systems, Bohemia, NY), and spiked with one of the two *E. coli* O104 strains. The samples were then stored at 4 °C for 48 h to stress the bacteria and simulate normal storage conditions. The enrichment was performed according to the BAM method (Feng et al., 2010). Briefly, 225 ml of mBPWp (Acumedia, Neogen Corporation, Lansing, Michigan) were added, and all samples were pummeled for 30 s with a Stomacher Lab-Blender 400 (Seward Laboratory Systems). Blended samples were incubated at 37 °C for 5 h, and then acriflavin hydrochloride (10 mg/l) (Sigma Aldrich, St. Louis, MO, USA), cefsulodin sodium salt (10 mg/l) (A. G. Scientific, Inc., San Diego, CA), and vancomycin hydrochloride (8 mg/l) (Sigma Aldrich) were added. The samples were incubated static at 42 °C for 18 h.

2.4. DNA extraction and multiplex real-time PCR

DNA extraction using 750 μl of the sprout enrichments was performed using the PrepSEQ Rapid Spin Sample Preparation kit (Life Technologies, Foster City, CA, USA) according to manufacturer's instructions. Primers and probes used for the PCR assays are shown in Table 1. Two multiplex real-time PCR assays with TaqMan® Exogenous Internal Positive Control Reagents (VIC Probe) (Life Technologies) were designed and optimized with the 7500 Fast Dx Real-time PCR instrument (Life Technologies). A 25-μl reaction contained the following: 1× TaqMan Environmental Master Mix 2.0 (Life Technologies), 1× Exo IPC Mix, 1× Exo IPC DNA, primers and probes (Integrated DNA Technologies, Coralville, IA, USA), and 2.9 μl of template DNA. The multiplex PCR assay used for samples contaminated with enteroaggregative STEC O104:H4 2011C-3493 targeted the stx_2, wzx_{104}, and *aggR* genes, and the assay for STEC O104:H7 RM9387 targeted $stx_{1\text{-}2}$, *ehxA* and wzx_{104}. The multiplex PCR assays were run using the following temperature cycling conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 59 °C for 1 min.

2.5. Determination of real-time multiplex PCR sensitivity

An overnight culture of each *E. coli* strain, prepared as described above, was serially diluted ten-fold in 0.1% peptone water to approximately 10 CFU/ml. Seventy-five microliters of each dilution were added to two vials containing 675 μl of mBPWp supplemented with ACV and to 675 μl of an uninoculated sprout enrichment. DNA extraction was performed using the PrepSEQ kit, and then 2.9 μl of template DNA were subjected to the real-time multiplex PCR assays.

2.6. Immunomagnetic separation

Twenty microliters of *E. coli* O104 IMS beads (Product No. 543060; Abraxis, Warminster, PA, USA) were mixed with 1 ml of the enrichment in a microcentrifuge tube, and the tubes were incubated at room temperature for 10 min with gentle and continuous agitation. The tube was then placed into a magnetic rack for 3 min to collect the beads, and the supernatant was removed. The beads were washed two times with PBST consisting of 0.01 M phosphate buffered saline, 0.138 M NaCl, 0.0027 M KCl, and 0.05% Tween 20 (Sigma Aldrich), pH 7.4. After the last wash, the beads were suspended in 100 μl of the same solution by gentle vortexing. The bead suspension was divided into two portions and introduced onto two selective and differential agar media. Each portion was spread with a swab over one half of the plate, and then streaking was continued with a sterile loop over the

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