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ABSTRACT

Serotyping of Shiga toxin-producing *Escherichia coli* (STEC) has been contingent upon the availability of antisera. Here we describe a 7-plex microbead-based immunoassay to simultaneously serotype seven STECs (i.e., belonging to serogroups O26, O45, O103, O111, O121, O145, and O157) by the Luminex xMAP® technology. This technology presents many advantages: Its multiplexed format (up to 100 analytes) saves time, reagents, and test sample, and many regulatory agencies currently utilize this platform for other assays. In this study, a total of seventy-nine STEC strains belonging to the 7 different serogroups of interest were tested. These strains had been previously serotyped and their serogroup was confirmed by PCR. Except for one strain belonging to the O111 serogroup, nearly all strains (i.e., 98.7%; 78/79) were correctly identified on the Bio-Plex 100 instrument in less than 4 h. This newly developed microbead-based immunoassay could be extended to include other STEC serogroups, virulence factors, and/or bacterial species.

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1. Introduction

Since the active surveillance of non-O157 STEC was implemented in the United States in 2001 (CDC, 2012), there has been an increase in number of reported cases of serotypes other than O157 mostly in part due to enhanced testing for the presence of Shiga toxin (Stx) in outbreak related strains (Hurd, 2007). Currently non-O157 infections account for approximately 50% of all confirmed STEC cases in Europe and in the United States they out number reported O157 cases (Bosilevac and Koohmaraie, 2011). Yet, the most virulent serotype commonly isolated in outbreaks in North America is *Escherichia coli* O157:H7, and is associated with bloody diarrhea, hemorrhagic colitis (HC), hemorrhagic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (Brooks et al., 2005; Willshaw et al., 2000). However, the last 10 years have produced an increasing number of outbreaks in the United States attributed to non-O157 STEC which can elicit infections and diseases similar to *E. coli* O157:H7 (Anonymous, 1995; Banatvala et al., 1996; Tarr et al., 1996; Fey et al., 2000; Brooks et al., 2004). It is estimated that 96,534 infections are caused by *E. coli* O157:H7, annually in the United States while non-O157 STECs serotypes cause 168,698 infections (Scallan et al., 2011). Approximately 20–50% of worldwide STEC infections are caused by non-O157 serotypes; incidence varies depending on geographical topology as well as climate conditions (Johnson et al., 2006; Nataro and Kaper, 1998) Cattle represent the prime reservoir of STEC strains, exhibiting a particularly high prevalence of non-O157 STEC strains (Bettelheim, 2001). The top six non-O157 STEC serogroups most commonly implicated in illnesses are O26, O111, O103, O121, O45, and O145 (Atkinson et al., 2006; Brooks et al., 2005; Gyles, 2006). STECs causes human illness by expressing at least one Shiga toxin gene (Stx) consisting of two forms; Stx1 and Stx2. Between these two isoforms, Stx2 exhibits higher virulence. Its variants are associated with an enhanced probability of HC and HUS (Beutin et al., 2008). Not all non-O157 STECs will cause human illness, therefore developing a rapid and highly sensitive method for identification of non-O157 STEC capable of pathogenesis would be of great benefit for regulatory agencies as well as in clinical medicine (Bettelheim, 2007).

We have developed a 7-plex microbead-based immunoassay to allow simultaneous serotyping of STEC strains belonging to serogroups O26, O45, O103, O111, O121, O145, and O157 by the Luminex xMAP® technology. Polyclonal antibodies that bind each bacterial serogroup were conjugated to microbeads used by the Luminex platform, thus allowing the identification of specific pathogenic bacteria. We tested the specificity

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of our 7-plex microbead-based immunoassay against a collection of 79 PCR typed *E. coli* strains belonging to the top 7 *E. coli*. Such an immunoassay presents significant advantages. The 96-well format allows high throughput, while the multiplexed format (up to 100 analytes) saves time, reagents, and test samples. Conventional serotyping methods for identification of non-O157 STECs are reliable forms of classification, but can be laborious as well as time consuming often requiring skilled technicians having access to sufficient high quality H antisera, which are mostly available in a few reference laboratories worldwide (Machado et al., 2000). Many affiliated Food Safety and Inspection Service (FSIS), Food and Drug Administration (FDA), and Food Emergency Response Network (FERN) laboratories currently utilize the Luminex platform for other assays, and our immunoassay would be directly transferable and could be implemented by these laboratories.

2. Materials and methods

2.1. Antibodies

The antibodies used in this study were previously described (Olsson-Allen et al., 2011; Hegde et al., 2012). These were prepared and purified by SDIX using proprietary methods. Several antibodies (goat anti-*E. coli* O26, O45, O103, O111, O121, O145, and O157; rabbit anti-*E. coli* O26, O45, O103, O111, O121, O145, and O157; and mouse anti-*E. coli* O157) were screened against one representative strain of each serogroup by indirect then by microplate sandwich ELISA, using a checkerboard strategy to determine the optimized concentrations of both capture and detector antibodies (data not shown). Except for O45, O145 and O157, the capture antibodies with the highest signal to noise ratio were from goats (data not shown). The best capture antibodies for O45 and O145 were from rabbit samples, and the ones chosen for O157 were of murine origin. Except for the capture antibodies for O157 which were used at a concentration of 50 μg/mL, all antibodies were used at a concentration of 5 μg/mL. Except for O157, the same antibodies were used for both capture and detection. The best detection antibodies for O157 were from goat samples. All detection antibodies were used at concentrations of 1 μg/mL. Detector antibodies were biotinylated with the EZ-Link Sulfo-NHS-Long Chain-Biotin kit (Pierce, Rockford, IL), according to the manufacturer's instructions.

2.2. Analytes

E. coli strains (Table 1) were inoculated in 5 mL of brain heart infusion broth (BHIB; Oxoid, Ltd., Basingstoke, UK). Samples were then incubated overnight (15 h) in a shaking incubator (100 rpm) at 37 °C. After enrichment, the samples were diluted to an OD_{600} of 0.01 in PBS (pH 7.4). Samples were tested by our 7-plex microbead-based immunoassay using the Luminex xMAP® technology (q.v.). The STEC strains used in this study were human and environmental isolates including the seven serogroups more commonly associated with outbreak of human illnesses. The serogroups of the strains used were previously confirmed by PCR (Lin et al., 2011). Appropriate Biosafety Level 2 precautions were used when handling pathogenic *E. coli* organisms.

2.3. Buffers and reagents

Phosphate-buffered saline (PBS) with a pH of 7.4 was used to prepare PBS-TB (PBS, 0.02% Tween 20, and 1% bovine serum albumin) and PBS-T (PBS and 0.1% Tween 20) that were used for washes. Reagent-grade chemicals (i.e., 2,29-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [ABTS], H_2O_2, KCl, Na_3 citrate, NaCl, and Tween 20) were purchased from Sigma Chemical Co. (St Louis, MO); immunoglobulin (Ig) G-free, protease-free bovine serum albumin from Jackson ImmunoResearch (West Grove, PA); streptavidin labeled with R-phycoerythrin from Invitrogen (Carlsbad, CA); and streptavidin-horseradish peroxidase conjugate from Zymed (San Francisco, CA).

Table 1
STEC strains tested by the 7-plex microbead-based immunoassay.

O serogroup	Strain identification	O serogroup	Strain identification	O serogroup	Strain identification
O26	H19[a]	O111	CL-37[a]	O157	5343[c]
	DEC10B[a]		DEC8B[a]		5354[c]
	DEC10C[a]		3007-85[a]		5618[c]
	DEC9F[a]		TB226A[a]		5718[c]
	TB285C[a]		928/91[a]		6411[c]
	VP30[a]		412/55[a]		6418[c]
	TB285A[a]		DEC8C[a]		6448[c]
	TB352A[a]		C412[a]		6515[c]
	EK29[a]		BCL19[a]		6577[c]
	97-3250[a]		ED-31[a]		6613[c]
O45	TWO7947[a]		BCL17[a]		6633[c]
	B8026-C1[a]		EK35[a]		6646[c]
	B8227-C8[a]		3215-99[a]		
	08-00017[b]		DA-18[a]		
	D88-28058[a]		88-4110H[a]		
	DEC11C[a]	O121	DA-69[a]		
	5431-72[a]		MT42[a]		
	4309-65[a]		3-524[a]		
	2566-58[a]		F6173[a]		
	B8227-C8[a]		DA-1[a]		
O103	TB154A[a]		DA-5[a]		
	87-2931[a]		DA-37[a]		
	MT#82[a]		MT#2[a]		
	MT#80[a]		87-2914[a]		
	EK30[a]		MT#11[a]		
	EK31[a]		MT#22[a]		
	EK32[a]	O145	GS-G5578620[a]		
	109-494[a]		IHIT0304[a]		
	107-226[a]		TB269C[a]		
	PMK5[a]		MT#66[a]		
	RW1372[a]		BCL73[a]		
	RW1374[a]		IH 16[a]		
	DA40[a]		02-3422[a]		
	D55[a]				

[a] Strains obtained from the STEC center at Michigan State University's National Food Safety and Toxicology Center, East Lansing, MI.
[b] Strain obtained from the Ohio State Department of Agriculture, Reynoldsburg, OH.
[c] Strains obtained from the Orange County Public Health Laboratory, Santa Ana, CA.

2.4. Indirect ELISA assay protocol

A representative strain of each *E. coli* serogroup was diluted to an OD_{600} of 0.01 in PBS (pH 7.4). The wells of a 96-well plate (Nunc, Rochester, NY) were coated with 100 μL of the diluted bacteria and incubated overnight at 4 °C. After removing the bacterial solution, the plates were washed three times with PBS-T and blocked with 200 μL of PBS-TB for 1 h at room temperature. After three washes with PBS-T, 100 μL of antibody solutions was added to the plate and incubated for 1 h at room temperature. After three washes with PBS-T, 100 μL of a 1/500 dilution of HRP-conjugated goat anti-rabbit, rabbit anti-goat, or rabbit anti-mouse polyclonal was added to each well and incubated for 1 h at room temperature. After three washes with PBS-T, 100 μL of ABTS (2,29-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma Chemical Co., St Louis, MO)-horseradish peroxidase substrate solution (Zymed, San Francisco, CA)) was added to each well to detect bound, conjugated antibodies. This substrate solution (pH 4.2) was made of 0.548 g/L of ABTS and 17.64 g/L of sodium citrate (Sigma Chemical Co.). A total of 40 μL of 1 M H_2O_2 was then added to 6 mL of ABTS citrate. After 15 min of incubation at room temperature, absorbance was read at 405 nm with the Wallac Victor 2 Multilabel Counter (PerkinElmer, Inc., Waltham, MA).

2.5. Sandwich ELISA assay protocol

Selected antibodies were previously tested by sandwich ELISA to optimize the assay conditions (i.e., concentrations of capture and detector antibodies) prior to incorporating those into a 7-plex microbead-based immunoassay on the Luminex platform for further specificity testing

(Bernard et al., 2012). A checkerboard analysis was conducted to assess which concentrations of capture and detector antibodies worked best.

One hundred microliters of a solution in PBS (pH 7.4) of capture antibodies (i.e., ranging from 0.5 to 2.0 μg/mL) was added to each well of a 96-well plate (Nunc, Rochester, NY) and incubated overnight at 4 °C. After removing the capture antibody solution, the plates were then blocked with 200 μL of PBS-TB for 1 h at room temperature. After three washes with PBS-T, 100 μL of analyte solutions was added to the plate and incubated for 1 h at room temperature. After three washes with PBS-T, 100 μL of a 0.5-mg/mL solution in PBS pH 7.4 of detector antibody (i.e., ranging from 0.125 to 2.0 μg/mL) was added to each well and incubated for 1 h at room temperature. After three washes with PBS-T, 100 μL of a 1/5000 dilution of streptavidin-horseradish peroxidase conjugate in PBS-TB was added to each well and incubated for 1 h at room temperature. After three washes with PBS-T, 100 μL of ABTS-horseradish peroxidase substrate solution was added to each well to detect bound, conjugated antibody. After 15 min of incubation at room temperature, absorbance was read at 405 nm with the Wallac Victor 2 Multilabel Counter (PerkinElmer, Inc., Waltham, MA). The optimal antigen, capture, and detector antibody concentrations were determined by serial dilutions. For all the pairs of antibodies tested, using the capture antibodies at a concentration of 1 μg/mL and detector antibodies at a concentration of 0.5 μg/mL worked best (data not shown).

2.6. Covalent coupling of antibodies to carboxylated magnetic

Seven different sets of spectrally unique carboxylated Magplex superparamagnetic microbeads (6.5 mm in diameter; Luminex Corp., Austin, TX) were coated with capture antibodies according to the manufacturer's instructions provided with the Bio-Plex Amine Coupling Kit (Bio-Rad, Hercules, CA). Bead region numbers 35, 51, 52, 53, 54, 55, and 56 were used to couple the capture antibodies for O157, O26, O45, O103, O111, O121, and O145, respectively. Briefly, each antibody was covalently coupled to a unique set of microbeads (1.25×10^6 beads per 100 μL coupling reaction) in a two-step carbodiimide-coupling protocol that utilized 50 mg/mL N-hydroxysulfosuccinimide sodium salt (Pierce Chemicals, Rockford, IL) and 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce Chemicals). After each reaction, the concentration of the microbead suspension was determined by a hemacytometer, and the coupled microbeads stored at 2 to 8 °C in the dark. Each coupling reaction was confirmed with phycoerythrin-labeled specie anti-species IgG antibodies (Invitrogen) at concentrations ranging from 0.5 to 32 μg/mL (Fig. 1).

2.7. 7-Plex microbead-based immunoassay protocol

The 7-plex microbead-based immunoassays were conducted in black, 96-well round bottom polystyrene microplates (Corning Costar, Lowell, MA), according to Luminex protocols (www.luminexcorp.com). All incubations were for 1 h at room temperature, protected from light, and shaken at 600 rpm. In brief, a 100-μl aliquot of each test sample (bacteria diluted in PBS to OD_{600} 0.01) was combined with 5000 microbeads (previously blocked for 30 min in PBS-TB) of each of the seven specificities: *E. coli* O26, O45, O103, O111, O121, O145, and O157 in a single microplate well. The microbeads were washed from unbound reaction components three times with PBS-TB using the Bio-Plex Pro Wash Station (Bio-Rad). Microbeads were then resuspended in 100 μL of the biotinylated detector antibody cocktail, containing 1 μg/mL of each antibody and the resultant mixture was incubated. The mixture was then washed three times as described earlier, resuspended in 100 μL of 4 μg/mL streptavidin labeled with R-phycoerythrin, and the resultant mixture was incubated, washed, and resuspended in 100 μL of PBS. The samples were analyzed by Bio-Plex Manager 6.0 standard software (Bio-Rad) with the Luminex 100 flow analyzer. Data were acquired using the following settings: 50 μL sample volume, 100 beads per region, 120 s reading, and DD gate 5000–25,000.

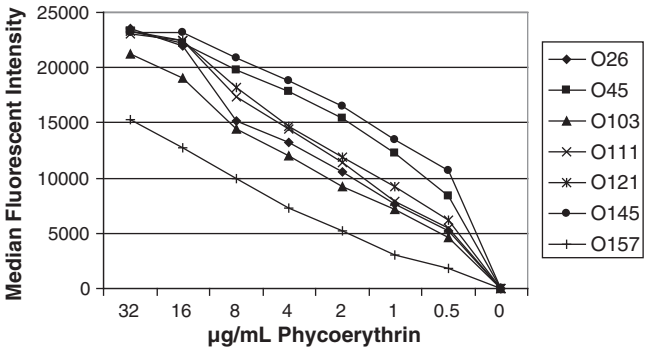

Fig. 1. Confirmation of antibody coupling to the Luminex microbeads using phycoerythrin (PE)-labeled anti-species.

The background fluorescence (i.e., blank samples) was measured on samples containing all immunoassay reagents except the analyte(s) of interest. In fact, every microbead has an inherent fluorescence because of the two fluorochromes embedded in each microbead.

2.8. Statistical analysis

For statistical analysis, the threshold for a positive result was defined as a net Median Fluorescent Intensity (MFI) value that was at least three times greater than the background MFI.

3. Results

3.1. Antibody coupling to microbeads

Once conditions were optimized by sandwich ELISA, the surface area of a microplate well was compared to the surface area of a microbead in order to calculate the amount of capture antibodies necessary for coupling. After coupling we used PE-labeled anti-species antibodies (in a single-plex format) to confirm that the antibodies had been coupled to the microbeads successfully. In this immunoassay, the background wells contained the beads, but not the anti-species-PE (i.e., MFI<30). In Fig. 1, the high MFI values, ranging from 15,338 for O157 to 23,532.5 for O26, indicated that the antibodies were successfully coupled to the beads.

3.2. 7-Plex microbead-based immunoassay specificity

The specificity of the 7-plex microbead-based immunoassay was tested by examining 79 STEC strains listed in Table 1, representing the seven O serogroups included in the assay. The resulting signal to background ratios is shown in Table 2 with positive samples and standard deviations indicated in bold. Except for one strain belonging to the O111 serogroup (i.e., BCL-19), all others were correctly identified. Thus the recovery of STEC by our newly developed 7-plex microbead-based immunoassay was 98.7%.

4. Discussion

A recent study by Verstraete et al. (2012) evaluated a method for the isolation of non-O157 STEC and sorbitol fermenting (SF) *E. coli* O157 in food (Possé et al., 2008), where samples were also submitted to a rapid predictive screening test for virulence genes (*stx*, *eae*, and *ehx*A [enterohemolysin]). The non-O157 STEC serotypes studied included O26, O103, O111, O145, non-sorbitol fermenting (NSF) O157, and SF O157. Food enrichments (i.e., minced beef, raw-milk cheese, and sprouted seeds) were inoculated with low numbers of bacterial cells of above mentioned serotypes. Bacterial cells were either non-stressed, cold stressed or freeze stressed. Of the analyzed serotypes only O111 and SF O157 strains resulted in an increased number of false negatives.

Table 2
Ranges of the signal to background ratio of O serogroup specific antibodies.

Target analyte	O serogroup specific antibodies						
	O26	O45	O103	O111	O121	O145	O157
O26	**26.3–56.6**	0.3–0.6	0.6–0.7	0.4–0.5	0.8–1.1	0.5–0.6	0.5–1.0
O45	0.8–1.1	**21.8–55.0**	0.9–1.0	0.9–1.1	0.7–0.9	0.9–1.0	0.8–1.3
O103	0.2–1.3	0.3–1.0	**6.9–54.4**	0.3–1.1	0.4–1.0	0.3–1.0	0.5–1.1
O111	0.9–1.1	0.8–1.1	0.8–1.0	**57.5–60.6**[a]	0.8–1.1	0.9–1.1	0.9–1.1
O121	0.7–1.2	0.8–1.0	0.5–1.1	0.7–1.1	**11.6–51.8**	0.8–1.1	0.9–1.1
O145	0.4–0.7	0.3–0.8	0.5–0.7	0.4–0.7	0.7–1.0	**3.8–61.8**	0.6–0.8
O157	0.4–0.6	0.3–0.5	0.6–0.8	0.4–0.7	0.9–1.3	0.5–0.8	**49.6–53.2**

[a] The strain that could not be correctly identified (MFI = 1.0 = background) was excluded from the range.

The false negatives could possibly be due to the stressed state of cells. Typically, stressed cells endure a longer lag-phase before exponential growth takes place (Jasson et al., 2007), therefore resulting in decreased growth in broth culture after an overnight incubation. In addition it is possible that certain serotypes grow more efficiently in some broths than others, and selective components may reduce the growth of target strains, as could be the case for O111 serogroup (Verstraete et al., 2012).

Classification of bacteria is typically done in terms of repeating chains of their O-antigen, capsular composition, and flagella antigens (Hitchcock et al., 1986). An important observation is that serotype classification involves the type, but not the number, of repeating O-antigen units. This allows for a level of enhanced variation within a strain of bacteria (Strauss et al., 2009). Strauss et al. (2009) conducted a study in which 5 *E. coli* strains that expressed O-antigen were compared to one another in terms of LPS length by atomic force microscopy. The strains compared in this study were *E. coli* O113:H4, O113:H21, O157:H7, O157:H12, and O157:H16. Out of these strains O113:H21 demonstrated a significantly greater LPS length compared to all other strains while O157:H7 exhibited the second greatest LPS length. This indicates that O-antigen exhibits great variability, and suggests a means for enhanced attachment of *E. coli* O113 and O157 to human intestinal cells and food surfaces, such as fresh produce (Manges et al., 2001). Such variation also explains why the MFI signals in our assay vary from strain to strain, even among a single serogroup.

Future directions include transferring the assay to the new, portable MagPix system. This would ultimately give us a portable, reproducible, and very sensitive assay that is capable of serotyping the top 7 *E. coli*. We also plan on developing monoclonal antibodies for those targets, and adding more antibodies to our 7-plex microbead-based immunoassay such as *E. coli* O91, O104, and O113 as well as other outer membrane proteins eliciting pathogenicity by adhering bacteria onto intestinal epithelial cells. Ultimately we would like to perform our microbead-based immunoassay on the KingFisher automated high throughput platform and test it in food enrichments.

Disclaimer

The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

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