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Rapid Detection of the Top Six Non-O157 Shiga Toxin-Producing *Escherichia coli* O Groups in Ground Beef by Flow Cytometry

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Running title: Detecting top six non-O157 *E. coli* by flow cytometry

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Rapid, sensitive, and highly specific flow cytometric assays were developed for the detection of the top six non-O157 STEC O groups in ground beef. The analytical sensitivity of the assays was $2x10^3$ target cells in a bacterial mixture of 10^5 CFU/ml, and the limit of detection in ground beef was 1-10 CFU following 8 h enrichment. The assays may be utilized for rapid detection of STEC O groups in meat.

Recently the Food Safety and Inspection Services declared six non-O157 Shiga toxin-producing *E. coli* (STEC) O groups (O26, O45, O103, O111, O121, and O145) as adulterants in meat. These top six STEC O groups were found to be associated with 83% of human infections (8). Current methods for detecting O groups by serotyping are labor and resource intensive, and can take from 5 to 9 days to complete. Recently, we and others have developed PCR methods for the identification of STEC O groups (1, 4, 9). Flow cytometry is one of the emerging techniques that may be exploited for rapid identification of *E. coli* serogroups for food safety, public health, medical diagnosis and environmental monitoring (3, 5, 7, 10). The objective of the present study was to develop flow cytometric assays for detecting the six major non-O157 STEC O groups that can be easily adopted for food safety testing.

Polyclonal antibodies were raised in rabbits against heat-killed, whole cell preparations of reference *E. coli* strains belonging to serogroups O26, O45, O103, O111, O121, and O145 and were further purified by SDIX (Newark, DE). Specificities of the antibodies were tested by agglutination assays against reference strains belonging to serogroups O1 through O181, except O31, O47, O67, O72, O93, O94, and O122 that are not designated, ten clinical isolates belonging to each of the top six STEC serogroups (n=60) and other bacterial species, including *Citrobacter freundii, Enterobacter cloacae, Hafnia alvei, Klebsiella pneumoniae, Proteus vulgaris, Salmonella enterica* serovars Enteritidis and Typhi, *Serratia marcescens, Shigella boydii,* and *Shigella flexneri.*

Ground beef (10% fat) samples purchased from a local store were spiked with reference strains belonging to top six STEC O groups, individually or with a mixture of all six strains, in duplicates at 1-10 CFU per strain. The ground beef samples (25g) were enriched in 225 ml Tryptic Soy Broth (TSB) containing vancomycin (16 mg/L), bile salts (1.5 g/ml), rifampicin (2

mg/L), and potassium tellurite (1 mg/L) as earlier reported (4, 6, 9). All samples were incubated at 37ºC for initial 4 h pre-enrichment followed by incubation at 42ºC for a total of 12 h enrichment. During enrichment, samples were collected at different time points (6, 8, and 12 h) and passed through filter paper to remove debris. Samples (1 ml) were centrifuged at 6000x *g* for 10 min and the cell pellets were washed once with 1 ml of Phosphate Buffered Saline (PBS) and resuspended in PBS (200 µl) for staining with respective labeled antibody, prepared as described below. Total numbers of bacterial cells in enriched samples were calculated by aerobic plate count (APC) method. Un-inoculated ground beef samples, enriched similarly, served as negative controls. Experiments were performed in duplicates and repeated three times.

Purified antibodies raised against all six STEC O groups were labeled using Zenon Rabbit IgG labeling kit (Molecular Probes, USA). Antibodies (1µg) were mixed with PBS (10 µl) and 5 µl of Zenon rabbit IgG labeling reagent (Alexa Fluor® 488) and incubated for 5 min at room temperature (RT). Zenon blocking reagent (5 µl) was added to the mixture and incubated for an additional 5 min. The labeled antibodies were mixed with enriched bacterial cells (200 µl) and incubated for 1 h at RT. They were washed three times with PBS (1 ml), resuspended in PBS (0.5 ml) and analyzed in a flow cytometer.

Cytometric analysis was performed on a Beckman Coulter FC500 flow cytometer equipped with an Argon ion blue 488 nm laser and a HeNe red 633 nm laser, each with 20 mW output. The instrument resolves 0.5 µm particles from background. Events (100,000) from the labeled bacterial cell suspension were analyzed with forward scatter discriminator set at 5. Bacterial cells were gated on the basis of forward versus side scatter profile, with typically >99% of all events being classified as bacterial cells. Listmode data files were collected using CXP software and analyzed using FlowJo version 7.6.5 (Tree Star, Inc., Ashland, Oregon).

Excellent correlation was observed between the percent of fluorophore labeled cells as measured by flow cytometry and the number of bacterial cells as determined by APC for all six serogroups (R2= 0.9809) (Fig. 1). The background flora in enriched cultures were low and the growth rates of target bacteria varied, with O103 and O45 strains showing faster growth than the others (data not shown). The flow cytometric assays could detect all six serogroups when spiked individually (Fig. 2) or in the mixture of strains belonging to all six O groups (data not presented) following 8 h enrichment. The flow cytometric assay could detect target serogroup unequivocally at $2x10^3$ cells without any cross reaction. Because of higher growth rate, 6 h enrichment was good for detecting strains belonging to O45 and O103 by flow cytometry, however, 8-12 h enrichment was required to distinguish all the six O groups by this method. At 12 h enrichment the target serogroup represented >15% of cells in the enriched culture. The limit of detection was established to be 1-10 CFU for the targeted O group in ground beef following 8-12 h enrichment.

Specificity of the antibodies against each O group was determined against reference strains for all other O serogroups and bacteria listed by agglutination reactions (2). There was no cross-reactivity of the top six STEC O groups with other O serogroups or bacterial species tested. Flow cytometer may be utilized for rapid detection of six non-O157 STEC O groups in conjunction with PCR assays for Shiga toxins and intimin genes for food testing and clinical diagnosis.

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Figure 1. Relationship between percent of fluorophore labeled cells detected by flow cytometry and cell number determined by aerobic plate count method.

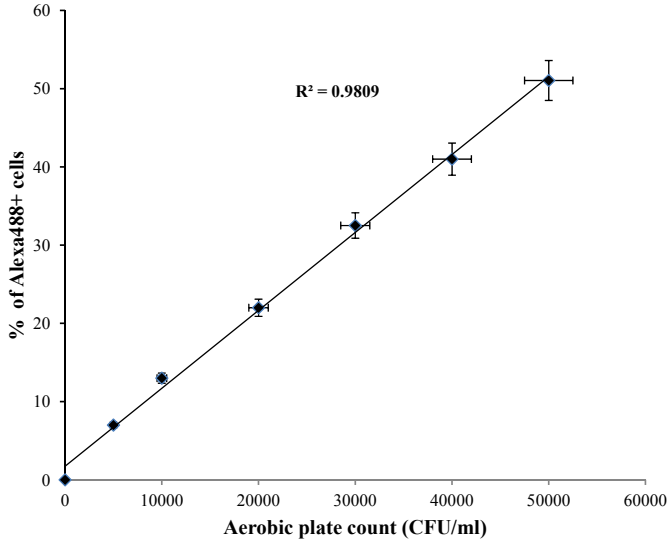

Figure 2. Detection of STEC O groups by flow cytometry in artificially inoculated ground beef. Individually spiked (1-10 CFU) top six serogroups in ground beef detected after 8 hour enrichment. Cells above horizontal bar represents % Alexa Fluor® 488+ cells for100, 000 events.