1 Title: Purification and characterization of lipopolysaccharides from six strains of
2 non-O157 Shiga toxin-producing *Escherichia coli*

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Abstract:

Certain Shiga toxin-producing *Escherichia coli* (STEC) are virulent human pathogens that are most often acquired through contaminated food. The United States Department of Agriculture, Food Safety and Inspection Service has declared several serogroups of STEC as adulterants in non-intact raw beef products. Hence, sensitive and specific tests for the detection of these STEC are a necessity for implementation in food safety programs. *E. coli* serogroups are identified by their respective O-antigen moiety on the lipopolysaccharide (LPS) macromolecule. We propose that the development of O-antigen-specific immunological assays can facilitate simple and rapid discriminatory detection of STEC in beef. However, the resources (antigens and antibodies) required for such development are not readily available. To overcome this, we extracted and characterized LPS and O-antigen from six STEC strains. Using hot phenol extraction, we isolated the LPS component from each strain and purified it using a series of steps to eliminate proteins, nucleic acids, and lipid A antigens. Antigens and crude LPS extracts were characterized using gel electrophoresis, immunoblotting, and modified Western blotting with commercially available antibodies, thus assessing the serogroup specificity and sensitivity of available ligands as well. The results indicate that, while many commercially available antibodies bind LPS, their activities and specificities are highly variable, and often not as specific as those required for serogroup discrimination. This variability could be minimized by the production of antibodies specific for the O-antigen. Additionally, the antigens generated from this study provide a source of characterized LPS and O-antigen standards for six serogroups of STEC.

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48 **Keywords:** antibody specificity, *E. coli*, lipopolysaccharide (LPS), O-antigen (O-ag),
49 serotyping, Shiga toxin-producing *E. coli* (STEC)

50

51 **Abbreviations:** Shiga toxin-producing *Escherichia coli* (STEC), lipopolysaccharide
52 (LPS), enzyme-linked immunosorbent assay (ELISA), O-antigen (O-ag)

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1. Introduction

Certain STEC are virulent human foodborne pathogens that are most often acquired through contaminated food. These organisms are the primary cause of hemolytic-uremic syndrome in children, and a leading cause of gastroenteritis worldwide (Quiñones and Swimley, 2011). In 2012, the United States Department of Agriculture, Food Safety and Inspection Service began testing beef manufacturing trimmings for six serogroups of non-O157 STEC, in addition to STEC O157:H7. All seven serogroups have been declared adulterants in raw, non-intact beef (USDA-FSIS, 2012), and the six non-O157 serogroups include O26, O45, O103, O111, O121, and O145. Additionally, the newly emergent O104:H4 strain has raised concerns about additional STEC serogroups contaminating food.

The emergence of STEC O157:H7 in the 1980's led to the development of a number of commercially available reagents and detection methods for this organism and more recently, several have become available for non-O157 STEC as well. These include differential agar for isolation of bacteria (Church et al., 2007; March and Ratnam, 1986); latex agglutination for O-group testing of isolates (Karmali et al., 1999); immunomagnetic separation for targeted enrichment in broth culture (Drysedale et al., 2004; Fu et al., 2005; Varela-Hernández et al., 2007); enzyme-linked immunosorbent assay (ELISA) for detection of target antigens (Fach et al., 2003; Ge et al., 2002; Hegde et al., 2012); and polymerase chain reaction (PCR) for detection of virulence factor genes, including targeted amplification of the O-antigen gene (Bai et al., 2012; Blanco et al., 2004; Settanni and Corsetti, 2007; Varela-Hernández et al., 2007). In general, the reagents and detection methods for STEC O157:H7 are

relatively sensitive and specific, but in contrast, those for non-O157 STEC are not (Carroll et al., 2003; Gerritzen et al., 2011; Hegde et al., 2012; Orskov et al., 1977). Cross-reactivity, batch-to-batch variability, excessive cost, limited shelf life, and lag time to available results are some of the immediate issues associated with such assays. PCR assays, while very sensitive, are laboratory intensive, may detect residual nucleic acid contaminants, and cannot discriminate whether target genes originated from one or more bacterial cells or types. All of the above are factors that can potentially lead to false positive results.

Historically, identification of serotype has been an important part of the *E. coli* diagnostic repertoire, and is part of the requirement for identification of STEC as adulterants. Serotyping is performed by identifying the unique chemical structures of O-, K-, and H- antigens, and combinations thereof present on the bacterial cell surface (Orskov et al., 1977; Wang et al., 2010). Due to the frequent problems associated with their identification, K-antigen testing is now only infrequently done. Current procedures primarily focus on identification of the O- and H- antigens or their associated genes. The detection of adulterant STEC in food products and distinguishing them from non-pathogenic species is problematic. Further, the number of existing O-, H-, and K- antigens is estimated to be 180, 60, and 80 respectively, resulting in hundreds of serotypes of *E. coli* (Eisenstein et al., 2000; Griffin and Tauxe, 1991; Johnson et al., 1996; Stenutz et al., 2006), more than 200 of which are known to possess Shiga toxin (Kaper et al., 2004).

Lipopolysaccharide (LPS) makes up approximately 70% of the outer membrane of Gram-negative bacteria (Nikaido, 2003; Raetz et al., 2008; Whitfield, 1995). It is a

110 virulence factor produced by several bacteria including *E. coli* (Alexander and
111 Rietschel, 2001; Raetz et al., 2002) and released by the bacterial cells during an
112 infection. The LPS molecules of various pathogens are structurally different. The
113 contribution of LPS to virulence, and the differences in this biomarker between
114 bacterial species contributes, not only to pathogenesis, but also makes LPS an ideal
115 target for pathogen detection (Bosshart and Heinzelmann, 2007) and serogroup
116 identification. The biochemical differences in LPS between different bacteria are
117 attributable to three major components of the LPS macromolecule (Whitfield, 1995).
118 Specifically, the molecule contains a highly conserved lipid A group attached to a
119 core polysaccharide and a hypervariable O-antigen (O-ag) polysaccharide chain
120 (Raetz et al., 2008).

121 Since many structural elements of LPS are largely conserved between strains of
122 *E. coli*, antibodies directed against the intact macromolecule exhibit cross-reactivity
123 between different serogroups of STEC. Serogroups of LPS primarily differ in the
124 chemical nature of the O-ag, while the other components remain relatively
125 conserved (Meredith et al., 2006; Whitfield, 1995). Thus, to ensure serogroup
126 discrimination, it is important that antibodies are targeted against the variable O-ag
127 of LPS, and not the conserved regions of the molecule. It naturally follows that some
128 research groups have developed antibodies against the specific O-ag epitopes of LPS
129 (Clark et al., 2006; Westerman et al., 1997) to facilitate serogroup-specific detection
130 of *E. coli*. However, in the absence of purified and well-characterized STEC LPS from
131 different serogroups, the evaluation of these antibodies is difficult. To overcome this
132 limitation, the objective of this work was to isolate and purify the O-ag from strains

of non-O157 STEC. These antigens can also be used to develop targeted and specific monoclonal antibodies, and consequently discriminatory detection assays for specific serogroups of STEC LPS. Isolation and characterization of LPS and O-ag from non-O157 STEC, including a comprehensive evaluation of commercially available antibodies against the same is presented herein.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and reagents were obtained from ThermoFisher Scientific, Inc. STEC strains DEC10B (O26:H11), B8227-C8 (O45:H2), MT#80 (O103:H2), 0201 9611 (O111:H11), and GS G5578620 (O145:NM) were obtained from Dr. Shannon Manning (STEC Center, Michigan State University). Serogroup was confirmed using latex agglutination kits (Abraxis) and PCR according to Bai et al. (Bai et al., 2012). TY-2482 (O104:H4) was obtained from Dr. John Luchansky (USDA, Agricultural Research Service, Eastern Regional Research Center) and serogroup was confirmed by PCR as previously described (Paddock et al., 2013). MDCH-4 (O113:H21) was obtained from the STEC Center at Michigan State University and serogroup was confirmed by PCR according to Bai et al. and subsequently retyped at the *E. coli* Reference Center at Pennsylvania State University (Bai et al., 2012; Son et. al, 2014). Twelve separate antibodies were used for characterization studies, and the identifying numbers used throughout this paper, as well as supplier information are listed in Table 1. LPS O111:B4 phenol extract, cetyltrimethylammonium bromide (CTAB), phenol (purified by re-

distillation), eosin B, acetic acid, and bovine serum albumin (BSA) were from Sigma-Aldrich. Precision Plus Dual Xtra Protein Standards, 40% acrylamide solution (19:1), polyvinylidene difluoride (PVDF) membrane, and nitrocellulose 0.2 μ m membrane, were from BioRad Laboratories. Regenerated cellulose dialysis tubing (MWCO 12-14000) was from VWR Scientific, LPS 0157:H7 was obtained from List Biological Labs, and sheep blood agar plates were from Remel. Nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (NBT-BCIP) came from Pierce-Thermo Scientific, and Trypticase soy broth (TSB), amino methylpersulfate (APS), and tetramethylethylenediamine (TEMED) were from ThermoFisher Scientific, Inc. FreeZone6 Freeze Dry System and accompanying freeze drying flasks and accessories were from LabConco. Water unless specified otherwise was 18.2 M Ω -cm, filtered through a 0.22 μ m membrane from a Barnstead™ Nanopure™ system.

Table 1.

Commercial Antibodies

Antibody # ^a	Antibody	Description	Source
1	Lipid A - 0157	polyclonal	Pierce/PA1-73178
2	LPS - 0157	polyclonal	LSBio/LS-C71709
3	<i>E. coli</i> 'O' & 'K'	polyclonal	Novus Biologicals/NB200-
4	<i>E. coli</i> LPS	polyclonal	Bioss/BS-2351R
5	O104	polyclonal	Abraxis/40120
6	O26	monoclonal	Abraxis/410000
7	O45	monoclonal	Abraxis/410010
8	O103	monoclonal	Abraxis/410020
9	O111	monoclonal	Abraxis/410030
10	O145	monoclonal	Abraxis/410050
11	O157	monoclonal	AbCam/ab75244
12	O121	monoclonal	Abraxis/410040
13	Anti-Goat AP	secondary	Thermo Scientific/31300
14	Anti-Rabbit AP	secondary	Pierce/31340
15	Anti-Mouse AP	secondary	Dako D0486

^a Numbers 1-5 indicate polyclonal antibodies and numbers 6-12 indicate monoclonal antibodies. Numbers 13-15 are secondary antibodies labeled with alkaline phosphatase (AP).

2.2. Growth of STEC

Pure cultures from each STEC strain were streaked for isolation onto sheep blood agar plates. Isolated colonies were inoculated into 20 mL of TSB (125 mL flasks) and incubated on a shaking incubator at 220 rpm, 37° C, overnight (approximately 18 h). Broth cultures were swabbed for confluency on 150 mm TSB with agar plates using sterile cotton swabs (K. Jann and Westphal, 1985; Westerman et al., 1997). Plates were allowed to dry for 3 min, then incubated for 24 h at 37° C. Bacterial paste was harvested with a sterile cell scraper, and suspended in water at an estimated 100% w/v ratio. Theoretical bacterial yields for 150 mm plates, based on data previously collected by our lab (Clark et al., 2006), was used to approximate an equivalent w/v ratio of wet bacterial cultures to water (Limjuco et al., 1978). Bacteria were dried under vacuum with -20° C acetone using a Büchner funnel apparatus (K. Jann and Westphal, 1985; Limjuco et al., 1978). Repeat washes were applied, and the bacteria were continually stirred and crushed using a glass rod.

2.3. Phenol extraction

Hot phenol-water extraction, following a modified procedure for high molecular weight polysaccharides was performed as outlined by Jann *et al.* (Clark et al., 2006; Darveau and Hancock, 1983; B. Jann et al., 1975; K. Jann and Westphal, 1985). Dried bacterial cultures were suspended in 68° C water (5% w/v) with stirring for 20 min. Equal volumes of 90% hot phenol, were prepared in water, heated to 68° C, added to the bacterial suspension, and stirred for 20 min at 68° C. The mixture was cooled on

ice to 10° C, and centrifuged at 5,100 x *g*, 10° C for 45 min. The resulting upper aqueous layer was collected and set aside on ice. Remaining organic interphase and phenol layers with bacterial pellet were rewashed with water, using equal v/v ratios. Heating, cooling and centrifugation were repeated and the aqueous layers collected and combined. Aqueous supernatant was dialyzed, in large volumes of water for 72 h (Parolis et al., 1997), with periodic water changes to ensure removal of low molecular weight compounds and phenol. The resulting solution was spun at 5,100 x *g* for 30 min to remove insoluble material. Material was lyophilized at -40° C and 0.133 mBar, until a dry crude extract was obtained.

2.4. Purification of LPS

Crude extract was dissolved in 50° C water (3% w/v) with stirring. Resulting opalescent solution was centrifuged at 109,000 x *g* for 4 h at 4° C. Supernatant was collected and lyophilized. The pellet was re-dissolved in warm water and the process repeated two more times until the supernatant was completely clear. Resulting purified extracts were lyophilized prior to CTAB precipitation.

2.5. Precipitation of nucleic acids from purified extracts

To remove nucleic acids, 4% w/v CTAB was dissolved at room temperature (RT, 25° C) into sterile 0.25 M NaCl solution as described by Jann *et al.* (K. Jann and Westphal, 1985). Lyophilized extracts were dissolved separately in 0.25 M NaCl with stirring, and 25 mL CTAB/NaCl solution was added to precipitate the nucleic acid-CTAB salt (K. Jann and Westphal, 1985). The solution was stirred for 15 min, RT, and centrifuged at 7,197 x *g* for 1 h, RT, to remove the precipitated pellet. Three equal volumes of water were added to the supernatant to form the LPS precipitate.

The mixture was placed on ice for 2 h, then centrifuged at 7,197 x *g*, 4° C for 1 h and supernatant was removed. The white LPS pellet was dissolved in 5 mL of 1.0 M NaCl, and purified LPS was precipitated using 40 mL of 100% ethanol, and repeated cooling and centrifugation (K. Jann and Westphal, 1985). The NaCl and ethanol precipitation was repeated and the ethanol supernatant was discarded. The final pellet was dissolved in 30 mL water and dialyzed (MWCO 12-14000) at 4° C for 24 h in 1.0 L of water with stirring. The resulting solution was then freeze dried to give the final purified LPS product.

2.6. Hydrolysis of lipid A

Lyophilized LPS was dissolved in glass vials using 1.5% acetic acid (Altman et al., 1986; Limjuco et al., 1978). Solution was heated to 100° C using an oil bath, (to adjust for the lower boiling point of water at altitude) for 5 h to separate core polysaccharide from lipid A. Samples were centrifuged for 20 min, 2000 x *g* (Altman et al., 1986; Limjuco et al., 1978; Yi and Hackett, 2000). The supernatant was collected, and lipid A precipitate was rewashed with acetic acid and centrifuged two more times (Limjuco et al., 1978). Acetic acid was removed from samples by rotary evaporation under high vacuum. Evaporated samples were brought up in water (Limjuco et al., 1978; Yi and Hackett, 2000), lyophilized on a Schlenk line and then stored at -20° C until further use.

2.7. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

Large polyacrylamide gels (16 cm x 16 cm) were prepared using a 15% resolving gel (Davies et al., 1994; Kim et al., 1996; Lesse et al., 1990; Palva and Mäkelä, 1980; Sutcliffe, 2000; Westerman et al., 1997). Gel was prepared by mixing a ratio of

15:15:6:4, acrylamide, gel buffer (3.0 M Tris, 0.3% SDS, pH 8.45), water, and glycerol, respectively (Lesse et al., 1990). Polymerization was induced by adding 100 μ L of 10% APS, then 10 μ L TEMED. Two milliliters of ethanol was added to level the air-gel interface, and the gel allowed to cure for 2 h at RT. Ethanol was removed by wicking and a 4% stacking gel was cast 8:1:1 (water, acrylamide, gel buffer) and allowed to cure for 1 h (Lesse et al., 1990). The apparatus was assembled and filled with anode buffer (0.2 M Tris-HCl, pH 8.9) and cathode buffer (0.1 M Tris-HCl, 0.1 M tricine, 0.1% SDS, pH 8.25). LPS extracts were prepared in phosphate buffered saline (PBS) at 5 mg/mL, then diluted to 2.5 mg/mL in Laemmli buffer (Laemmli, 1970), and heated at 96° C for 5 min. Ten μ L of protein standard was loaded into several lanes to monitor potential distortion patterns across the gel. Twenty five micrograms of each LPS sample was loaded, and the gel was run for 1.5 h to allow samples to enter the resolving matrix (BioRad, 2012; Lesse et al., 1990). Voltage was increased to 90 V, and optimized to a period of 15 h, after which the gel was removed from the apparatus.

2.8. Inverse staining with eosin B

Gels were removed and fixed with 3 rinses of 400 mL, 50% methanol for 7 min each (Zhu et al., 2012). A solution of 0.4% eosin B and 50% methanol (Zhu et al., 2012) was prepared by first dissolving eosin in tap water and titrating with 1.0 M NaOH until all eosin was in solution. Gels were stained for 40 min, RT, with shaking. 400 mL of 5% acetic acid was used to develop the gels for 2 min. Gels were scanned at 800 dpi using an HP Scanjet 4890, and adjusted for brightness and contrast (Zhu et al., 2012).

2.9. Antibodies and immunoblotting

Several commercially available antibodies (Table 1) were tested against LPS antigens. Antibodies were evaluated for specificity to whole LPS extracts, O-ag, and lipid A subgroups. In each case, nitrocellulose membranes were blotted two times with 2 μ L blots of 5 mg/mL LPS (20 μ g), or concentrated solutions of O-ag, and lipid A. Standard commercial preps of LPS O111:B4 and LPS O157 were used as comparative standards for determining activity. PBS and 5% BSA blots were used as negative controls. Antigen and control blots were allowed to dry and the membrane was blocked with 5% BSA for 2 h, 37° C in a shaking incubator. Membranes were rinsed three times each, first with PBS/0.1% Tween 20 (PBS-T), then with PBS, for 5 min/each, RT, and shaking. Primary antibodies (1-12) were prepared 1:2000 in PBS and incubated with the membranes for 4 h, 37° C. Membranes were rinsed again as previously described. Appropriate secondary antibodies (13-15) were diluted 1:5000 in PBS and incubated for 1 h, RT with shaking, and the membrane was again rinsed as described. NBT-BCIP substrate was added, incubated for 30 min with shaking and rinsed twice with water. Blots were photographed with a Nikon CoolPix P50 against a dark purple background to increase contrast in the photos.

2.10. Western-type blotting of lipopolysaccharides

To further characterize the LPS extracts, we used a Western-type blotting method as an alternative to SDS-PAGE. Alternate lanes were loaded with 25 μ g of LPS, and run as described above. Gels were removed from the electrophoresis apparatus, then equilibrated in Bjerrum Schafer-Nielson buffer (BSN) (48 mM Tris, 39 mM Glycine, pH = 9.2), (BioRad, 2012; Bjerrum and Schafer-Nielson, 1986) for 2

h on an orbital shaker, RT, prior to transfer. PVDF membrane was cut to width of 1.5 lanes and strips were aligned to match up with lanes on the gel. Gels were transferred to PVDF using a Trans-Blot® SD Semi-Dry electrophoretic transfer device (BioRad) in BSN buffer at 24 V for 30 min. PVDF was then rinsed twice in tris-buffered saline (TBS, pH 7.5) for 5 min each on an orbital shaker at RT. Membranes were blocked with 5% milk/TBS for 2 h at 37° C (Westerman et al., 1997), then overnight at 4° C. Membranes were subjected to a rinse cycle of 3 times with TBS + 0.05% Tween 20 for 5 min, and then 3 times with TBS, 5 min. Each strip of PVDF was individually blotted with the respective primary antibody, diluted 1:2000 in 1% milk/TBS (Westerman et al., 1997), and incubated with membranes for 4 h at 37° C with shaking, then rinsed as described. Secondary antibodies were prepared 1:5000 in 1% milk/TBS, and incubated 1 h, RT with shaking. Again PVDF was rinsed 3 times each with TBS-Tween and TBS, then developed with NBT-BCIP substrate for 30 min, RT, shaking. Membranes were then rinsed with water two times for 5 min each and photographed.

2.11. Image processing

Images of Western-type blots and immunoblots were processed using Adobe Photoshop CS5, and gels (Fig. S2) were processed using ImageJ. All images were converted to black and white, then brightness and contrast were optimized and compared to the original images. All attempts were made to conserve the integrity of the original images.

3. Results and Discussion

3.1. Bacterial growth and purification of lipopolysaccharides

The resulting yields from harvest of bacterial strains (Table S1) spanned a range of 11.1 – 53.3 g of dried bacteria. According to previous studies, LPS crude extract should make up 8-10% of dry bacterial weight (Gamian et al., 1992; K. Jann and Westphal, 1985). The crude extracts of only two strains, DEC10B and GS G55078620, fell within the expected range, but the products of B8227-C8 and MDCH-4 exceeded theoretical yields. Calculated yields of extracts from ultracentrifugation were obtained by using a theoretical yield of 200-250 mg LPS per gram of dry bacteria (B. Jann et al., 1975; K. Jann and Westphal, 1985). However, yields of extracts isolated were significantly lower than this predicted range, as were the yields from CTAB precipitation (K. Jann and Westphal, 1985). This was attributed to the low initial low yields from the LPS crude extracts, and also from withholding 100 mg of LPS crude extracts for assay development and antigen characterization. Upon hydrolysis of lipid A, the resulting low amounts of O-ag and lipid A in each vial inhibited our ability to gravimetrically estimate the mass of isolated O-ag and lipid A subgroups.

3.2. Gel electrophoresis

Gel electrophoresis was performed to characterize the relative size of isolated antigens and test for the presence of lipid A contamination in the sample preps. In Fig. S2, lane 3 and 4 show the resulting ladder-like banding patterns of core polysaccharide LPS O157, indicated by black arrows 1 and 2 (Parolis et al., 1997; Lesse et al., 1990; Palva and Mäkelä, 1980). Concentrations of LPS below 2.50 mg/mL were not visible in lanes 5 and 6, due to poor sensitivity of staining methods

(Zhu et al., 2012). Additionally, O-ag and lipid A from LPS O157 were also not visible in lanes 7 and 8. Good visualization of the typical O-ag banding pattern (Palva and Mäkelä, 1980; Rezania et al, 2011) of LPS O111:B4 were visible in lanes 9-11, and very faintly in lane 12, as indicated by arrow 3. Also seen, were strong banding patterns in the core polysaccharide region, indicated by arrow 4. In lanes 9 and 10, there was also a faint band, as indicated by arrow 5 that we identified as lipid A, as it has the lowest molecular weight of the subgroups, and should migrate the furthest on the gel.

3.3. Western-type and immunoblotting

Since we were unable to detect the presence of O-ag and lipid A in our LPS control groups with SDS-PAGE, we began investigating the specificity of antibodies to O-ag and whole LPS to confirm binding activity. Results of the immunoblots are summarized in Table 2, and original pictures of the blots, inclusive of lipid A antigen blots, are shown in Fig. S1 and Table S2. As expected, polyclonal antibodies 1-4 were the most cross-reactive due to the presence of multiple antibody paratopes in polyclonal antibody preparations. These antibodies seemed to have the lowest affinity for antigens from the O104 and O145 groups. As TY-2482 O104:H4 is a newly emergent strain (Muniesa et al., 2012), available polyclonal antibodies against conserved epitopes of LPS would be expected to bind lipid A, or core polysaccharide present in whole LPS O104. This raises the question of whether the molecular structures of LPS O104 are similar to other LPS serogroup counterparts, which nuclear magnetic resonance spectroscopy would be needed to confirm.

Table 2
Immunoblotting antibody activity against LPS antigens

Antigen	ID #	Antibody	Antigen Serogroup								
			0111:B	0157	026	045	0103	0104	0111	0113	0145
LPS O-ag	1	Lipid A - 0157	+	+	+++	+	+++	+	++	++	+
	2	LPS - 0157	++	++	+++	+	++	+	+++	+++	+
	3	<i>E. coli</i> 'O' & 'K'	+	+	+++	+	++	+	++	++	+
	4	<i>E. coli</i> LPS	+	+	+++	+	+++	+	++	++	+
	5	0104	-	-	++	-	++	-	+	+	-
	6	026	+	-	+	+	+	+	+	+	+
	7	045	-	-	-	++	-	-	+	-	-
	8	0103	-	-	-	-	-	-	-	-	-
	9	0111	-	-	-	-	-	-	+++	-	-
	10	0145	-	-	-	-	-	-	-	-	+
	11	0157	-	-	-	-	-	-	-	-	-
	12	0121	-	-	-	-	-	-	-	-	-
Whole LPS	1	Lipid A - 0157	+++	+++	+++	++	++	+	++	-	-
	2	LPS - 0157	+++	+++	+++	++	+++	++	+++	++	-
	3	<i>E. coli</i> 'O' & 'K'	+	++	+++	++	++	+	++	+	+
	4	<i>E. coli</i> LPS	+++	+++	+++	++	++	+	+++	+	+
	5	0104	-	+	++	+	+	+++	-	-	-
	6	026	+++	+	++	+++	+	+++	+++	++	++
	7	045	++	-	+	+++	-	+	+	-	-
	8	0103	-	-	-	-	+	-	-	-	-
	9	0111	++	-	-	-	-	-	+++	-	-
	10	0145	-	-	-	-	-	-	-	-	+++
	11	0157	-	++	-	-	-	-	-	-	-
	12	0121	-	-	-	-	-	-	-	-	-

+++ = intensely positive, ++ = moderately positive, + = weakly positive, - = negative result.

The results of the monoclonal antibodies were highly varied in regards to binding response against the antigens. We note that in all these cases, surfaces developed and optimized for protein estimation (e.g. nitrocellulose, PVDF) are being

utilized for the detection of a lipoglycan antigen, LPS. These methods are therefore, sub-optimal due to the lack of adsorption efficiency demonstrated with LPS (Davies et al., 1991, 1994; Pyle and Schill, 1985). Antibody 6 had the highest degree of non-specificity amongst all of the monoclonal antibodies tested, as it reacted with all groups of whole LPS, many groups of O-ag, and was faintly reactive to some groups of lipid A (Table S2). Of note, antibodies 8 and 10 were exclusively specific to whole LPS O103 and O145, respectively, with no cross-reactive binding to other serogroups. However, no response signal was seen with either O-ag or lipid A, indicating poor sensitivity at the concentration of antigens used here, or that the epitope for this antibody is only present on whole LPS. Additionally, reduced adsorption of the antigens to nitrocellulose as well as conformational presentation to the antibody may negatively affect the binding of the antibody (Davies et al., 1994). Antibody 9 was also highly specific to both O111 serogroups, and reactivity against the commercial O111:B4 whole LPS as well as the extracted O111 O-ag can be seen. Interestingly enough, this antibody did not bind the O-ag or lipid A components of commercial LPS, but did bind these targets when tested with the extracted LPS O111 antigens. As shown in Table 2, Antibody 12 did not react with any of the serogroups, either in the immunoblots or the Western-type blots (data not shown). Originally, the O121:H21 strain tested negative for O121 by latex agglutination, but had an amplicon similar to that of the O121 serogroup by PCR (Bai et al., 2012). As such, the strain was sent to be retested by the *E. coli* Reference Center (Pennsylvania State University) and subsequently identified as O113:H21; a result previously seen by Son et al (Son et al., 2014). Since antibody 12 was not

directed against the LPS antigens tested in this study, we have used it as a negative antibody control. Overall most antibodies exhibited some cross-reactivity indicating the need for more specific reagents for targeting O-ag, in addition to optimized surfaces for LPS detection. To address these issues, our team has been working on the development of targeted assays specifically designed for the detection of amphiphilic moieties like LPS (Mukundan et al., 2012; Sakamuri et al.; 2014).

Due to poor results of our control antigens obtained with eosin staining, we decided to investigate the regions that antibodies would bind to by using a method similar to Western blotting for proteins. Since coating the LPS molecule with SDS does not result in the same effect of charge per mass unit of protein, it does not migrate through the gel based exclusively on size as proteins do (Davies et al., 1994). The molecule, however, has been characterized by many to separate into size discriminate regions when subjected to a high percentage of acrylamide and extended run times (Kim et al., 1996; Lesse et al., 1990; Palva and Mäkelä, 1980; Sutcliffe, 2000; Westerman et al., 1997). Interestingly, in contrast to SDS-PAGE, LPS O111:B4 was not well represented on the blot (Fig. 1) when incubated with antibody 1 (Fig. 1, lane B), indicating perhaps that transfer of the O-ag region of LPS to PVDF membrane was diminished (Pyle and Schill, 1985). However, antibody 1 did bind the antigen in the core polysaccharide region, suggesting that lipid A attached to core polysaccharide was most likely present. The strongest binding responses in the Western-type blot (lane C and G) were seen with antibodies 5 and 11. This contrasted with the immunoblots where a very poor response was recorded with antibody 11 against LPS O-ag, but it did bind specifically to whole LPS O157.

409 Additionally, antibody 5 reacted with whole LPS O104, but not against LPS O-ag
 410 O104.

411
 412 **Fig. 1.** Western-type blotting of LPS purified extracts. Lane A is protein ladder to ensure even migration through
 413 the gel. (B). LPS O111:B4/antibody # 1 (C). LPS O157/# 11 (D). LPS O26/# 6 (E). LPS O45/# 7 (F). LPS O103/#
 414 8. (G). LPS O104/# 5 (H). LPS O111/# 9 (I). LPS O145/# 10.

415
 416 This discrepancy could be due to a variety of factors, the first being the
 417 choice of membrane (nitrocellulose in immunoblotting and PVDF in Western-type
 418 blotting) or blocking agent employed (Davies et al, 1994). Additionally, the effect of
 419 electrophoretic charge separation on the conformation of LPS transferred to
 420 membranes should be considered when sensitivity of antibodies is evaluated
 421 (Davies et al. 1994; Pyle and Schill, 1985). Indeed, antibodies are known to exhibit
 422 differential performance in different assays (e.g. immunohistochemistry, ELISA,
 423 Western blot and others) as often indicated on their product information sheets.
 424 This may also explain the weak binding activity exhibited by antibody 6, (lane D) in

Western-type assays, when compared to the indiscriminate binding seen in immunoblots.

Overall, we see that most antibodies appear to target the core polysaccharide region as demonstrated by bands in lanes, B-E, and arguably lanes G and I. We cannot, however, overrule the occurrence of trace O-ag in these preparations. Of all the antibodies tested, antibodies 8-10 have the most consistent results between immunoblotting and Western-type blotting, with the exception that antibody 8 did not bind O-ag in the dot blots. These studies indicate that some commercial antibodies that are designed to be serogroup specific for different serogroups of STEC demonstrate significant cross-reactivity as evidenced with monoclonal antibodies 6 and 7, as well as polyclonals 1, 2, and 5.

4. Conclusions

The overall goal of this work was to develop methods to contribute to the rapid and discriminatory detection among non-O157 STEC and non-pathogenic *E. coli* in beef products. Serotyping is often performed using antibodies that are not only expensive, but are also poorly effective as they cross-react between multiple serotypes of bacteria. However, this does not mean that antibody detection is a poor method for serotyping. Antibodies with high specificity and affinity for their epitopes can produce very rapid and discriminate results if the cognate antigens are available for screening the antibodies. Unfortunately, in the case of several non-O157 STEC, the O-ag for screening antibodies are not commercially available and must be prepared by extracting and purifying them from bacteria. We have

448 extracted and chemically purified the LPS, lipid A, and O-ags from six non-O157
449 STEC strains and characterized them against 11 commercially available antibodies
450 for cross-reactivity and specificity. These studies demonstrate that while some
451 antibodies are specific and discriminatory, many of them are not, and there is still a
452 need for improvement in monoclonal antibodies against STEC. As such, to our
453 knowledge, this is the first time that these non-O157 STEC antigens have been
454 prepared for the purposes of developing antibodies and detection tools against
455 STEC. Future studies will involve screening monoclonal antibodies against O-ags,
456 which we intend to implement into multiple detection schemes for more rapid and
457 accurate serotyping of STEC.

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