2 Evaluation of ELISA Tests Specific for Shiga Toxin 1 and 2 in 3 Food and Water Samples

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17 **Abstract:** Two enzyme-linked immunosorbent assay (ELISA) kits were evaluated for their
18 effectiveness in detecting and differentiating between Shiga toxin 1 and 2 (Stx1 and Stx2)
19 produced by Shiga toxin-producing *E.coli* (STEC) inoculated into food and water samples.
20 Each kit incorporated monoclonal antibodies previously determined to bind all known Stx1
21 or Stx2 subtypes with the exception of Stx2b. Four different sample types, including
22 ground beef, Romaine lettuce, pond water, and pasteurized milk were inoculated with
23 Stx1a-, Stx2a-, or Stx1a- and Stx2a-producing STEC strains, enriched using modified
24 tryptic soy broth (containing mitomycin C) for 6, 16, and 22 h, and tested using the ELISA
25 kits in the presence of a bacterial protein extraction reagent (B-PER™). The two Shiga
26 toxin types were readily detected and distinguished for all tested sample types. There was
27 good overall sensitivity, specificity, variance, and reproducibility for the two ELISA kits
28 and they should prove useful for application in food testing.

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31 **Keywords:** B-PER™; colorimetric; detection; ELISA; *E. coli*; food; Shiga toxin; STEC;
32 Stx1; Stx2
33

34 1. Introduction

35 Foodborne illnesses continue to be an ongoing concern in the United States, resulting in
36 approximately 9.4 million illnesses, 56,000 hospitalizations, and 1,350 deaths every year, of which
37 Shiga toxin-producing *Escherichia coli* (STEC) are estimated to cause approximately 175,000 of
38 domestic cases annually (Scallan, et al., 2011). Infections may lead to gastrointestinal illness,
39 including hemorrhagic colitis and hemolytic uremic syndrome (Gyles, 2007). The *E. coli* O157
40 serogroup has been the most commonly recognized, but non-O157 STEC cause an estimated twice the
41 number of infections as O157, though the illnesses caused may not be as severe (Luna-Gierke, et al.,
42 2014; Scallan, et al., 2011). Six other STEC serogroups that include O26, O45, O103, O111, O121,
43 and O145, are implicated in the majority of these non-O157 infections (Fratamico, et al., 2011). In
44 addition to serotyping, STEC may be further characterized by their virulence factors, including
45 production of two Shiga cytotoxin types, Stx1 and Stx2 (Gyles, 2007), of which Stx2 has an LD₅₀
46 about 400 times lower than that of Stx1, and they share 56% amino acid sequence homology (Tesh, et
47 al., 1993). Additionally, Stx1 and Stx2 can be differentiated into subtype ss. Stx1 has been shown to
48 have subtypes 1a, 1c, and 1d, while Stx2 has 2a, 2b, 2c, 2d, 2e, 2f, and 2g (Scheutz, et al., 2012).
49 Therefore, rapid, sensitive detection methods that can distinguish between the two Stx types, without
50 missing detection of any of the subtypes, are vital for proper diagnosis and treatment of infected
51 patients.

52 Based on the distinct antigenicity of Stx1 and Stx2 (Nataro & Kaper, 1998), a number of
53 monoclonal and polyclonal type-specific antibodies have been developed. Some of those have been
54 characterized in terms of reactivity and binding specificity (Luz, et al., 2015; Skinner, et al., 2014).
55 There are also a few commercially produced Stx1 or Stx2 antibodies and associated ELISA assay kits,
56 such as ImmunoCard STAT! EHEC (Meridian Bioscience, Cincinnati, OH) and Premier EHEC test
57 (Meridian Bioscience), ProSpecT Shiga toxin *E. coli* (STEC) Microplate Assay (Remel Inc., Lenexa,
58 KS), Ridascreen (r-Biopharm AG, Darmstadt, Germany), Verotoxin Enzyme Immunoassay (r-
59 Biopharm AG), Shiga Toxin Chek T30696 (TECHLAB, Inc., Blacksburg, VA), and VTEC-PRLA
60 “Seiken” (Denka Seiken, Japan). These Stx antibody-based immunoassays are not inclusive for
61 detection of all the subtypes of Stxs produced by different serotypes of STEC, such as Stx2c, Stx2e,
62 Stx2f, and Stx2g (Feng, et al., 2011). This becomes problematic as it may raise questions during
63 analysis, particularly related to detection of unknown STECs in food samples (Willford, Mills, &
64 Goodridge, 2009).

65 Recently, Abraxis, Inc. (Warminster, PA) developed separate enzyme-linked immunosorbent assay
66 (ELISA) kits to differentiate between the Stx1 and Stx2 toxins and detect all subtypes using specific,
67 monoclonal antibodies. The sensitivity and specificity of the antibodies in these kits for 10 subtypes of
68 Stx1 and Stx2 in phosphate buffer and bacterial culture were evaluated in our laboratories (Kong,
69 2016). In that investigation the following number of STEC strains with their associated subtypes were
70 demonstrated to yield correct responses with the new Abraxis ELISA tests for Stx1 and Stx2: Stx1a-
71 18, Stx1d-3, Stx2a-12, Stx2b-1, Stx2c-1, Stx2d-1, Stx2e-2, Stx2f-2, Stx2g-1, Stx1a & Stx2a-5, and
72 Stx1a & Stx2d-1. In addition, the following PCR confirmed for *stx* gene subtype (Scheutz, et al.,
73 2012) strains were also tested (in 3 separate experiments; data not shown) to correctly respond to the

74 two Abraxis ELISA tests: USDA-ARS Eastern Regional Research Center (USDA-ARS-ERRC) in-
75 house STEC strains 1.2673 (O104:H12; Stx1c), 6.0830 (O104:H7; Stx1c), 04-1450 (O113:H21; Stx2c
76 and Stx2d), 85-489 (O91:H21; Stx2d), SJ88 (O63:H6; Stx2f), CB9765 (O63:H6; Stx2f), CB10019
77 (O63:H6; Stx2f), and 7V (O2; Stx2g; Stx2 ELISA positive 2 of 3 tests). Furthermore, non-targets Stx-
78 negative *E. coli* strains including: ATCC25922 and RM7103 (Kong, 2016), 3056-85 (O121:H19;
79 USDA-ARS-ERRC in-house), and RM4876 (O157:H7; watershed isolate; (He, et al., 2011)) also
80 correctly tested negative for both of the two Abraxis ELISA Stx1 and Stx2 tests (data not shown). The
81 purpose of this study was to evaluate the effectiveness of the kits with different sample matrices, by
82 testing four inoculated sample types: ground beef, Romaine lettuce, pond water, and pasteurized milk,
83 all of which have all been associated with non-O157 STEC outbreaks in the United States (Luna-
84 Gierke, et al., 2014). The *E. coli* inoculation strains were selected from among the Big Six non-O157
85 serogroups according to their Stx1 or Stx2 production. The Abraxis kits accurately differentiated
86 between Stx1- and Stx2-positives for all sample types investigated.

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88 2. Results and Discussion

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90 2.1. Preliminary ELISA Detection of Stx in Ground Beef

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92 Initial investigation focused on balancing the concentration of mitomycin C-induced Shiga toxin
93 (Stx) with bacterial growth time, release of any cell-associated Stx using bacterial protein extraction
94 reagent (B-PER™), and the ELISA limit of detection. As with all the food or environmental samples
95 tested, the background flora present in uninoculated samples was determined via total aerobic plate
96 counting (Table 1). The background levels (30-160 CFU/g; 8-100 CFU/mL) were generally low and
97 ultimately did not appear to be the cause of any growth competition for the relatively low inoculum
98 levels of STEC targeted and achieved (0.08-1.5 CFU/g; 0.2-0.96 CFU/mL; Table 1). For the short
99 induction and enrichment time of 6 h, no Stx was detected for any of the ground beef samples
100 inoculated with any of the STEC expressing the selected Stx subtypes—1a, 2a, or 1a, 2a. Hence, it is
101 deduced that not enough Stx was generated and released during 6 h enrichment and induction for
102 detection with this immunoassay. Compared to observations in cursory studies (He, et al., 2016;
103 Kong, 2016), this result suggests that ≤ 10 to 50 pg/mL of Stx was present after 6 h of
104 enrichment/induction. Such a low level of detection would be a prerequisite if testing (including
105 sample preparation, enrichment and detection) is desired to be conducted within an 8 h work shift.

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107 2.2. ELISA Detection of Stx1 and Stx2

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Based on preliminary tests, it was determined that longer, overnight antibiotic induction and growth enrichment would be necessary for reproducible detection of STEC-generated Stx. The food and water samples were inoculated and held at 4°C for 24 h prior to 1:4 dilution in modified tryptone soya broth (mTSB) containing mitomycin C and then subjected to ELISA-based detection using polystyrene 96-well plates precoated with capture antibody followed by enzyme-amplified colorimetric detection in the presence of Stx-releasing B-PER™. Wells containing the Abraxis kit negative control and those containing small amounts (0.05 ng per well) of purified Stx, underwent exposure to all ELISA reagents, and were respectively designated “negative” and “positive” controls. Absorbency responses for assayed samples, tested in duplicate (technical repeats) with all repeats also performed as duplicate, technical repeats as well [the summation of combined replicates was designated “n”], were averaged and standard deviations (STDEV; 99% confidence level) are presented for all the tested samples (ground beef, Romaine lettuce, pond water, milk) employed in this investigation (Tables 2 and 3). The absorbance values were transformed to a semi-quantitative scale ranging from non-detectable (-) to one of four levels of increasing detection response (+ to ++++). The cutoff limit was a conservative S/N (quotient of averaged sample absorbance and averaged negative control) of 4 (6 for lettuce) for both anti-Stx1 and anti-Stx2 testing. In practicality, a lower S/N cutoff factor may be employed, however, it is expected that this value may be unique to the sample matrix. In other words, in food production and environmental testing, it is expected that sample matrices will be similar from day-to-day in the respective operations suggesting that a unique matrix effect factor will eventually evolve to a stable, minimum level. However, this factor may very well fluctuate with potential seasonal variations.

Results for the two ELISAs, testing for either Stx1 or Stx2, are presented in Tables 2 and 3, respectively. Detection responses were generally strong for all Stx1 samples as demonstrated in Table 2. The same was true for STEC expressing Stx1a, 2a subtypes when testing for Stx2 (Table 3). However there was a slight decrease in overall response for all Stx2a samples perhaps indicative that the STEC strain expressing both Stx1a, 2a, expresses higher levels of Stx2a than the other strain that expresses Stx2a only (Table 3). In comparing the matrices, the overall detection response generally neither increased nor decreased when enrichment time was increased from 16 to 22 h. The uninoculated sample controls were recorded as negative since their associated responses were demonstrated to not be significantly above negative control levels. Variation in responses as indicated by associated standard deviations was overall higher for anti-Stx1 testing, but was generally at (or well below) a variance of 15-20%.

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Table 1. Background and Inoculum levels for anti-Stx ELISAs

Sample	Background*	Inoculum levels for STEC with designated STX types		
		Stx1a	Stx2a	Stx1a, 2a
Ground Beef (6 h)	3×10^1 CFU/g	0.3 CFU/g	0.20 CFU/g	0.08 CFU/g
Ground Beef, repeat	3.6×10^1 CFU/g	1.5 CFU/g	1.1 CFU/g	1.0 CFU/g
Ground Beef, repeat 2	3×10^1 CFU/g	0.60 CFU/g	0.64 CFU/g	0.60 CFU/g
Milk	1×10^1 CFU/mL	0.52 CFU/mL	0.84 CFU/mL	0.64 CFU/mL
Milk, repeat	8×10^0 CFU/mL	0.72 CFU/mL	0.76 CFU/mL	0.52 CFU/mL
Pond Water	1.0×10^2 CFU/mL	0.96 CFU/mL	0.68 CFU/mL	0.4 CFU/mL
Pond Water, repeat	9.2×10^1 CFU/mL	0.56 CFU/mL	0.44 CFU/mL	0.2 CFU/mL
Lettuce	1.5×10^2 CFU/g	0.96 CFU/g	0.68 CFU/g	0.4 CFU/g
Lettuce, repeat	1.6×10^2 CFU/g	0.56 CFU/g	0.44 CFU/g	0.2 CFU/g

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* Average of duplicate measurements of background flora on aerobic plate count agar.

146 **Table 2.** Food and Environmental Sample Testing, anti-Stx1 ELISA

Sample	Stx type	Enrichment Time (h)	Average Response (Abs)	Standard Deviation	Replicates ¹ (n=)	Response ²
Ground Beef	Uninoculated control	6	0.0634	0.0057	6	-
		16	0.1120	0.0175	8	-
		22	0.1098	0.0316	12	-
	1a	6	0.0647	0.0070	6	-
		16	2.2848	0.5998	7	+++
		22	2.0523	0.6203	14	+++
	2a	6	0.0616	0.0054	6	-
		16	0.1155	0.0047	6	-
		22	0.1127	0.0542	12	-
	1a, 2a	6	0.0618	0.0056	6	-
		16	2.3465	0.4088	8	+++
		22	2.4953	0.7753	14	+++
Lettuce	Uninoculated control	16	0.3846	0.1965	8	-
		22	0.1069	0.0076	4	-
	1a	16	2.8847	1.0715	8	+++
		22	2.9677	0.8270	8	+++
	2a	16	0.2578	0.1458	8	-
		22	0.1571	0.0648	6	-
	1a, 2a	16	2.9728	0.6648	8	+++
		22	1.9901	0.8596	8	++
Milk	Uninoculated control	16	0.1062	0.0112	7	-
		22	0.1571	0.1046	7	-
	1a	16	2.5666	0.9177	8	+++
		22	3.1355	0.9393	8	++++
	2a	16	0.1360	0.0582	8	-
		22	0.1318	0.0886	7	-
	1a, 2a	16	2.9195	0.6858	8	+++
		22	2.9779	0.8630	8	+++
Pond Water	Uninoculated control	16	0.1429	0.0539	6	-
		22	0.0968	0.0088	7	-
	1a	16	3.0405	1.1470	8	++++
		22	2.9142	0.5160	8	+++
	2a	16	0.0974	0.0060	6	-
		22	0.1084	0.0156	8	-
	1a, 2a	16	2.4424	1.0896	8	+++
		22	2.2919	0.7438	8	+++
Negative control			0.1357	0.0810	32	
Positive control			0.6283	0.1514	33	

147 ¹Residual replicates following treatment with Dixon’s Q-test at Q_{90%}.
148 ²Semi-quantitative response of “-” for < 4x (6x for lettuce samples) S/N (average sample/negative control
149 absorbencies), otherwise average sample absorbencies ≥ 4x (6x for lettuce samples) S/N to 1 were “+”, > 1 to 2,
150 “++”, > 2 to 3 “+++”, and ≥ 3, “++++”.

152 **Table 3. Food and Environmental Sample Testing, anti-Stx2 ELISA**

Sample	Stx type	Enrichment Time (h)	Average Response (Abs)	Standard Deviation	Replicates ¹ (n=)	Response ²
Ground Beef	Uninoculated control	6	0.0887	0.0063	6	-
		16	0.1519	0.0133	8	-
		22	0.1576	0.0479	14	-
	1a	6	0.0927	0.0120	6	-
		16	0.4555	0.0918	8	-
		22	0.3906	0.1201	13	-
	2a	6	0.0932	0.0043	5	-
		16	1.7016	0.4742	8	++
		22	2.6358	0.2182	13	+++
	1a, 2a	6	0.1074	0.0221	6	-
		16	2.0218	0.3897	8	+++
		22	1.7774	0.2399	14	++
Lettuce	Uninoculated control	16	0.3183	0.0610	8	-
		22	0.2155	0.0431	6	-
	1a	16	0.5877	0.1087	8	-
		22	0.5293	0.0696	8	-
	2a	16	1.2141	0.4593	8	++
		22	1.3147	0.8360	8	++
	1a, 2a	16	2.3602	0.7027	8	+++
		22	2.2414	0.6059	8	+++
Milk	Uninoculated control	16	0.0742	0.0073	8	-
		22	0.0800	0.0026	6	-
	1a	16	0.2611	0.0272	8	-
		22	0.2444	0.0183	8	-
	2a	16	0.8862	0.0920	8	+
		22	1.3787	0.3935	8	++
	1a, 2a	16	3.1604	0.2219	8	++++
		22	3.8256	0.3298	7	++++
Pond Water	Uninoculated control	16	0.2631	0.0422	7	-
		22	0.2342	0.0830	8	-
	1a	16	0.3990	0.1519	8	-
		22	0.3260	0.0998	8	-
	2a	16	0.7155	0.3196	8	+
		22	0.8293	0.2783	8	+
	1a, 2a	16	2.4162	0.8129	8	+++
		22	2.4063	0.4400	8	+++
Negative control			0.1149	0.0209	33	
Positive control			0.5113	0.1209	33	

153 ¹Residual replicates following treatment with Dixon’s Q-test at Q_{90%}.
154 ²Semi-quantitative response of “-” for < 4x (6x for lettuce samples) S/N (average sample/negative control
155 absorbencies), otherwise average sample absorbencies ≥ 4x (6x for lettuce samples) S/N to 1 were “+”, > 1 to 2,
156 “++”, > 2 to 3 “+++”, and ≥ 3, “++++”.

3. Experimental Section

3.1. Materials

Beef chuck roast, milk (2% fat), and Romaine lettuce samples were purchased from local grocery stores. Replicated tests were conducted with these foods sourced from different lots and/or different manufacturers. Pond water was obtained from a nearby public park reservoir on multiple days for replicate testing. *E. coli* O103:H25 (Stx1a-producing strain 03-2444), O145:H28 (Stx2a-producing strain 07865), and O111:H8 (Stx1a-, Stx2a-producing strain EE5) strains were obtained from in-house stocks, and the inoculum was grown in Bacto peptone water (Becton Dickinson and Company, Sparks, MD) in 15mL Falcon tubes (Corning, Corning, NY) and diluted in 16 mm x 150 mm Fisher glass dilution tubes (Pittsburgh, PA). Tryptic soy agar for inoculum plate counts was also from Becton Dickinson and Company. Samples were enriched in an Innova 42 growth chamber (Eppendorf, Inc., Hamburg, Germany) and aliquots were subsequently stored for analysis in 1.5 mL microcentrifuge tubes (Eppendorf, Inc.). ELISA tests were performed using Abraxis, Inc. Shiga-toxin 1 and Shiga-toxin 2 detection kits (cat #542000 and cat #542010, respectively; Warminster, PA, USA) and buffered protein extraction reagent (B-PER™; Thermo Scientific, Rockford, IL, USA). Absorbance values were collected using a Tecan Safire2 microplate reader (Männedorf, Switzerland).

3.2. Preparation and Enrichment of Samples

All cutting surfaces, grinding implements, and utensils were sanitized with 70% ethanol prior to use. Ground beef was aseptically prepared from chuck roast by cutting away the exterior, trimming excess fat, and grinding in a Megrindul commercial duty meat grinder (Buffalo Corp., O'Fallon, MO, USA). The final fat content of the ground beef was estimated to be 10-20%. Romaine lettuce was chopped into ~1 cm² pieces using a knife. Samples, 25 g ground beef, 25 g chopped Romaine lettuce, 25 mL pond water, or 25 mL milk, were weighed or measured into separate Seward Ltd. Classic 400 Stomacher strainer bags (Davie, FL, USA) and kept at 4°C until enrichment. Enrichment broth comprised of mTSB made from an Oxoid mTSB base (Basingstoke, England) with 10 g/L casamino acids (Neogen, Lansing, MI) and 66.7 ng/mL mitomycin C (Sigma-Aldrich, St. Louis, MO) was added immediately before enrichment.

Inocula of *E. coli* strains that carried the *stx1a*, *stx2a*, or *stx1a* and *stx2a* genes (all isolates confirmed using a PCR protocol (Scheutz, et al., 2012) were created by serially diluting an overnight axenic culture made from frozen stock, shaken and grown at 37°C, into peptone water to a desired CFU/mL of 10 CFU per 25 g (or mL) of sample. Actual inoculum levels were determined by spread plating 1 mL of cultures onto TSA and, after absorption of excess liquid, incubating overnight at 37°C.

One mL of the prepared dilutions or peptone water control was added to the samples, and kept at 4°C. Enrichment broth (mTSB) was then added to dilute 25 g (or mL) of each sample 1:4, and the sample-broth solution was mixed by hand-massaging. Enrichment was then performed statically in the growth chamber for 6, 16 or 22 h at 42°C.

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3.3. Execution and Analysis of ELISA Tests

Post-enrichment, 1.5 mL sample aliquots were removed from the Stomacher bags and pipetted into microcentrifuge tubes. ELISA reagents and buffers were prepared according to Abraxis kit instructions. A portion (100 µL) of each sample aliquot was added to the ELISA microtiter plates, followed by 100 µL of B-PER™, and allowed to react statically for 1 h at room temperature. Testing proceeded according to kit instructions, with no shaking. Absorbance values for samples as well as negative and positive controls were read on the Tecan microplate reader 5 min after addition of the stop solution. Values for replicates across different data collection times were averaged. The average absorbencies and associated standard deviations were calculated; in addition an overall optimized signal-to-noise (S/N) ratio for each sample type was evaluated. The S/N ratios were represented as a semi-quantitative analysis response (- to +++) for each sample condition tested.

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4. Conclusions

Food legislation (e.g., in the European Union) does not require producers to differentiate Stx1 and Stx2 in the current regulatory framework. However, distinction of the two toxin types with immunoassay kits may be beneficial since strains that carry some variants of the *stx2* gene cause more severe disease ((Bielaszewska, et al., 2006; Orth, et al., 2007). The Abraxis ELISA kits were used to detect Stx1 and Stx2 as inoculated into all of the samples tested including ground beef, pond water, lettuce, and milk. The kits effectively demonstrated the ability to detect and differentiate the presence of the two toxins in all of the tested samples using an average signal-to-noise (background or negative control) of at least 4x (6x for lettuce samples). This ratio was a conservative, empirically-derived factor that may be reduced dependent upon the sample matrix. These test kits may effectively detect Shiga toxin in food and environmental samples after an enrichment period as short as 16 h with a high degree of confidence (100% accurate in this study). In addition, the dose response performance for the Abraxis kits were exhibited to compare favorably with that of the Meridian EHEC Premiere kit for the detection of two toxoids representing each Stx subtype (Kong, 2016). These new ELISA kits hold promise in testing for Shiga toxins while in the presence of food matrices, as well as samples similar in composition, by food producers and regulators alike. Testing of clinical specimens, such as stool, may also be possible. However, since this ELISA protocol essentially relies on the culture enrichment-based active production and antimicrobial-mediated release of Stx, it may not be reliable for conclusive detection of low levels of STECs amidst exceedingly large numbers of potentially growth competitive background flora.”

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