International Journal of Food Microbiology 173 (2014) 99–104

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

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

Gian Marco Baranzoni [b], Pina M. Fratamico [a,*], Fernando Rubio [c], Thomas Glaze [c], Lori K. Bagi [a], Sabrina Albonetti [b]

[a] USDA, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA, United States
[b] Department of Veterinary Medical Science, University of Bologna, 50 via Tolara di Sopra, Ozzano dell'Emilia (BO), Italy
[c] Abraxis, LLC, 54 Steamwhistle Drive, Warminster, PA, United States

ARTICLE INFO

Article history:
Received 13 September 2013
Received in revised form 19 November 2013
Accepted 22 December 2013
Available online 29 December 2013

Keywords:
Shiga toxin-producing *E. coli* O104
Sprouts
Detection
Immunomagnetic separation
Antibodies
Latex beads

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_{1-2}, *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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* Corresponding author at: Eastern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, 600 East Mermaid Lane, Wyndmoor, PA 19038, United States. Tel.: +1 215 233 6525; fax: +1 215 233 6581.
E-mail address: pina.fratamico@ars.usda.gov (P.M. Fratamico).

0168-1605/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.12.020

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*$_{1-2}$, *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*$_{1-2}$, *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

Table 1
PCR primers and probes used in the multiplex PCR assays for detection of enteroaggregative STEC O104:H4 and STEC O104:H7.

	Sequence 5′ → 3′[a]	Reference	Conc.[b] O104:H7	Conc.[b] O104:H4
Stx 1/2-F	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	Wasilenko et al. (2012)	0.1	0.1
Stx 1/2-R	CCC CAG TTC ARW GTR AGR TCM ACR TC	Wasilenko et al. (2012)	0.1	0.1
Stx1-P	/56FAM/ CTG GAT GAT /ZEN/ CTC AGT GGG CGT TCT TAT GTA A /3IAbkFQ/	Wasilenko et al. (2012(	0.2	–
Stx2-P	/56FAM/ TCG TCA GGC /ZEN/ ACT GTC TGA AAC TGC TCC /3IAbkFQ/	Wasilenko et al. (2012)	0.2	0.2
ehxA F	GTG TCA GTA GGG AAG CGA ACA	Bugarel et al. (2010a)	1.25	–
ehxA R	ATC ATG TTT TCC GCC AAT G	Bugarel et al. (2010a)	1.25	–
ehxA P	/56-TAMN/ CGT GAT TTT GAA TTC AGA ACC GGT GG /3BHQ_2/	Bugarel et al. (2010a) (dyes modified)	0.2	–
aggR-333f	CAG CGA TAC ATT AAG ACG CCT AAA G	Hidaka et al. (2009)	–	1
aggR-448r	CGT CAG CAT CAG CTA CAA TTA TTC C	Hidaka et al. (2009)	–	1
aggR-pro	/56-TAMN/ AGA TGC TTG CAG TTG TCC GAA TTG GTC[c] /3BHQ_2/	Modified — Hidaka et al. (2009)	–	0.2
wzx_{O104} F	TGT CGC GCA AAG AAT TTC AAC	Bugarel et al. (2010b)	1.25	1
wzx_{O104} R	AAA ATC CTT TAA ACT ATA CGC CC	Bugarel et al. (2010b)	1.25	1
wzx_{O104} P	/5Cy5/ TTG GTT TTT TTG TAT TAG CAA TAA GTG GTG TC /3BHQ_2/	Bugarel et al. (2010b) (dyes modified)	0.2	0.2

[a] Degenerate nucleotide codes are as follows: Y (C,T), W (A,T), R (A,G), M (A,C), S (C,G).
[b] Concentration of primers and probes used in the PCR (μM).
[c] The underlined bases show the modification made to the probe described by Hidaka et al. (2009) for the purpose of changing the melting temperature to be similar to that of the other probes used in the multiplex PCR assay, and the dyes were modified.

remaining two quadrants. Plates were incubated at 37 °C for 24 h. Modified Rainbow Agar O157 (mRBA) (Biolog, Hayward, CA, USA) with 0.05 mg/l cefixime trihydrate (Sigma Aldrich), 5 mg/l novobiocin sodium salt (Sigma Aldrich) and 0.15 mg/l potassium tellurite hydrate (Sigma Aldrich) (Tillman et al., 2012) were used for both strains. CHROMagar STEC O104 (CHROMagar, Paris, France) was used for samples contaminated with *E. coli* O104:H4, and CHROMagar STEC (CHROMagar, Paris, France) was used for *E. coli* STEC O104:H7.

2.7. Latex agglutination

Two presumptive positive colonies, one from each agar medium, were picked and tested using the *E. coli* O104:H4 Latex Test Kit (Product No. 541060; Abraxis, Warminster, PA, USA) following the manufacturer's instructions. The multiplex PCR assays described above were performed on latex agglutination-positive colonies for further confirmation. DNA from colonies was extracted by resuspending in 100 μl of nuclease free water and heating at 99 °C for 10 min, and 1 μl of template DNA was used in the multiplex PCR assays.

3. Results and discussion

The aim of this study was to enhance the ability to detect and isolate STEC O104 by incorporating IMS for concentration of the target pathogen and latex agglutination to confirm presumptive positive colonies from selective and differential agars. The O104 IMS and latex agglutination kits are available from Abraxis, LLC. The same antibodies raised in rabbits were attached to both the immunomagnetic and the latex beads. In addition, multiplex PCR assays were designed to target the *E. coli* O104:H4 enteroaggregative STEC strain that caused the large outbreak associated with sprouts in 2011 in Europe, as well as STEC O104 strains. The assay for the enteroaggregative STEC O104:H4 targeted the stx_2, wzx_{104}, and *aggR* genes, and the assay for STEC O104 (non-enteroaggregative) targeted $stx_{1\text{-}2}$, *ehxA*, and wzx_{104}.

The enrichment medium used in the current study is the medium recommended in the FDA Bacteriological Analytical Manual (BAM) for detection of *E. coli* O157:H7 and other STEC (Feng et al., 2010) and used by Jinneman et al. (2012) for detection of STEC O104 from sprouts. Jinneman et al. (2012) stated that identification of colonies on the selective agars they employed was challenging due to the high background microflora. They were able to isolate the O104 strains on Rainbow Agar O157 without novobiocin and tellurite, but only when the samples were plated at high dilutions, since the sprouts contained a high level of background microflora. The use of immunomagnetic separation would likely have eliminated much of the background flora, facilitating isolation of the pathogens; however, IMS and latex reagents for O104 only recently became commercially available.

3.1. Multiplex PCR assays of sprout enrichments

Aerobic plate counts (APCs) were performed to evaluate the background flora of the sprouts used in this study. The APCs were determined using 25 g of sprouts diluted in 225 ml of TSB, as well as after incubating the sprouts for 48 h at 4 °C and adding mBPWp, and both were followed by plating onto TSA. Results using both treatments (TSB and mBPWp) were similar and they ranged from 5.82×10^6 to 1.45×10^7 CFU/ml and from 7.91×10^6 to 1.33×10^7 CFU/ml, respectively. The results obtained for sprouts inoculated at the different levels of contamination are shown in Table 2. All samples inoculated with a relatively higher level (24–160 CFU/25 g) were positive by the PCR assays, and presumptive positive colonies were identified as the target pathogen by latex agglutination and the PCR assays. Samples inoculated with lower levels down to 4 CFU/25 g were also positive; however, a number of inoculated samples were negative by the PCR (Table 2). For example, 1 out of 5 samples inoculated with 14 CFU of *E. coli* O104:H4 per 25 g, 2/2 inoculated with 13 CFU, and 1/3 inoculated with 6 CFU were negative by the PCR. For samples seeded with *E. coli* O104:H7, 3/4, 1/3, and 1/3 samples inoculated with 22, 9, and 6 CFU/25 g, respectively were negative by the real-time multiplex PCR assay. Negative results were obtained for all of the uninoculated samples, indicating that the sprouts were not naturally contaminated with STEC O104. No mauve colonies were found on either selective agars in these samples. Several non-mauve colonies were also tested by latex agglutination and by multiplex PCR, and positive results were never obtained.

It is unclear why some inoculated samples and not others seeded with low levels of the pathogens gave negative results; samples inoculated with 4 and 9 CFU of O104:H4 were positive by the PCR. It is possible that the incubation at 4 °C for 48 h may have injured the bacteria in some of the samples, and they did not recover during enrichment. Another reason may be that some samples inoculated with very low levels did not receive any bacteria, which may also have been caused by pipetting errors. Tzschoppe et al. (2012) found that in salad samples inoculated with low levels (1–10 CFU/g) of three different enterohemorrhagic *E. coli* (EHEC) strains and stored for 72 h at less than 6 °C, the pathogens became undetectable. They found that although the EHEC became undetectable, the background flora in the salad samples grew at the low temperature.

Use of the TaqMan® Exogenous Internal Positive Control Reagents generated a signal in all multiplex PCR assays, including the uninoculated control enrichments, confirming that there were no false negative results due to PCR inhibition. The sensitivity of the real-time multiplex real-time PCR for detection of *E. coli* O104:H4 was $\leq 10^3$ CFU/ml, and

Table 2
Results of detection and isolation of *E. coli* O104 from sprout samples, and identification and confirmation of presumptive colonies.

Contamination level,		*E. coli* strain	Positive sprout samples		Presumptive colonies[a]	
CFU/g,	25 g		Detection by real-time multiplex PCR[b]	No. of samples from which presumptive colonies were isolated from agars[c]	No. of colonies positive by latex agglutination	No. of colonies confirmed by real-time multiplex PCR
3.6,	90	O104:H4	1/1	1/1	2/2	2/2
2.7,	68		2/2	2/2	4/4	4/4
2.4,	60		1/1	1/1	2/2	2/2
1.8,	45		2/2	2/2	4/4	4/4
0.76,	19		3/3	3/3	6/6	6/6
0.56,	14		4/5	4/5	8/8	8/8
0.52,	13		0/2	0/2	–	–
0.36,	9		4/4	4/4	8/8	8/8
0.24,	6		2/3	2/3	4/4	4/4
0.16,	4		3/3	3/3	6/6	6/6
6.4,	160	O104:H7	1/1	1/1	2/2	2/2
4.4,	110		2/2	2/2	4/4	4/4
3.8,	95		2/2	2/2	4/4	4/4
3.2,	80		1/1	1/1	2/2	2/2
1.72,	43		3/3	3/3	6/6	6/6
0.96,	24		5/5	5/5	10/10	10/10
0.88,	22		1/4	1/4	2/2	2/2
0.52,	13		2/2	2/2	4/4	4/4
0.36,	9		2/3	2/3	4/4	4/4
0.24,	6		2/3	2/3	4/4	4/4

[a] For each sprout sample, two presumptive positive colonies, one from each agar medium, were tested by latex agglutination and real-time multiplex PCR. Since *E. coli* O104:H7 RM9387 does not grow on CHROMagar STEC, the two presumptive colonies were both taken from mRBA.
[b] Number of positive samples out of number of total sample enrichments tested by PCR.
[c] Number of positive samples out of number of total sample enrichments where presumptive colonies were isolated from an agar medium.

for the PCR assay targeting *E. coli* O104:H7, the detection limit was $\leq 10^4$ CFU/ml, although some samples tested showed detection limits that were ten-fold lower for both strains. The results were similar in samples consisting of mBPWp + ACV to which the bacteria were added and uninoculated sprout enrichments into which the *E. coli* strains were seeded at the different dilutions. The results of the current study are similar to those of Fratamico et al. (2011) and to those of Hidaka et al. (2009) who reported detection limits in the range of 7×10^2 to 1.1×10^4 CFU/ml.

3.2. IMS and plating of sprout enrichments and confirmation of isolated colonies

Regardless of the PCR screening results, all sprout samples underwent IMS, and presumptive positive mauve colonies were tested by latex agglutination and the PCR assays. In all samples that were positive by the PCR screening assays, the *E. coli* O104 strain was isolated on CHROMagar and/or mRBA, and the colonies were confirmed. Moreover, no presumptive mauve colonies were found on any of the agars when the PCR screening result was negative. To determine if samples treated with IMS had fewer background colonies compared to samples not treated by IMS, sprouts inoculated with ca. 100 CFU/25 g and enriched in mTSB with novobiocin (Wasilenko et al., 2012) and in mBPWp + ACV were tested with and without IMS. Samples tested without IMS showed a higher level of background flora, thus isolation and identification of the target pathogen would have been more difficult (data not shown).

Both *E. coli* O104:H4 and O104:H7 were easily isolated and identified on mRBA, which contains 0.05 mg/l cefixime trihydrate, 5 mg/l novobiocin sodium salt, and 0.15 mg/l potassium tellurite hydrate (Fig. 1). In all cases, a colony picked from a plate was confirmed as the target pathogen by both latex agglutination and the multiplex PCR assays (Table 2). *E. coli* O104:H7 did not grow on CHROMagar STEC even when pure cultures were streaked onto the plates. Tzschoppe et al. (2012) showed that STEC strains that did not grow on CHROMagar STEC lacked *terB* (tellurite resistance); therefore the O104:H7 strain used in the current study may lack *terB*. Similar results were described by Jinneman et al. (2012) when *E. coli* O104:H21 was plated onto CHROMagar O157. For this reason, all of the *E. coli* O104:H7 colonies tested by the latex agglutination kit were isolated from mRBA, which is modified to contain lower concentrations of tellurite and novobiocin than is recommended by the manufacturer (Tillman et al., 2012). On the contrary, CHROMagar STEC O104 was successfully used for the isolation of *E. coli* O104:H4 after IMS. However, when colonies from a pure culture of this strain were enumerated on both TSA and CHROMagar STEC O104 plates, there was about a two log higher number of bacteria on TSA compared to CHROMagar O104 (data not shown). The same comparison was made using TSA and mRBA, and there was no notable difference in the number of colonies on the two plates. Recently a rapid detection procedure for aggregative enterohemorrhagic *E. coli* (EHEC) O104:H4 based on a 6-h enrichment followed by three monoplex real-time PCR assays was described (Tzschoppe et al., 2012). The investigators found that the level of O104:H4 reached 5.8×10^4 CFU/ml in salad enrichments; therefore, it may be possible to use the multiplex PCR assays optimized in the current study in conjunction with the enrichment protocol described by Tzschoppe et al. (2012), thus saving time and reagents.

3.3. Conclusions

This study evaluated two multiplex PCR assays targeting STEC and enteroaggregative-STEC belonging to serogroup O104, as well as commercially available O104 IMS and latex agglutination reagents for detection, isolation, and identification of the pathogens from artificially contaminated sprouts. The multiplex PCR assays determine the presence of specific virulence genes of enteroaggregative-STEC and STEC O104 and the O104 serogroup. The pathogens were detected and isolated in sprout samples inoculated at a level of less than 1 CFU/g and subjected to a cold stress treatment for 48 h using a combination of enrichment in mBPWp + ACV, the real-time multiplex PCR assays, IMS, and isolation on selective agars (mRBA and CHROMagar STEC O104). The beads were able to concentrate the target bacteria, and presumptive colonies were easily identified and confirmed on selective agars using O104 latex particles and the multiplex PCR assays despite a high level of background flora in the sprouts based on the aerobic plate counts. Thus, the IMS beads and the latex reagents linked with antibodies

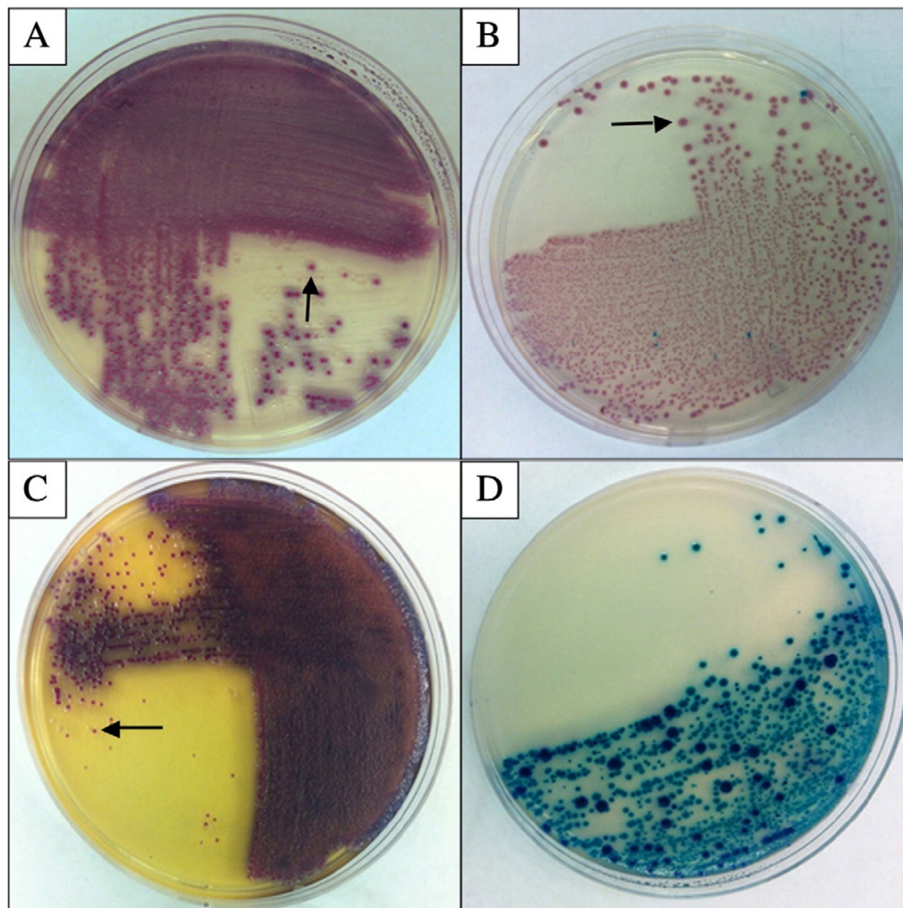

Fig. 1. Colonies formed after IMS of sprout enrichment cultures inoculated with *E. coli* O104:H4 (A and B) and *E. coli* O104:H7 RM9387 (C and D) grown onto mRBA (A and C), CHROMagar STEC 104 (B) and CHROMagar STEC (D). Arrows indicate O104:H4 2011C-3493 or O104:H7 RM9387 colonies. No typical O104:H7 colonies were visible on CHROMagar STEC.

against *E. coli* O104 are useful for enhancing the ability to detect *E. coli* belonging to this serogroup.

Acknowledgments

The authors thank James Smith for critical reading of the manuscript and Joe Sites for his assistance in preparing the photographs of the agar plates.

References

Berger, C.N.C., Sodha, S.V.S., Shaw, R.K.R., Griffin, P.M.P., Pink, D.D., Hand, P.P., Frankel, G.G., 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ. Microbiol. 12, 2385–2397.

Bettelheim, K.A., 2007. The non-O157 Shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. Crit. Rev. Microbiol. 33, 67–87.

Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., Peters, G., Karch, H., 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. Lancet Infect. Dis. 11, 671–676.

Brooks, J.T., Sowers, E.G., Wells, J.G., Greene, K.D., Griffin, P.M., Hoekstra, R.M., Strockbine, N.A., 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. J. Infect. Dis. 192, 1422–1429.

Bugarel, M., Beutin, L., Fach, P., 2010a. Low-density macroarray targeting non-locus of enterocyte effacement effectors (*nle* Genes) and major virulence factors of Shiga toxin-producing *Escherichia coli* (STEC): a new approach for molecular risk assessment of STEC isolates. Appl. Environ. Microbiol. 76, 203–211.

Bugarel, M., Beutin, L., Martin, A., Gill, A., Fach, P., 2010b. Micro-array for the identification of Shiga toxin-producing *Escherichia coli* (STEC) seropathotypes associated with hemorrhagic colitis and hemolytic uremic syndrome in humans. Int. J. Food Microbiol. 142, 318–329.

Centers for Disease Control and Prevention (CDC), 1995. Outbreak of acute gastroenteritis attributable to *Escherichia coli* serotype O104:H21. Morb. Mortal. Wkly Rep. 44, 501–503 (http://www.cdc.gov/mmwr/PDF/wk/mm4427.pdf (accessed 9.11.13)).

European Centre for Disease Prevention Control (ECDC), European Food Safety Authority (EFSA), 2011. Shiga toxin/verotoxin-producing *Escherichia coli* in humans, food and animals in the EU/EEA, with special reference to the German outbreak strain STEC O104. http://www.ecdc.europa.eu/en/publications/Publications/1106_TER_EColi_joint_EFSA.pdf (accessed 8.12.13).

Feng, P., Weagant, S.D., Monday, S.R., 2001. Genetic analysis for virulence factors in *Escherichia coli* O104:H21 that was implicated in an outbreak of hemorrhagic colitis. J. Clin. Microbiol. 39, 24–28.

Feng, P., Weagant, S.D., Jinneman, K.G., 2010. Diarrheagenic *Escherichia coli*. Bacteriological Analytical Manual, 8th edition. U.S. Food and Drug Administration (Revised, Chapter 4A, http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm (accessed 8.16.13)).

Fratamico, P.M., Bagi, L.K., Cray Jr., W.C., Narang, N., Yan, X., Medina, M., Liu, Y., 2011. Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 in ground beef. Foodborne Pathog. Dis. 8, 601–607.

Gould, L.H., Mody, R.K., Ong, K.L., Clogher, P., Cronquist, A.B., Garman, K.N., Lathrop, S., Medus, C., Spina, N.L., Webb, T.H., White, P.L., Wymore, K., Gierke, R.E., Mahon, B.E., Griffin, P.M., Emerging Infections Program FoodNet Working Group, 2013. Increased recognition of non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States during 2000–2010: epidemiologic features and comparison with *E. coli* O157 infections. Foodborne Pathog. Dis. 10, 453–460.

Hidaka, A., Hokyo, T., Arikawa, K., Fujihara, S., Ogasawara, J., Hase, A., Hara-Kudo, Y., Nishikawa, Y., 2009. Multiplex real-time PCR for exhaustive detection of diarrhoeagenic *Escherichia coli*. J. Appl. Microbiol. 106, 410–420.

Jinneman, K.C., Waite-Cusic, J.G., Yoshitomi, K.J., 2012. Evaluation of shiga toxin-producing *Escherichia coli* (STEC) method for the detection and identification of STEC O104 strains from sprouts. Food Microbiol. 30, 321–328.

Rasko, D.A., Webster, D.R., Sahl, J.W., Bashir, A., Boisen, N., Scheutz, F., Paxinos, E.E., Sebra, R., Chin, C.-S., Iliopoulos, D., Klammer, A., Peluso, P., Lee, L., Kislyuk, A.O., Bullard, J., Kasarskis, A., Wang, S., Eid, J., Rank, D., Redman, J.C., Steyert, S.R., Frimodt-Møller, J., Struve, C., Petersen, A.M., Krogfelt, K.A., Nataro, J.P., Schadt, E.E., Waldor, M.K., 2011. Origins of the *E. coli* strain causing an outbreak of hemolytic–uremic syndrome in Germany. N. Engl. J. Med. 365, 709–717.

Robert Koch Institute, 2011. Final presentation and evaluation of epidemiological findings in the EHEC O104:H4 outbreak, Germany 2011. http://www.rki.de/EN/Home/EHEC_final_report.pdf?__blob=publicationFile (accessed 8.16.13).

Rohde, H., Qin, J., Cui, Y., Li, D., Loman, N.J., Hentschke, M., Chen, W., Pu, F., Peng, Y., Li, J., Xi, F., Li, S., Li, Y., Zhang, Z., Yang, X., Zhao, M., Wang, P., Guan, Y., Cen, Z., Zhao, X., Christner, M., Kobbe, R., Loos, S., Oh, J., Yang, L., Danchin, A., Gao, G.F., Song, Y., Li, Y., Yang, H., Wang, J., Xu, J., Pallen, M.J., Wang, J., Aepfelbacher, M., Yang, R., 2011. Open-source genomic analysis of Shiga toxin-producing *E. coli* O104:H4. N. Engl. J. Med. 365, 718–724.

Rump, L.V., Bodeis-Jones, S., Abbott, J., Zhao, S., Kase, J., Lorenz, S., Fischer, M., Brown, E., Meng, J., 2012. Genetic characterization of *Escherichia coli* O104 isolates from different sources in the United States. Appl. Environ. Microbiol. 78, 1615–1618.

Taormina, P.J., Beuchat, L.R., Slutsker, L., 1999. Infections associated with eating seed sprouts: an international concern. Emerg. Infect. Dis. 5, 626.

Tillman, G.E.G., Wasilenko, J.L.J., Simmons, M.M., Lauze, T.A.T., Minicozzi, J.J., Oakley, B.B.B., Narang, N.N., Fratamico, P.P., Cray, A.C.A., 2012. Isolation of Shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 from ground beef using modified rainbow agar and post-immunomagnetic separation acid treatment. J. Food Prot. 75, 1548–1554.

Tzschoppe, M., Martin, A., Beutin, L., 2012. A rapid procedure for the detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O26, O103, O111, O118, O121, O145 and O157 strains and the aggregative EHEC O104:H4 strain from ready-to-eat vegetables. Int. J. Food Microbiol. 152, 19–30.

Wasilenko, J.L., Fratamico, P.M., Narang, N., Tillman, G.E., Ladely, S., Simmons, M., Cray Jr., W.C., 2012. Influence of primer sequences and DNA extraction method on detection of non-O157 Shiga toxin-producing *Escherichia coli* in ground beef by real-time PCR targeting the *eae*, *stx*, and serogroup-specific genes. J. Food Prot. 75, 1939–1950.

Weagant, S.D., Bound, A.J., 2001. Evaluation of techniques for enrichment and isolation of *Escherichia coli* O157:H7 from artificially contaminated sprouts. Int. J. Food Microbiol. 71, 87–92.