Comparison of the Presence of Shiga Toxin 1 in Food Matrices as Determined by an Enzyme-Linked Immunosorbent Assay and a Biological Activity Assay

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ABSTRACT

This study was conducted to compare the identification of Shiga toxin 1 (Stx1) based on its specific biological activity and based on results of a commercial enzyme-linked immunosorbent assay (ELISA) kit. Stx1 was thermally treated for various periods in phosphate-buffered saline, milk, and orange juice. The residual Stx1 concentration was determined with the commercial ELISA kit, and its residual enzymatic activity (amount of adenine released from a 2,551-bp DNA substrate) was determined with a biological activity assay (BAA). Regression analysis indicated that the inactivation of Stx1 as a function of time followed first-order kinetics. The half-lives determined at 60, 65, 70, 75, 80, and 85°C were 9.96, 3.19, 2.67, 0.72, 0.47, and 0.29 min, respectively, using the BAA. The half-lives determined by the ELISA with thermal treatments at 70, 75, 80, and 85°C were 40.47, 11.03, 3.64, and 1.40 min, respectively. The Z, Q10, and Arrhenius activation energy values derived by both assays were dissimilar, indicating that the rate of inactivation of the active site of Stx1 was less sensitive to temperature change than was denaturation of the epitope(s) used in the ELISA. These values were 10.28°C and 9.40 and 54.70 kcal/mol, respectively, with the ELISA and 16°C and 4.11 and 34 kcal/mol, respectively, with the BAA. Orange juice enhanced Stx1 inactivation as a function of increasing temperature, whereas inactivation in 2% milk was not very much different from that in phosphate-buffered saline. Our investigation indicates that the ELISA would be a reliable method for detecting the residual toxicity of heat-treated Stx1 because the half-lives determined with the ELISA were greater than those determined with the BAA (faster degradation) at all temperatures and were highly correlated ($R^2 = 0.994$) with those determined with the BAA.

Shiga toxin is listed as a select agent by the National Select Agent Registry (15) because of the likelihood that the toxin could be used as an oral biological warfare agent. One scenario is the intentional introduction of a toxin into a farmer’s milk tank or into the transport tanker truck taking milk to a processing facility. Most states require testing for beta-lactam proteins in milk upon arrival to detect the presence of allergenic penicillin derivatives. This test takes 20 to 30 min and could be combined with other tests for various toxic agents. However, most of these tests are more time-consuming and could disrupt the supply chain timing if the unloading of milk is delayed. Thus, if results obtained 1 to 2 h after milk was tested at the plant facility indicated that a milk batch (e.g., a full silo, 40,000 gal [151,360 liters]) was positive for a specific toxin at levels that could be toxic to children, that batch of milk would have to be pulled from the food supply and destroyed. Although destruction protocols have not been completely clarified, the disposal of a full silo of milk into a city’s water treatment system or onto land is not an option. The U.S. Environmental Protection Agency requires that the toxin content of the contaminated food be reduced to a safe level before disposal. The National Center for Food Protection and Defense (U.S. Department of Homeland Security) has provided funding for study of the inactivation kinetics of select bioterror agents (e.g., anthrax, ricin, and Shiga toxin) as a function of temperature combined with chemical disinfectants and development of decontamination guidelines (24).

Shiga toxins 1 and 2, abrin, and ricin belong to a group of proteins known as ribosome-inactivating proteins (RIPs). RIPs inactivate ribosomes by enzymatically cleaving adenine residues, leading to cessation of protein synthesis and eventually cell death (2, 6, 16, 19). RIPs also can remove adenine residues from polynucleotides in vitro (1, 2). In a recent study (14), we addressed the question of whether enzyme-linked immunosorbent assay (ELISA) results accurately reflect the toxicity or biological activity of ricin. This evaluation became necessary because in recent reports (4, 20) researchers have questioned the suitability of ELISAs for determining residual ricin toxicity because an ELISA cannot directly identify biological activity or toxicity but rather provides an assessment of ricin concentration via antibody-antigen interactions involving specific epitopes or binding sites on the toxin and paratopes on commercial proteins.
antibodies. However, this antibody-antigen interaction is gradually lost during thermal treatment because of denaturation of the epitopes, resulting in a negative ELISA result. Thus, researchers were unsure whether a negative ELISA result would accurately reflect the absence of biological activity or toxicity of the RIP because the epitope(s) or binding site(s) recognized by antibodies may be inactivated at a different rate than the active site of the RIP. Our findings indicated that the ELISA was suitable for residual ricin toxicity determination as a result of the strong correlation between immunodetection and biological activity.

In the present study, we attempted to address the same question with respect to Shiga toxin: would an ELISA be suitable for routine determination of residual Shiga toxin toxicity especially if the test could be done within a 20- to 30-min time frame? In other words, would there be agreement between immunodetection and the biological activity of Shiga toxin after a thermal treatment? An answer to this question would be helpful because accurate determination of Shiga toxin activity or toxicity is necessary before decontaminated food, equipment, or surfaces are declared free of residual Shiga toxin. The suitability of the ELISA also has implications for serological testing of blood and bloody stool samples of patients for Shiga toxin–producing Escherichia coli.

We compared a biological activity assay (BAA) with an ELISA for determination of residual Shiga toxin 1 (Stx1) after a series of thermal treatments in various media. The BAA measured the ability of an RIP (Stx1 or ricin) to remove adenine residues from a 2,551-bp DNA substrate as previously described (14). The release of adenine residues from polynucleotides has been used by previous investigators as an indicator of RIP biological activity (1, 2, 5). Results obtained with the ELISA were compared with those obtained with the BAA, and the correlation between the results was evaluated.

MATERIALS AND METHODS

Reagents and kits. Stx1 was purchased from Toxin Technologies (Sarasota, FL), and the Stx1 ELISA kit (Premier EHEC) was purchased from Meridian Biosciences (Cincinnati, OH). Chloroacetaldehyde and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Thermal treatment of Stx1. A series of 100-μl microcentrifuge tubes containing 5 μl of Stx1 (0.5 mg/ml in phosphate-buffered saline [PBS]) were thermally treated (55 to 85°C) in a water bath for up to 60 min. After treatment, the samples were immediately cooled in a water bath (4°C), and residual Stx1 concentration and biological activity for each treatment was determined with the ELISA and the BAA, respectively. The control was unheated Stx1. The percentage of Stx1 or Stx1 activity remaining after each treatment was plotted against time for both assays, and kinetic parameters were determined using the statistical procedures of Xu et al. (24). All reactions were performed in duplicate.

BAA. The 2,251-bp DNA substrate was obtained by amplifying the 731 to 2,981 position section of the pBR322 plasmid (New England Biolabs, Beverly, MA) using a modification of the method described by Brigotti et al. (5). The forward primer was 5′-ATGACTGTTCTTTATCAT-3′, and the reverse primer was 5′-TACTGTCTTCTTAGTGTAGCC-3′. PCR amplification was performed in a total volume of 25 μl containing 0.6 μg/ml BamHI-linearized pBR322 plasmid, 0.012 mM concentrations of each primer, and 12.5 μl of Promega PCR Mastermix (400 μM concentrations of each deoxynucleoside triphosphate and 2 U of Taq polymerase). The conditions for amplification were an initial hold at 95°C for 5 min, 45 cycles of 95°C for 30 s (denaturation), 46°C for 30 s (annealing), and 72°C for 150 s (extension), and a final extension period of 5 min. At the end of the reaction, primers and unincorporated nucleotides were removed with the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Chalfont St. Giles, UK), and the 2,551-bp DNA product was verified by gel electrophoresis and stored at 4°C.

BAA: digestion of 2,551-bp DNA with Stx1. A modification of the method described by Brigotti et al. (5) was used for Stx1 digestion of DNA. The enzymatic reaction was performed in duplicate in microcentrifuge tubes (50 μl) containing 0.05 mg/ml Stx1 (active or thermally treated) and 80 μg/ml 2,551-bp DNA substrate in 50 mM sodium acetate buffer (pH 7.0, 100 mM KCl). After incubation for 3 h at 50°C, the reaction products were diluted fivefold with distilled water and applied to Bond Elut-NH2 columns (Varian, CA) pre-equilibrated with distilled water. Free adenine was eluted from the columns by spining at 100 × g for 3 min. The elution step was repeated after the addition of 150 μl of distilled water to the columns, and eluates from both washes were pooled.

BAA: quantification of released adenine. Adenine was quantified using a modification of the method described by Barrio et al. (3). The adenine released from the 2,551-bp DNA fragment was converted to 1,N2-ethenoadenine (εA) by reacting with 40 mM chloroacetaldehyde at 80°C for 1 h in distilled water (pH 6.1). The fluorescence intensity of εA was quantified on a luminescence spectrophotometer (LS 50B, Perkin Elmer, Waltham, MA) with an excitation wavelength of 275 nm and an emission wavelength of 410 nm. The fluorescence intensity reading was repeated four times for each replicate, the average was calculated, and the mean fluorescence intensity of εA was used as a measure of biological activity. Residual Stx1 activity was calculated by dividing the mean fluorescence intensity of each treatment by the mean fluorescence intensity obtained for nonheated Stx1 (time zero). All reactions were performed in duplicate, and mean values were recorded.

ELISA. Residual Stx1 concentration was determined with the commercial Premier EHEC ELISA kit (Meridian Biosciences). A standard curve (slope = 0.633; R2 = 0.99) was constructed using various concentrations (0.5 to 200 ng/ml) of Stx1. Stx1 samples (treated and nontreated) were diluted to 100 ng/ml with PBS for the assay, and absorbance was read at 450 nm on a Synergy HT plate reader (BioTek, Winooski, VT) using KC4 software (BioTek). Residual Stx1 concentration was determined by dividing the average absorbance of each treatment by the average absorbance of the nontreated Stx1 (time zero). All reactions were performed in duplicate, and the mean values were used.

Statistical analysis. Statistical analysis was performed by calculating the upper and lower 95% confidence limits for each half-life according to the methods of Labuzza and Kamman (13). When the 95% confidence limits of any set of conditions did not overlap, the values were considered significantly different at α ≤ 0.05.
The release of adenine from polynucleotides by RIPs has been reported (1, 2, 5, 14). In the present study, the adenine released by Stx1 was reacted with chloroacetaldehyde to form the fluorescent eA (8–10), which was quantified and used as a measure of Stx1 biological activity. Preliminary investigations revealed that Stx1 was most active at pH values greater than 6.0 (data not shown) but lost significant activity at lower pH values. Therefore, the temperature dependence of the biological activity of Stx1 (0.05 mg/ml) also was investigated (Fig. 1). The calibration curve is the difference between reactions performed with Stx1 (Rxn w/ Stx1) or without Stx1 (Rxn w/o Stx1). The error bars represent standard deviations.

RESULTS AND DISCUSSION

BAA. The release of adenine from polynucleotides by RIPs has been reported (1, 2, 5, 14). In the present study, the adenine released by Stx1 was reacted with chloroacetaldehyde to form the fluorescent eA (8–10), which was quantified and used as a measure of Stx1 biological activity. Preliminary investigations revealed that Stx1 was most active at pH values greater than 6.0 (data not shown) but lost significant activity at lower pH values. Therefore, the temperature dependence of the biological activity of Stx1 (0.05 mg/ml) also was investigated (Fig. 1). The calibration curve is the difference between reactions with Stx1 and without Stx1 (control). Stx1 activity was highest from 40 to 50°C but decreased above 55°C. Subsequent biological activity reactions were therefore performed at pH 7 and 50°C.

Determination of residual Stx1 concentration and activity. Figure 2 shows semilog plots of residual Stx1 biological activity (Fig. 2A) and residual Stx1 concentration as determined with the ELISA (Fig. 2B) as a function of time at different temperatures in PBS. These figures clearly indicate that the inactivation of Stx1 followed first-order kinetics with high R² values. Residual Stx1 concentration and activity were calculated by dividing the mean concentration or mean activity of Stx1 at each time interval by their respective values at time zero. The relationship between the dependent and independent variables is described by equation 1, where [A/A₀] × 100 is the percentage of Stx1 remaining, k is the first-order rate constant per minute, which equals the slope of ln[A/A₀] versus time, and t is time (minutes). The half-life (t½) was determined using equation (2):

\[
[A/A₀] × 100 = e^{-kt}
\]

\[
t_{1/2} = \ln 2/k
\]

The half-life values determined from the semilog plots (Fig. 2) are presented in Table 1. The values obtained with the ELISA were greater than those obtained with the BAA when compared at the same temperatures, indicating that the active site of Stx1 was inactivated at a faster rate than was that of the epitope(s) or the binding site(s) on Stx1 responsible for ELISA determination. For example, the half-lives at 70°C were 40.47 and 2.67 min from the ELISA and BAA, respectively. The difference between the two assays was significant at the temperatures investigated in this study, as indicated by the lack of overlap of the 95% confidence limits (Table 1). This observation suggests that the BAA was more sensitive than the ELISA for residual Stx1 determination. This finding is in agreement with that of a previous study (14) in which the thermal inactivation rate of the active site of ricin was compared with that of the epitope(s) responsible for immunodetection. These results also suggest that a negative Stx1 determination with an ELISA should be considered reliable because a negative outcome also would be obtained with a BAA.

The temperature dependence of the rates of inactivation of the active site of Stx1 and the epitope(s) responsible for the ELISA determination was calculated by fitting half-lives obtained from the semilog plots of residual Stx1 against time (Fig. 2) to the Arrhenius equation (equation 3), where k₀ is the preexponential factor, Eₐ is the activation energy (calories per mole), R is the gas constant (1.987 cal/K mol), and T is the absolute temperature in degrees Kelvin. The activation energy was derived from the slope (EₐR) of a semilog plot of k against 1/T. The increase in reaction rate per 10°C increase in temperature (Q₁₀) was calculated from equation 4 using the slope derived from a semilog plot of k (from Table 1) against temperature (equation 5) as previously reported (11, 12), where b is the slope, T is the temperature (degrees Celsius), and k₀ is a constant. The Z-value, i.e., the temperature required for the inactivation rate (or half-life) to increase by 1 log cycle was determined using equation 6:

\[
k = k₀e^{-Eₐ/RT}
\]

\[
Q₁₀ = e^{10b}
\]

\[
t_{1/2} = t₀e^{-bT}
\]

\[
Z = 2.303/b
\]
FIGURE 2. Residual Stx1 loss (in PBS) as a function of temperature as determined by (A) the biological activity assay (BAA) and (B) the ELISA.

TABLE 1. Half-life values for inactivation of Stx1 in PBS determined by BAA and ELISA after heat treatment at various temperatures and respective Z, Q_{10}, and Arrhenius activation energy (E_a) values^a

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Value</th>
<th>Upper CL</th>
<th>Lower CL</th>
<th>Value</th>
<th>Upper CL</th>
<th>Lower CL</th>
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</thead>
<tbody>
<tr>
<td><strong>Half-life (min)</strong></td>
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<tr>
<td>60 °C</td>
<td>9.96</td>
<td>18.06</td>
<td>6.88</td>
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<tr>
<td>65 °C</td>
<td>3.19</td>
<td>3.82</td>
<td>2.75</td>
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<tr>
<td>70 °C</td>
<td>2.67 A</td>
<td>3.20</td>
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<tr>
<td>75 °C</td>
<td>0.72 A</td>
<td>0.92</td>
<td>0.59</td>
<td>40.47 B</td>
<td>77.26</td>
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<td>80 °C</td>
<td>0.47 A</td>
<td>0.55</td>
<td>0.41</td>
<td>11.03 B</td>
<td>14.47</td>
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<td>85 °C</td>
<td>0.29 A</td>
<td>0.31</td>
<td>0.28</td>
<td>3.64 B</td>
<td>4.43</td>
<td>3.10</td>
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<td><strong>Inactivation parameter</strong></td>
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<tr>
<td>Z (°C)</td>
<td>15.99 A</td>
<td>30.90</td>
<td>14.72</td>
<td>10.28 B</td>
<td>11.14</td>
<td>10.03</td>
</tr>
<tr>
<td>Q_{10}</td>
<td>4.11 A</td>
<td>4.78</td>
<td>2.11</td>
<td>9.40 B</td>
<td>9.93</td>
<td>7.52</td>
</tr>
<tr>
<td>E_a (kcal/mol)</td>
<td>34.00 A</td>
<td>37.20</td>
<td>18.5</td>
<td>54.70 B</td>
<td>56.00</td>
<td>49.50</td>
</tr>
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</table>

^a BAA, biological activity assay; ELISA, enzyme-linked immunosorbent assay; CL, 95% confidence limit. Within a row, values with the same letter are not significantly different (α = 0.05).
4,000 Da larger than the A subunit (32,000 Da), which is a monomer. The different sizes of the subunits likely contributed to the difference in sensitivity between the two assays, hence their different $Z$, $Q_{10}$, and Arrhenius activation energy values (Table 1), all of which are significantly different (no overlap of the 95% confidence limits). We therefore conclude that the ELISA overestimated Stx1 toxicity, i.e., a longer time was needed to achieve a 50% reduction in Stx1 concentration as determined with the ELISA than it did to reach a 50% reduction in Stx1 toxicity as determined with the BAA. Therefore, a negative ELISA result cannot be considered false negative because the measurement obtained with the BAA would have a negative value much sooner. Solving for $T$ by equating the expressions of the curves, a temperature of 102.56°C obtained, at which there would be no difference between the rates of inactivation of the active site of Stx1 and epitopes recognized by the ELISA. Plotting the half-lives of Stx1 obtained with the ELISA against those obtained with the BAA gave a correlation coefficient ($R^2$) of 0.994. This result suggests that the ELISA is suitable for routine estimation of residual Stx1 toxicity at least in foods decontaminated by thermal treatment, even though the amount of active Stx1 present in samples in the present study was overestimated. Because the concentration of Stx1 at which there would be no adverse effect in the most sensitive population remains classified, the number of half-life reductions needed for a given initial contamination level remains unknown.

We also investigated the effects of food matrices on thermal inactivation of Stx1 (Fig. 3). Before inactivation, Stx1 was introduced into orange juice (OJ) and 2% milk at 0.05 mg/ml. The half-lives and temperature-dependent kinetic parameters are summarized in Table 2. At 70, 75, and 80°C, there was no significant difference in half-life among the PBS, milk, and OJ systems (the 95% confidence limits overlapped). However, as indicated in Figure 4 and
by the temperature sensitivity values in Tables 1 and 2 the OJ system was much more temperature sensitive (lower \( Z \) value and higher \( Q_{10} \) and activation energy) than the others. We had expected the low pH (3.86) of OJ to have a significant effect on the rate of Stx1 inactivation because there was significant reduction (~70%) in the biological activity of the toxin when it was treated at 50°C for 3 h at pH 4.0 compared with a treatment at pH 7.0 in our preliminary investigation. However, the OJ system was affected by only temperature. Figure 4 does suggest that at higher temperatures (>80°C) there would be a significant difference in the rates of Stx1 inactivation in OJ relative to PBS.

Higher half-life values were expected for Stx1 inactivation in 2% milk compared with PBS because milk contains proteins and carbohydrates that could limit the effectiveness of the thermal treatment. However, the half-life of Stx1 inactivation was consistently shorter (faster rate) in milk than in PBS (Fig. 4), although this difference was not significant. Milk proteins could have influenced the results in one or two ways. First, milk proteins could have masked Stx1 epitopes as a result of intimate associations or hydrophobic interactions between the proteins (17). This masking would generate a false-negative result, i.e., the effective binding between Stx1 antibody paratopes and Stx1 (antigen) epitopes would be reduced, giving rise to a reduction in absorbance readings of the end products of the ELISA. In this case, ELISA results would lead to an underestimation of the amount of active Stx1 antigen remaining after the inactivation process.

Second, the slightly faster rate of inactivation of Stx1 in 2% milk could be attributed to nonspecific binding of Stx1 antibodies (primary or secondary) to the walls of the ELISA wells during incubation through hydrophobic protein interactions and ionic and electrostatic interactions (18). This binding would lead to a high background signal, resulting in an increase in intensity of the ELISA reading or an overestimation of the amount of antigen (Stx1) present in the sample. Nonspecific binding of antibodies can be prevented (blocked) by pretreatment with bovine serum albumin, casein, or skim milk (7, 23).

### TABLE 2. Half-life values for Stx1 in orange juice and 2% milk as determined by ELISA after treatment at various temperatures and respective \( Z \), \( Q_{10} \), and Arrhenius activation energy (\( E_a \)) values\(^a\)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Orange juice</th>
<th>2% milk</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Upper CL</td>
</tr>
<tr>
<td>Half-life (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70°C</td>
<td>46.22 ( \alpha )</td>
<td>95.77</td>
</tr>
<tr>
<td>75°C</td>
<td>8.67 ( \alpha )</td>
<td>11.50</td>
</tr>
<tr>
<td>80°C</td>
<td>1.62 ( \alpha )</td>
<td>2.34</td>
</tr>
<tr>
<td>85°C</td>
<td>1.26</td>
<td>1.66</td>
</tr>
<tr>
<td>( Z ) (°C)</td>
<td>6.87 ( \alpha )</td>
<td>7.19</td>
</tr>
<tr>
<td>( Q_{10} )</td>
<td>28.53 ( \alpha )</td>
<td>40.93</td>
</tr>
<tr>
<td>( E_a ) (kcal/mol)</td>
<td>80.60 ( \alpha )</td>
<td>89.40</td>
</tr>
</tbody>
</table>

\(^a\) CL, 95% confidence limit. Within a row, values with the same letter are not significantly different (\( \alpha = 0.05 \)).

**FIGURE 4.** Comparison of the effect of temperature on the rate (indicated by half-life) of Stx1 inactivation as determined with the biological activity assay (BAA) in PBS (■), with the ELISA in PBS (●) and with the ELISA in orange juice (OJ; ▲) and in 2% milk (○). The error bars represent upper and lower 95% confidence limits.
Because PBS does not contain milk proteins that would block nonspecific antibody binding, Stx1 inactivation in PBS was expected to be slightly slower than that in 2% milk. Milk proteins, especially casein, are superior blocking agents (7). The avidity with which milk proteins bind to solid phases has been attributed to their various sizes, amino acid sequences, degrees of glycosylation and sulfation, and surface charges (21). We are unsure what type of blocking agent was contained in the antigen diluent or washing buffer supplied by the manufacturer of the ELISA kit, if any agent was included. The proteins in the 2% milk further reduced nonspecific antibody binding slightly compared with that in PBS, assuming that the manufacturer included a blocking agent in the diluent or the washing buffer. Therefore, as a result of nonspecific antibody binding the ELISA likely overestimated the amount of active Stx1 remaining in the PBS samples.

This study, like the previous one (14), revealed that (i) ELISA results provide an overestimation of the biological activity (or toxicity) of Stx1 at the molecular level and (ii) the ELISA can discriminate between active and inactive Stx1. Based on these findings, the ELISA can be considered a conservative estimator of Stx1 biological activity and thus would be a reliable and rapid tool for routine assessment of residual Stx1 toxicity, i.e., when the ELISA result is negative for Stx1 (i.e., no active Stx1), the decontaminated food is definitely safe for disposal. Our findings also indicate that the inactivation rate as measured with the ELISA in OJ and 2% milk was not significantly different from that in PBS at 70 to 80°C (Table 2 and Fig. 4).

These findings are applicable only to the ELISA kit used in this study because different manufacturers may use different epitopes to generate antibodies. Thorn et al. (22) found that different outcomes are to be expected when different epitopes are targeted. Manufacturers of ELISA kits must present comparisons with a BAA to assure the user that the results are meaningful. This comparison also should be made for any ELISA kits used for analysis of Shiga toxins in blood or bloody stools.

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