Detection and Recovery of *E. coli* O157:H7 in Alfalfa sprouts by Real-time PCR Combined with Immunomagnetic Separation With and Without an Acid Treatment

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SUMMARY

E. coli O157:H7 has been implicated in foodborne disease outbreaks with alfalfa sprouts. However, detection in sprouts by standard cultural methods can be difficult due to the high background microflora. This study evaluated an improved procedure for rapid detection and isolation of *E. coli* O157:H7 from sprout samples.

Alfalfa sprouts were artificially contaminated with *E. coli* O157:H7 at low (~0.2 cfu/g) and high (~2 cfu/g) levels and enriched in modified buffered peptone water + pyruvate (mBPWp) with cefsulodin and vancomycin at 42°C with shaking. After 5h and 24h, the target organisms were concentrated by IMS using PATHATRIX™ or Dynabeads® MAX *E. coli* O157 (MS beads were screened by real-time PCR for simultaneous detection of *stx*1, *stx*2 and *uidA* genes using the SmartCycler. Additionally, broth cultures and IMS beads were streaked onto selective agar plates (Rainbow® agar, R&F® *E. coli* O157 Chromogenic medium and TC-SMAC agar) for isolation of *E. coli* O157:H7. Both broth cultures and IMS beads were also acid treated to improve upon cultural recovery.

After 5 h enrichment and PATHATRIX™ IMS, *E. coli* O157:H7 was detected by real-time PCR in **23/25 samples inoculated at the low level**, **isolated from 7/25 inoculated samples on selective agars** , but from 22/25 following acid treatment of the beads. *E. coli* O157 was detected in 22/25 samples and isolated from 0/25 following IMS with the Dynabeads® MAX *E. coli* O157 system, but from 15/25 following acidification of the beads. After 24h enrichment, cultural recovery was improved for enrichment broths and both types of IMS beads following acid treatment of the samples. Acidification of *E. coli* O157 enrichment broths and IMS beads improved isolation by eliminating competing organisms that make isolation difficult.

INTRODUCTION

A procedure for the enrichment of Shiga toxin producing *E. coli* in foods using selective enrichment of samples in modified Buffered Peptone Water (mBPWp) (Weagant & Bound, 2001) was combined with a rapid, real-time PCR assay specific for *stx*1, *stx*2, and the +93 *uidA* mutation to screen for the presence of *E. coli* O157:H7 in foods (Jinneman et al., 2003; Yoshitomi et al., 2003) and is capable of detecting < 1 CFU/g of STEC in foods.

Enrichment in mBPWp at 42˚C under shaking with antibiotics (cefsulodin and vancomycin) followed by IMS was shown to be an effective method to detect EHEC in alfalfa sprouts (Wendakoon et al., 2008). However, alfalfa sprout enrichment broths present a challenge in isolating EHEC, even with the use of IMS, because competing microorganisms that grow in the enrichment, are co-extracted with *E. coli* O157 by IMS and subsequently grow on the selective agar plates.(Weagant et al, 2008). We evaluated the optimized enrichment with alfalfa sprouts artificially contaminated with five EHEC strains and continued to encounter difficulty in recovering the inoculated EHEC on the selective plates (Jinneman et al., 2009). An acid treatment of IMS beads and enrichment broth cultures was found to improve recovery of EHEC from artificially contaminated cilantro (Fedio, et al, 2009).

The Pathatrix™ (Matrix Microscience, CO, USA), and the Invitrogen Bead Retriever with Dynabeads® MAX *E. coli* O157 were used for detection and isolation of *E. coli* O157. The IMS system uses magnetic beads coated with antibodies to capture the target bacteria. The beads with captured target organisms are ready for further use in PCR detection or cultural isolation methods. In addition, an acidification treatment was employed to improve cultural recovery on selective agars.

MATERIALS AND METHODS

Bacterial Strains

Five E. coli O157:H7 strains were used for inoculation of the alfalfa sprouts: *E. coli* O157:H7 ATCC 35150 (*stx*1, *stx*2), ATCC 43895 (*stx*1, *stx*2), ATCC 43894 (*stx*1, *stx*2), ATCC 43889 (*stx*2 only), and TT7 (*stx*1 only). Stationary phase cultures were diluted in Butterfield's Phosphate Buffer and used to inoculate the alfalfa sprouts.

Preparation of Alfalfa sprouts samples

Alfalfa sprouts were weighed (25 g portions) and placed in sterile, polythene filter bags (7.5" x 15", NASCO). The sample was inoculated with 1 ml diluted culture to achieve the low (~0.2 cfu/g) and high (~2 cfu/g) high inoculum levels. Uninoculated control samples were also prepared. All the samples were mixed thoroughly. The inoculum levels were determined by plate counts of the dilutions.

Enrichment procedure

Modified Buffered Peptone Water with pyruate (mBPWp) was used to enrich *E. coli* O157:H7 in the sprout samples. For each 25 g sample, 225 ml of pre-warmed mBPWp was added. Samples were stomached for 2 min and antibiotics were added. Antibiotics: (final concentration in mBPWp): Cefsulodin (10mg/l), Vancomycin (8mg/l). Samples were incubated at 42°C with shaking (100 rpm) for 5h or 24h.

Detection

After 5 h enrichment, samples were immunoconcentrated by the Pathatrix and Bead Retriever systems. The IMS beads were plated onto selective media or acid treated prior to plating as outlined in Figure 1. A portion of the beads were also used for real time PCR detection with the primers as probes listed in Table 1 on the Smart Cycler II as shown in Figure 2. After 24 h enrichment, enrichment broth samples and IMS beads were used for PCR and both non-acid treated and acid treated samples were plated onto selective agars.

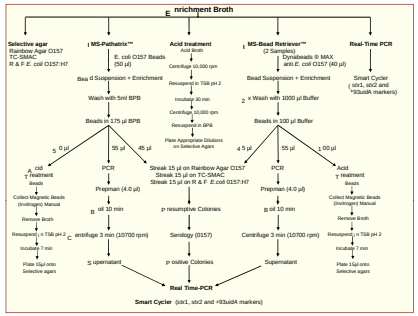

Figure 1. Procedure for analyzing enrichment cultures by real time PCR, Pathatrix IMS and Invitrogen IMS for detection of *E. coli* O157:H7 and isolation on selective agars.

Pathatrix system

Bead Retriever System

Table 1. Primer/probe sequences for use on SmartCycler II platform.

Primers[1]	GenBank #	Bases	5'→3' Sequence
Stx1F934	M19473	26	gTg gCA TTA ATA CTg AAT TgT CAT CA
Stx1R1042	M19473	21	gCg TAA TCC CAC ggA CTC TTC
Stx2F1218	X07865	24	gAT gTT TAT ggC ggT TTT ATT TgC
Stx2R1300	X07865	26	Tgg AAA ACT CAA TTT TAC CTT Tag CA
UidAF241	AF305917	21	CAg TCT ggA TCg CgA AAA CTg
UidAR383	AF305917	22	ACC AgA CgT TgC CCA CAT AAT T
Probes[1]			
Stx1P990	M19473	31	TxRd-TgA TgA gTT TCC TTC TAT gTg TCC ggC AgA T-BHQ2
Stx2P1249	X07865	25	6FAM-TCT gTT AAT gCA ATg gCg gCg gAT T-BHQ1
UidAP266	AF305917	15	TET-ATT gAg CAg CgC Tgg-MGB/NFQ

[1] Primer/Probe name composed of target gene (*stx*1, *stx*2 or *uidA*), forward primer (F), reverse primer (R) or probe (P), 5' base position of oligonucleotide in the respective gene sequence specified in column 2.

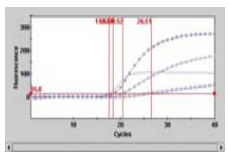

Figure 2. Real-time PCR detection of *E. coli* O157:H7 *stx*1, *stx*2, and +93 *uid*A markers. Smart cycler II equipment (left) and graph of amplification (right).

Table 2. Detection of *E. coli* O157:H7 in alfalfa sprouts artificially contaminated at a low level (0.1-0.3 cfu/g), enriched in mBPWp with cefsulodin (10mg/L) and vancomycin (8mg/L) and incubated with shaking at 42°C for 5h. Detection by real time PCR, and cultural isolation with or without IMS and an acid treatment.

E.coli O157 (inoculum level)	Incubation Time	Pathatrix beads					Dynabeads® MAX *E.coli* O157				
		PCR	Cultural isolation	Rainbow	TC-SMAC	R & F	PCR	Cultural isolation	Rainbow	TC-SMAC	R & F
ATCC 35150 (0.18 cfu/g)	5h	5/5	3/5	3/5	1/5	1/5	4/5	0/5	0/5	0/5	0/5
ATCC 43894 (0.2 cfu/g)	5h	5/5	5/5	5/5	5/5	4/5	3/5	3/5	2/5	1/5	0/5
ATCC 43895 (0.18 cfu/g)	5h	3/5	4/5	2/5	4/5	1/5	2/5	0/5	0/5	0/5	0/5
ATCC 43889 (0.27cfu/g)	5h	5/5	5/5	4/5	4/5	5/5	5/5	5/5	5/5	1/5	1/5
TT7 (0.18 cfu/g)	5h	5/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Total		23/25	18/25	15/25	14/25	11/25	14/25	8/25	7/25	2/25	1/25
Acid Treated											
ATCC 35150 (0.18 cfu/g)	5h		0/5	0/5	0/5	0/5		0/5	0/5	0/5	0/5
ATCC 43894 (0.2 cfu/g)	5h		0/5	0/5	0/5	0/5		0/5	0/5	0/5	0/5
ATCC 43895 (0.18 cfu/g)	5h		0/5	0/5	0/5	0/5		0/5	0/5	0/5	0/5
ATCC 43889 (0.27cfu/g)	5h		0/5	0/5	0/5	0/5		0/5	0/5	0/5	0/5
TT7 (0.18 cfu/g)	5h		0/5	0/5	0/5	0/5		0/5	0/5	0/5	0/5
Total			0/25	0/25	0/25	0/25		0/25	0/25	0/25	0/25

Table 3. Detection of *E. coli* O157:H7 in alfalfa sprouts artificially contaminated at a high level (1-3 cfu/g), enriched in mBPWp with cefsulodin (10mg/L) and vancomycin (8mg/L) and incubated with shaking at 42°C for 5h. Detection by real time PCR, and cultural isolation with or without IMS and an acid treatment.

E.coli O157 (inoculum level)	Incubation Time	Pathatrix beads					Dynabeads® MAX *E.coli* O157				
		PCR	Cultural isolation	Rainbow	TC-SMAC	R & F	PCR	Cultural isolation	Rainbow	TC-SMAC	R & F
ATCC 35150 (1.8 cfu/g)	5h	5/5	4/5	1/5	3/5	1/5	5/5	2/5	2/5	0/5	0/5
ATCC 43894 (2.0 cfu/g)	5h	5/5	5/5	2/5	5/5	3/5	5/5	2/5	0/5	1/5	1/5
ATCC 43895 (1.8 cfu/g)	5h	5/5	5/5	0/5	5/5	1/5	5/5	0/5	0/5	0/5	0/5
ATCC 43889 (2.7cfu/g)	5h	4/5	2/5	0/5	2/5	0/5	5/5	0/5	0/5	0/5	0/5
TT7 (1.8 cfu/g)	5h	5/5	4/5	4/5	3/5	0/5	5/5	2/5	2/5	1/5	0/5
Total		24/25	20/25	7/25	18/25	5/25	25/25	6/25	4/25	2/25	1/25
Acid Treated											
ATCC 35150 (1.8 cfu/g)	5h		5/5	5/5	3/5	4/5		5/5	5/5	4/5	0/5
ATCC 43894 (2.0 cfu/g)	5h		5/5	5/5	4/5	4/5		5/5	4/5	5/5	5/5
ATCC 43895 (1.8 cfu/g)	5h		5/5	4/5	5/5	5/5		4/5	1/5	3/5	2/5
ATCC 43889 (2.7cfu/g)	5h		5/5	5/5	0/5	0/5		4/5	3/5	4/5	2/5
TT7 (1.8 cfu/g)	5h		5/5	5/5	4/5	4/5		5/5	5/5	4/5	4/5
Total			25/25	24/25	16/25	17/25		23/25	18/25	20/25	13/25

Table 4. Detection of *E. coli* O157:H7 in alfalfa sprouts artificially contaminated at a high level (0.1-3.3 cfu/g) with EHEC and enriched in mBPWp with cefsulodin (10mg/L) and vancomycin (8mg/L) and incubated with shaking at 42°C for 24 h. Detection by real time PCR, and cultural isolation with or without IMS and an acid treatment.

E.coli O157 (inoculum level)	Incubation Time	Broth					Pathatrix beads					Dynabeads® MAX *E.coli* O157				
		PCR	Cultural isolation	Rainbow	TC-SMAC	R & F	PCR	Cultural isolation	Rainbow	TC-SMAC	R & F	PCR	Cultural isolation	Rainbow	TC-SMAC	R & F
ATCC 35150 (1.8 cfu/g)	24h	2/5	5/5	5/5	5/5	3/5	4/5	5/5	5/5	5/5	4/5	4/5	5/5	5/5	5/5	5/5
ATCC 43894 (2.0 cfu/g)	24h	5/5	3/5	1/5	1/5	1/5	4/5	4/5	3/5	4/5	4/5	5/5	2/5	2/5	0/5	1/5
ATCC 43895 (1.8 cfu/g)	24h	2/5	1/5	1/5	1/5	0/5	5/5	1/5	0/5	0/5	1/5	5/5	0/5	0/5	0/5	0/5
ATCC 43889 (2.7cfu/g)	24h	5/5	5/5	5/5	4/5	4/5	5/5	5/5	4/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5
TT7 (1.8 cfu/g)	24h	5/5	5/5	5/5	5/5	2/5	5/5	5/5	5/5	5/5	2/5	5/5	1/5	5/5	5/5	1/5
Total		19/25	19/25	17/25	16/25	10/25	23/25	20/25	17/25	19/25	15/25	24/25	13/25	17/25	15/25	17/25
Acid Treated																
ATCC 35150 (1.8 cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
ATCC 43894 (2.0 cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
ATCC 43895 (1.8 cfu/g)	24h		5/5	1/5	5/5	1/5		5/5	4/5	5/5	5/5		5/5	5/5	5/5	5/5
ATCC 43889 (2.7cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
TT7 (1.8 cfu/g)	24h		5/5	5/5	5/5	5/5		4/5	4/5	4/5	4/5		5/5	5/5	5/5	5/5
Total			25/25	21/25	25/25	21/25		24/25	23/25	24/25	24/25		25/25	25/25	25/25	25/25

Table 5. Detection of *E. coli* O157:H7 in alfalfa sprouts artificially contaminated at a high level (1-3 cfu/g) with EHEC and enriched in mBPWp with cefsulodin (10mg/L) and vancomycin (8mg/L) and incubated with shaking at 42°C for 24 h. Detection by real time PCR, and cultural isolation with or without IMS and an acid treatment.

E.coli O157 Strain (inoculum level)	Incubation Time	Broth					Pathatrix beads					Dynabeads® MAX *E.coli* O157				
		PCR	Cultural isolation	Rainbow	TC-SMAC	R & F	PCR	Cultural isolation	Rainbow	TC-SMAC	R & F	PCR	Cultural isolation	Rainbow	TC-SMAC	R & F
ATCC 35150 (0.18 cfu/g)	24h	4/5	5/5	4/5	3/5	3/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	3/5	3/5	4/5
ATCC 43894 (0.2 cfu/g)	24h	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5	5/5	5/5
ATCC 43895 (0.18 cfu/g)	24h	5/5	5/5	5/5	4/5	3/5	5/5	4/5	1/5	4/5	3/5	5/5	4/5	1/5	4/5	1/5
ATCC 43889 (0.27cfu/g)	24h	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5	5/5	5/5
TT7 (0.18 cfu/g)	24h	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Total		24/25	25/25	24/25	22/25	23/25	25/25	24/25	21/25	24/25	23/25	25/25	24/25	17/25	22/25	20/25
Acid Treated																
ATCC 35150 (0.18 cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
ATCC 43894 (0.2 cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	4/5		5/5	5/5	5/5	5/5
ATCC 43895 (0.18 cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	2/5		5/5	5/5	5/5	5/5
ATCC 43889 (0.27cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
TT7 (0.18 cfu/g)	24h		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5		5/5	5/5	5/5	5/5
Total			25/25	25/25	25/25	25/25		25/25	25/25	24/25	21/25		25/25	25/25	25/25	25/25

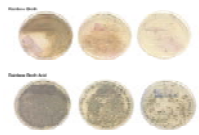

Figure 4. Isolation of *E. coli* O157 from alfalfa sprouts on Rainbow agar. Top row: 24h enrichment broth streak plate (10 µl of undiluted enrichment broth) and spread plate (50 µl of 10^{-3} and 10^{-5} dilution). Bottom row: Spread plates of acid treated alfalfa sprout enrichment broth (50 µl of undiluted, 10^{-1} and 10^{-2} dilutions of acid treated enrichment).

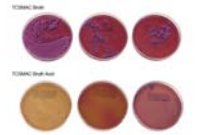

Figure 5. Isolation of *E. coli* O157 from alfalfa sprouts on TC-SMAC agar. Top row: 24h enrichment broth streak plate (10 µl of undiluted enrichment broth) and spread plate (50 µl of 10^{-3} and 10^{-5} dilution). Bottom row: Spread plates of acid treated alfalfa sprout enrichment broth (50 µl of undiluted, 10^{-1} and 10^{-2} dilutions of acid treated enrichment).

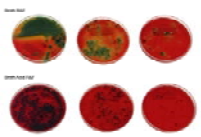

Figure 6. Isolation of *E. coli* O157 from alfalfa sprouts on R & F Chromogenic medium for *E. coli* O157. Top row: 24h enrichment broth streak plate (10 µl of undiluted enrichment broth) and spread plate (50 µl of 10^{-3} and 10^{-5} dilution). Bottom row: Spread plates of acid treated alfalfa sprout enrichment broth (50 µl of undiluted, 10^{-1} and 10^{-2} dilutions of acid treated enrichment).

Figure 7. Isolation of *E. coli* O157 from alfalfa sprout enrichment broths following Pathatrix IMS. Top row: Pathatrix IMS bead streak plates (15 µl of IMS beads from 24 h enrichment broth) on Rainbow agar (left), TC SMAC (center) and R&F Chromogenic medium for *E. coli* O157 (right). Bottom row: Acid treated Pathatrix IMS beads on Rainbow agar (left), TC SMAC (center) and R&F Chromogenic medium for *E. coli* O157 (right).

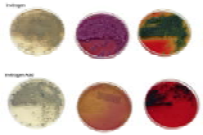

Figure 8. Isolation of *E. coli* O157 from alfalfa sprout enrichment broths following Invitrogen IMS. Top row: Dynabeads® MAX anti-*E. coli* O157 beads streak plates (15 µl of IMS beads from 24 h enrichment broth) on Rainbow agar (left), TC SMAC (center) and R&F Chromogenic medium for *E. coli* O157 (right). Bottom row: Acid treated Dynabeads® MAX anti-*E. coli* O157 beads on Rainbow agar (left), TC SMAC (center) and R&F agar(right).

CONCLUSIONS

- Low levels of EHEC can be reliably detected by real-time PCR in alfalfa sprouts following enrichment in mBPWp with CV added at the onset of incubation, incubating the samples at elevated temperature (42°C) with shaking at 100 rpm throughout the enrichment.
- Isolation on selective agar can be difficult due to the presence of competing microorganisms on the selective agar plates even in samples where the EHEC are concentrated by immunomagnetic separation.
- Cultural recovery of *E. coli* O157 was easier when the acid treatment was used either directly or with IMS beads.

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