Detection and Isolation of Shiga Toxin-Producing *Escherichia coli* (STEC) O104 and Other STEC Serogroups of Public Health Concern

Gian Marco Baranzoni[1], Pina Fratamico[2], Fernando Rubio[3], Thomas Glaze[3], Natalie Launchi[2], Lori Bagi[2] and Sabrina Albonetti[1]

[1]Department of Veterinary Medical Science, University of Bologna, 50 via Tolara di Sopra, Ozzano dell'Emilia (BO), Italy; [2]USDA, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA, United States; [3]Abraxis, LLC, 54 Steamwhistle Drive, Warminster, PA, United States

Contac information:
Gian Marco Baranzoni, PhD
phone: (215) 836-6929
email: gianmarco.baranzoni@ars.usda.gov

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are important food-borne pathogens that cause outbreaks and serious cases of food-borne illness. Methods for detection and isolation of STEC, particularly the non-O157 STEC, are needed to prevent their transmission through contaminated food. The objectives of this study were to develop antibodies against different STEC serogroups and produce immunomagnetic separation (IMS) and latex reagents. Furthermore, methodologies were developed to detect and isolate STEC O104 and enteroaggregative STEC (EAEC-STEC) O104:H4 in sprouts, utilizing multiplex PCR assays and the IMS and latex reagents. Antibodies against the top six non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145), as well as STEC O104 and O157 were generated in mice and used to produce IMS and latex beads for the different pathogens. Specificity testing was performed using the target pathogens and non-target *E. coli*. A method was developed for detection of STEC O104 and EAEC-STEC O104:H4 in sprouts, consisting of a selective enrichment, two real-time multiplex PCR assays (stx_2, *aggR*, and wzx_{104} genes for detection of EAEC-STEC O104:H4 and stx_{1-2}, *ehxA*, and wzx_{104} for STEC O104), followed by IMS, isolation from selective agars, and confirmation by latex agglutination and PCR. The IMS and latex reagents against all of the STEC serogroups showed good specificity and were useful for isolating the target pathogens, as shown by plating onto selective and non-selective agars. Cold stressed STEC and EAEC-STEC O104 were detected and isolated from sprout samples inoculated at a level of less than 1 CFU/g. All presumptive colonies were confirmed by agglutination using the O104 latex particles and the multiplex PCR assays. The IMS and latex reagents and the methods developed in this study improve the ability of the food industry and regulatory agencies worldwide to detect and isolate important STEC from food.

Introduction

Various STEC serotypes are emerging pathogens that are responsible for food-borne outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS). STEC O157:H7 is the most common serotype associated with serious illness, and it was declared an adulterant in beef in 1994. However, other STEC serogroups cause similar illnesses, and in 2000, non-O157 STEC infections were included in the list of National Notifiable Diseases. From 2000 to 2010, serogroup O157 was associated with 80% of all STEC infections when the O-antigen was identified, while O26, O45, O103, O111, O121 and O145 (top six) were responsible for 83% of non-O157 STEC infections. In the same period, O157 together with the top six non-O157 STEC serogroups caused 92% of the total STEC infections. Moreover, due to improvements in laboratory diagnostic techniques, the frequency of non-O157 STEC cases has steadily increased reaching the same level as that of O157 STEC (Gould et al, 2013). The occurrence of infections due to non-O157 STEC pathogenic strains is always a potential danger. In 2011, an EAEC O104:H4 strain acquired the genes encoding antibiotic resistance genes and for Shiga toxin 2 by phage recombination. In Europe, the EAEC-STEC O104:H4 strain caused one of the largest outbreaks ever recorded characterized by thousands of hemorrhagic colitis cases, over 50 deaths, and 855 patients with HUS (Robert Koch Institute, 2011). Recently, the U.S. Department of Agriculture, Food Safety and Inspection Services (USDA-FSIS) declared STEC O26, O45, O103, O111, O121 and O145 a raw beef adulterant and described a method for detection and isolation of these non-O157 STEC (USDA-FSIS, 2014).

Due to the heterogeneity of non-O157 STEC strains and the increasing number of reported cases, it is important to develop rapid methods to rapidly detect and identify each serogroup. In this study, specific antibodies against the top six non-O157 STEC serogroups, as well as STEC O104 and O157 were generated and used to produce reagents for immunomagnetic separation (IMS), latex bead agglutination, and ELISA methods. Furthermore, methodologies were designed to detect and isolate STEC O104 and EAEC-STEC O104 in sprouts, including multiplex PCR assays, and IMS and latex bead agglutination assays.

Methods and Materials

- **Monoclonal antibodies inclusivity and exclusivity** - Sandwich type ELISA formats were developed using Costar 96-well microtiter plates and affinity-purified monoclonal antibodies against O26, O45, O103, O111, O121, O145 and O157. Each antibody was tested against pure cultures of 64 *E. coli* strains and 29 non-*E. coli* bacteria. Overnight cultures of each bacterial strain (200 mL) were centrifuged, washed, and resuspended in 13 mL of PBS. The bacteria were irradiated with 6 kGy by a Cesium-137 gamma source. The inactivated cells were used to coat a microtiter plate. ELISA assays were performed according to Abraxis LLC's instructions.
- **Immunomagnetic Separation** - Affinity purified antibodies (Abraxis LLC) prepared against O26 , O45, O103, O104, O111, O121, O145 and O157 were coupled to 3 µm super-paramagnetic particles as described by Fratamico et al. (2011) with some modifications. Sensitivity and cross reactivity of IMS beads specific for O45, O103, O111, O121, O145 and O157 were evaluated by IMS of pure cultures of *E. coli* O26:H11 05-6544, O45:H2 05-6545, O103:H2 04-2446, O111:H8 01387, O145:H8 07865 and O157:H7 06E20128. One milliliter of 10^4 CFU/mL were used for the non-target *E. coli* strains while 10^3 CFU/mL and three volumes of beads (5, 10 and 20 µL) were used for the target bacteria. The procedure was performed according to the manufacturer's instructions. The beads were then resuspended in 1 mL of washing buffer, and 100 µL were plated onto tryptic soy agar (TSA).
- **Latex Bead Agglutination Assay** – Affinity-purified polyclonal antibodies (Abraxis, LLC) prepared against O26, O45, O103, O111, O121 and O145 were covalently coupled to red latex particles according to the procedure described by Medina et al (2012). Latex bead agglutination assays were tested on pure cultures of *E. coli* strains (serogroups O26, O45, O103, O104, O111, O121, O145, O157, and K12).

Method for detection of STEC O104 and EAEC-STEC in sprouts (Baranzoni et al, 2014)

Sample Preparation
- Artificial contamination with 10 or 100 CFU of EAEC-STEC O104:H4 or STEC O104:H7 in 25g of alfalfa and dill sprouts and 48h incubation at 4 °C.
- Enrichment: 225 ml of modified buffered peptone water with pyruvate and 5 h incubation at 37°C.
- Selective enrichment: addition of acriflavin hydrochloride (10 mg/l), cefsulodin sodium salt (10 mg/L), and vancomycin hydrochloride (8 mg/L) and static incubation at 42°C for 18h.

Screening
- DNA extraction from enriched sprout samples using PrepSEQ Rapid Spin Sample Preparation kit (Thermo Fisher).
- Real-time PCR assay for EAEC-STEC O104 (stx_2, *aggR* and wzx_{104}) or STEC O104 (stx_{1-2}, *ehxA*, wzx_{104}) with internal positive control.

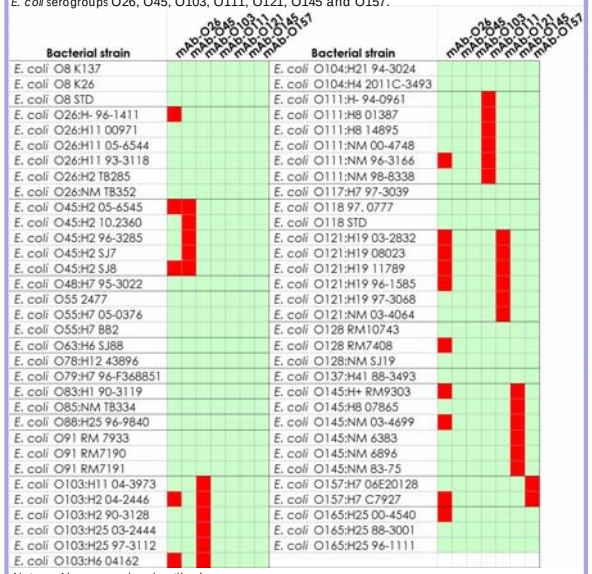
Isolation
- Immunomagnetic separation following Abraxis LCC's instructions.
- Plating the bead suspension onto modified Rainbow Agar O157 (0.05 mg/l cefixime, 5 mg/l novobiocin and 0.15 mg/L potassium tellurite) and CHROMagar STEC or STEC O104.

Identification of Presumptive Colonies
- Mauve colonies identified using latex agglutination following Abraxis LCC's instructions and real-time PCR assay for EAEC-STEC O104 (stx_2, *aggR* and wzx_{104}) or general STEC O104 (stx_{1-2}, *ehxA*, wzx_{104}) with internal positive control.

Results and Discussions

Monoclonal antibodies: inclusivity and exclusivity

Results of inclusivity and exclusivity testing using 64 *E. coli* strains and monoclonal antibodies against *E. coli* serogroups O26, O45, O103, O111, O121, O145 and O157.



Note: mAb = monoclonal antibody
Cubes are red when the antibody recognized the specific strain and green when the reaction was negative.

Monoclonal antibodies resulted negative when tested with non-*E. coli* strains, including *Enterobacter* B-199, *Salmonella* Kentucky, *Salmonella* Heidelberg, *Salmonella* Hadar, *Salmonella* Thompson, *Salmonella* Newport, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Muenster, *Salmonella* Reading, *Salmonella* Montevideo, *Salmonella Saintpaul*, *Salmonella* Derby, *Salmonella* Cerro, *Yersina enterocolitica* P+ GER O:3, *Bacillus cereus* DCMS 27, *Bacillus brevis* SMS 13, *Bacillus subtilis* DCMS 22, *Staphylococcus aureus* FRI 100, *Klebsiella pneumoniae* 709, *Citrobacter freundii* ATCC 33128, *Citrobacter freundii* ATCC 8090, *Citrobacter brakii*, *Shigella dysenteriae* ATCC 29029, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 25931, *Shigella boydii* ATCC 9207, *Listeria monocytogenes* ATCC 1513 and *Serratia liquefaciens*.
Results: Monoclonal antibodies against O26 showed cross reactivity.

Immunomagnetic separation: sensitivity and cross-reactivity

Recovery after IMS from pure cultures of target *E. coli* (10^3 CFU/mL) and non-target *E. coli* (10^4 CFU/mL) are shown below.

Number (CFU/mL) of target bacteria recovered

Volume of beads	O103	O111	O121	O145	O157
5 µl	345	835	435	370	655
10 µl	785	1,005	1,160	660	1,105
20 µl	1,230	1,555	900	1,145	1,540

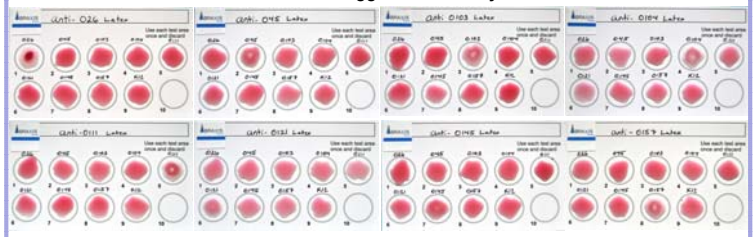
Antigen of monoclonal antibodies coupled to magnetic beads

Number (CFU/mL) of non-target bacteria recovered

Serogroups of non-target *E. coli*	O103	O111	O121	O145	O157
O157	25	0	0	0	
O145	15	245	0		0
O121	0	0		5	0
O111	0		5	0	0
O103		230	5	885	15
O45	5	20	5	5	0
O26	65	50	50	0	0

Monoclonal antibodies coupled to magnetic beads

Latex bead agglutination assays

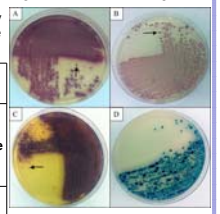


Results of the latex agglutination assays for detection of *E. coli* serogroups O26, O45, O103, O104, O111, O121, O145 and O157. Positive reactions were observed only on target *E. coli* serogroups.

Detection and isolation of STEC O104 and EAEC-STEC O104 in artificially contaminated sprouts

Number of positive samples out of number of total samples tested for every experimental group. Samples in which some negative results were observed are highlighted in red. There was agreement between screening and isolation results.

EAEC-STEC O104:H4				STEC O104:H7			
Contamination level		Multiplex real-time PCR screening	Isolation of presumptive colonies	Contamination level		Multiplex real-time PCR screening	Isolation of presumptive colonies
CFU/g	CFU/25g			CFU/g	CFU/25g		
3.6	90	1/1	1/1	6.4	160	1/1	1/1
2.7	68	2/2	2/2	4.4	110	2/2	2/2
2.4	60	1/1	1/1	3.8	95	2/2	2/2
1.8	45	2/2	2/2	3.2	80	1/1	1/1
0.76	19	3/3	3/3	1.72	43	3/3	3/3
0.56	14	4/5	4/5	0.96	24	5/5	5/5
0.52	13	0/2	0/2	0.88	22	1/4	1/4
0.36	9	4/4	4/4	0.52	13	2/2	2/2
0.24	6	2/3	2/3	0.36	9	2/3	2/3
0.16	4	3/3	3/3	0.24	6	2/3	2/3



Colonies isolated after IMS from enriched sprouts artificially contaminated with EAEC-STEC O104:H4 (A and B) and STEC O104:H7 (C and D) grown onto modified Rainbow agar (A and C), CHROMagar STEC 104 (B) and CHROMagar STEC (D). Arrows indicate typical mauve colonies. No typical STEC O104:H7 colonies were visible on CHROMagar STEC.

Conclusions

- Very specific monoclonal antibodies for the detection of *E. coli* belonging to serogroups O45, O103, O111, O121, O145 and O157 were successfully developed. Further studies are needed for production of monoclonal antibodies for O26.
- Sensitive and specific IMS beads specific for *E. coli* serogroups O103, O111, O121, O145 and O157 were designed. Development of IMS beads specific for *E. coli* serogroups O26 and O45 is in progress.
- Simple and rapid latex agglutination assays were developed for the identification of *E. coli* belonging to serogroups O26, O45, O103, O104, O111, O121, O145 and O157, demonstrating that the antibodies for the target serogroup are specific.
- A notable observation was that the detection method for EAEC-STEC and STEC O104 could be used to screen and isolate the target bacteria when inoculated at a concentration lower than 1 CFU/g in sprouts, despite the high level of background flora and the cold stress treatment.

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