Improved Recovery of Outbreak-Associated *Escherichia coli* O157:H7 from Farm Environmental Samples in Hawaii Using Recirculating Immunomagnetic Separation System (RIMS)

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Abstract

A novel use of the Pathatrix Recirculating Immunomagnetic Separation System (RIMS) coupled with optimal enrichment conditions was demonstrated in the recovery of low levels of *E. coli* O157:H7 from leafy produce, Moore swabs and surface water samples during the 2006 trace-back investigations in California. The recovery of *E. coli* O157:H7 from highly suspect materials using the standard U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) method proved to be difficult in the early part of an *E. coli* O157:H7 outbreak investigations that occurred in Hawaii in May 2007. Building on the success of the RIMS in these types of specimens, the Hawaii State Laboratories Division adapted the system to analyze farm soil and manure specimens that were believed to be the source of the *E. coli* O157:H7 outbreak in Hawaii. Out of a total of 168 samples of soil, sediment, surface water, cow and pig manure analyzed, *E. coli* O157:H7 was found in 18 out of 168 (11%) cow manure samples tested using the RIMS method in contrast to 1 (0.6%) sample detected by the BAM method. The RIMS led to superior isolation of *E. coli* O157:H7 from manure, which was identified by both real-time PCR and conventional culture methods. Molecular typing data by Pulsed-Field-Gel Electrophoresis (PFGE) confirmed that environmental isolates were indistinguishable from outbreak-related clinical isolates. This investigation validates the application of the RIMS method in improving the recovery of outbreak-associated *E. coli* O157:H7 from farm environmental samples.

Introduction

Cattle are generally considered the primary reservoir of *Escherichia coli* O157:H7, although this bacteria has also been isolated from the intestines of healthy deer, goats and sheep. Further, *E. coli* O157:H7 has been shown to remain viable in bovine feces for up to 9 weeks, depending on environmental conditions (1)(2). In the 2006 California outbreaks, clinical cases of *E. coli* O157:H7 were associated with consumption of contaminated baby spinach that was traced back to farms in California.

Genetic analysis of isolates confirmed that the environmental *E. coli* O157:H7 was the likely source of the outbreaks, although mechanisms of contaminations were still unclear (3).

The California investigation set the stage for the first use of RIMS for the recovery of *E. coli* O157:H7 in environmental samples. The Food and Drug Laboratory from the California Department of Public Health demonstrated that this method was a simple and sensitive alternative to BAM for the recovery of *E. coli* O157:H7 in fresh leafy produce, Moore swabs and surface water (4).

In March of 2007, seven U.S. mainland visitors to Kauai and one resident became ill after eating locally grown organic greens from area restaurants. Although denied permission to sample gardens directly, investigators collected feral pig, horse, and cow manure, compost fertilizer, surface water, soil, and lettuce, from regions near the farms that supplied the restaurants. One hundred and sixty-eight (168) samples were tested at the Hawaii State Laboratories Division (SLD). Standard BAM methods with and without RIMS technology were used in attempts to recover the outbreak strain of *E. coli*.

Materials and Methods

Sample processing and enrichment:
Sample processing and enrichment of 168 environmental swabs and fecal material were conducted using the BAM methods (5) and the California RIMS Protocol for the Detection and Isolation of *E. coli* O157:H7 (4) with some modifications. Briefly, samples were enriched overnight in EHEC Enrichment Broth supplemented with Cefixime, Cefsulodin and Vancomycin (EEB) for BAM, while modified Buffered Peptone Water with Acriflavin, Cefsulodin and Vancomycin (mBPW + ACV) was used for the RIMS protocol. The strength of mBPW used was dependent on the type of matrix (2x for surface water; 1 X for produce, manure, environmental swabs, and Moore swab samples). Prior to the RIMS concentration, homogenates were incubated with constant shaking at 42ºC for exactly 5 hours. For the BAM method, EEB was used to incubate the homogenates overnight (18-24 hours) at 35ºC without agitation.

Recirculating Immunomagnetic Separation (RIMS) using Pathatrix:
Following enrichment, *E. coli* O157 cells were further concentrated by re-circulating the cells via an immunomagnetic bead-capture system on the Pathatrix instrument for 30 minutes. The beads were washed and re-suspended in 80uL of mBPW. Aliquots of the suspension were screened for STEC by rti PCR, and plated onto Rainbow Agar and Sorbitol-MacConkey agar with potassium tellurite and cefixime (TC-SMAC).

Detection by Multiplex Real-Time (rti) PCR:
Approximately 25uL of the RIMS bead suspension was centrifuged at 12000 x g for 3 minutes. The supernatant was removed and the remaining pellet was re-suspended in sterile water. DNA extraction was a simple boil preparation. Multiplex rti PCR detection of *stx*1, *stx*2, and the +93 *uid*A genes were modified for the Smart Cycler II (Cepheid) using Texas Red (instead of Rox) on the *stx*1 probe, a PCR reagent bead (containing polymerase, dNTPs, Mg ++ and buffer), and an internal control (IC) (6). BAM-enriched samples were screened by real-time BAX.

Isolation of *E. coli* O157:H7:
Following BAM or RIMS enrichment, and in parallel with rti PCR screening, isolation of *E. coli* O157:H7 was performed by BAM or Food Emergency Response Network (FERN) Methods respectively (6). Typical colonies were confirmed biochemically and serologically using standard methods.

Pulsed-Field-Gel Electrophoresis (PFGE):
Molecular DNA fingerprinting by PFGE was performed on the confirmed isolates using standard PulseNet protocols (7).

Results and Discussion

Homogenates incubated at higher temperatures (42ºC) overnight using mBPW +ACV for enrichment, followed by Immunomagnetic Separation (IMS) has been shown to increase the recovery of *E. coli* O157:H7 in the presence of competing microflora (4,8,9). Our results indicate that these combined methods can be further enhanced if broths were shaken, if even for the shorter (5 hours) incubation time. Further, using a larger sample volume (225 mL) for the RIMS step may have contributed to the improved recovery of the captured target, compared with other IMS systems that use 1mL of the enrichment broth (10). Although recovery of *E. coli* O157:H7 was successful in 17 of 42 cow and a single horse manure samples, we were not able to recover the bacteria in other environmental samples tested (Table 1). Importantly, BAM methods only detected 1 positive sample whereas modified RIMS detected 18.

Table 1. Sample types

Sample Type	N	RIMS Pos (%)	BAM Pos (%)	PFGE Match
Cattle Manure	42	17 (10%)	1 (0.6%)	14 (EXHX01.1860) 2(EXHX01.0975) 1(unresolved)
Horse Manure	1	1 (0.6)%	0%	1 (EXHX01.4080)
Bird Droppings	2	0%	0%	
Non Feral Pig-Fecal	9	0%	0%	
Feral Pig-Fecal	12	0%	0%	
Turkey Manure	3	0%	0%	
Lettuce	5	0%	0%	
Moore Swab	56	0%	0%	
Watercress	3	0%	0%	
Plot by goat	2	0%	0%	
Silt	2	0%	0%	
Soil	29	0%	0%	
Water	2	0%	0%	

In the 2006 California outbreaks, investigators linked the outbreak strains of *E. coli* O157:H7 to consumption of fresh produce (2,4). Our inability to isolate *E. coli* from epidemiologically associated lettuce grown from regions near the farms that supplied the restaurants may be attributed to sampling limitations rather than a false negative. Improved recovery of *E. coli* in produce (lettuce, sprouts, spinach) has been reported when incubation time was increased to 24-hours (8). A six-month study conducted by Himathongkham *et.al*. (4) described a 5-hour enrichment at 42ºC followed by rti PCR detection of RIMS-enriched samples had potential use as a rapid screening tool.

Table 2. PCR Cycle Threshold (Ct) values.

Source	PFGE ID	Pathatrix beads				Isolate			
		TxR stx1	FAM stx2	TET uid	Cy5 IC	TxR stx1	FAM stx2	TET uid	Cy5 IC
COW	N07 141	0.00	0.00	0.00	26.65	0.00	0.00	0.00	26.49
COW	N07 129	0.00	0.00	0.00	26.89	25.73	25.56	27.40	25.58
COW	N07 130	32.49	31.47	0.00	26.41	19.89	19.74	21.40	0.00
COW	N07 131	0.00	34.84	0.00	25.99	25.23	25.09	27.34	24.48
COW	N07 132	0.00	34.50	0.00	26.69	17.10	17.00	18.90	0.00
COW	N07 133	30.98	30.11	34.29	26.40	23.95	24.14	25.97	25.11
COW	N07 134	0.00	36.45	0.00	26.54	19.57	19.69	21.29	0.00
COW	N07 135	0.00	34.55	0.00	26.68	24.36	24.19	26.03	25.43
COW	N07 136	0.00	0.00	0.00	26.48	22.69	22.49	24.32	0.00
COW	N07 142	0.00	0.00	0.00	26.65	19.62	19.28	21.07	0.00
COW	N07 143	32.40	30.70	0.00	26.38	17.76	17.50	19.47	0.00
COW	N07 144	30.15	28.19	35.69	26.30	18.21	18.09	19.74	0.00
HORSE	N07 145	25.81	25.03	29.53	25.81	17.95	17.57	19.90	0.00
COW	N07 146	27.52	26.84	29.97	26.13	19.11	18.56	20.50	0.00
COW	N07 147	0.00	33.35	0.00	26.78	18.94	18.57	21.15	0.00
COW	N07 148	39.94	36.91	0.00	26.51	18.16	17.94	19.89	0.00
COW	N07 149	0.00	36.87	0.00	26.22	17.67	17.62	19.32	0.00
COW	N07 150	0.00	34.72	0.00	26.21	18.77	18.32	20.37	0.00

Although rti PCR was positive for 7/18 (39%) samples obtained from the 5-hour enrichment/RIMS, only 4/7 (57%) were positive for all three target markers (*stx*1, *stx*2 and *uid*A; Table 2). When PCR was conducted on typical colonies, 17/18 (94%) of the isolates were positive for all 3 target genes. The PCR results from the 5-hour samples reinforces the California study indicating the potential use of *stx*1 and *stx*2 genes for screening *E. coli* O157:H7 in environmental samples.

Figure 1

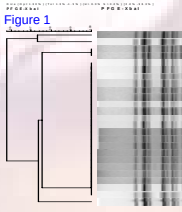
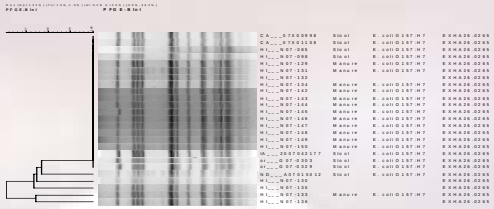


Figure 2



Molecular typing by Pulsed-Field-Gel Electrophoresis (PFGE) proved that *E. coli* O157:H7 isolates from manure were the source of the outbreak strains isolated from clinical cases (Figures 1 & 2). CDC identified 3 patterns with *Xba*I (Fig 1) and 1 pattern with *Avr*II/*Bln*I enzymes (Fig 2).

Conclusions

1. A combination of enrichment buffer (mBPW + ACV), incubation at higher temperature (42ºC) for a shorter time (5 hrs vs overnight) in a shaking incubator, followed by RIMS, outperformed BAM in the recovery of *E. coli* O157:H7 in environmental manure samples.
2. Environmental isolates were critical to locate the source of the outbreak strain.
3. The RIMS protocol followed by rti PCR in screening for *E. coli* O157:H7 has potential to improve recovery of organisms during outbreak source or other environmental investigations.
4. Additional studies are needed to assess the performance characteristics of the RIMS protocol in various matrices.

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Supported by the USDA Cooperative Agreement No. FSIS-C-35-2005/02, CDC Cooperative Agreement PHEP, Opinions, findings, conclusions, or recommendations expressed are those of the authors, and do not reflect the views of the USDA, CDC, or the State of Hawaii. We would also like to thank Dr. Paul Fox for all his help in editing this poster.

Background image http:vvm.edu/~empact/water/water_img/ecoli1jpg