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PRESENCE OF ANTIBODIES TO SHIGA TOXIN IN RANDOM POPULATION IN IDAHO

By

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Committee Approval

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Abbreviations

Stx1	Shiga toxin 1
Stx2	Shiga toxin 2
STEC	Shigatoxigenic group of Escherichia coli
MID	Minimum infectious dose
HUS	Hemolytic-uremic syndrome
EHEC	Enterohemorrhagic E. coli
TTP	Thrombotic thrombocytopenic purpura
PFGE	Pulsed field gel electrophoresis
PCR	Polymerase chain reaction
ELISA	Enzyme linked immunosorbent assay
HRP	Horseradish peroxidase
TMB	Tetramethylbenzidine

Thesis Abstract

Shiga toxins are a family of related toxins with two major groups, Stx1 and Stx2[1]. The most common sources for Shiga toxin are the bacteria *S. dysenteriae* and the Shigatoxigenic group of *Escherichia coli*(STEC), which includes serotypes O157:H7, O104:H4, and other enterohemorrhagic *E. coli* (EHEC) [2][3]. STEC infections often cause diarrhea, sometimes bloody. Some patients with STEC infection develop hemolytic uremic syndrome (HUS), a severe complication characterized by renal failure, hemolytic anemia, and thrombocytopenia that can be fatal[4]. The overall incidence rate of STEC infection in the United States in 2011 was 1.8 per 100,000 population. Idaho had the third highest reported incidence rate (6.6 per 100,000) in 2011. The incidence rate of STEC O157 infection in 2011 is 0.76 per 100,000 population. Idaho was in the highest incidence rate area[5]. In general, not all persons ill with STEC infection seek medical care, healthcare providers may not obtain a specimen for laboratory diagnosis, or the clinical diagnostic laboratory may not perform the necessary diagnostic tests. Accounting for under-diagnosis and under-reporting, an estimated 96,534 STEC O157 and 168,698 non-O157 infections occur each year[6]. Detection of antibodies to Shiga toxin in random populations can provide information for estimated STEC incidence rate accounting for under-diagnosis and under-reporting of STEC. In this study, we used an ELISA technique to detect antibody to Shiga Toxin in samples from a random population in Idaho to confirm the ELISA method can be used to detect antibody to toxins directly in serum and provide evidence for estimated STEC infection in Idaho.

Chapter I: Introduction

Shiga toxin was named for Kiyoshi Shiga, a Japanese bacteriologist. It consists of two major groups , Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) [1]. Stx1 consists of an A subunit and 5 B subunits. The sequence of the B subunit from *Shigella dysenteriae* type 1 is same as the B subunit of Stx1. The B subunit binds specifically to a glycolipid in microvillus membranes, and the released A subunit stops protein synthesis by inactivating the 60S ribosomal subunit. N-glycosidase activity of the toxin results in this inactivation. An adenine molecule(A-4323) is cleaved off the 28S ribosomal RNA, and as a result the structure of the 60s subunit is modified, resulting in a reduced affinity for EF-1 and, thus, an inhibition of aminoacyl-tRNA binding. The end result of toxin action is a cessation of protein synthesis, the sloughing off dead cells, and a bloody diarrhea. It is worthy to mention that Sxt1 carries out the same reaction as the plant toxins ricin and abrin. Stx2 is biologically similar to Stx1. They are genetically distinct since there is only 50% to 60% homology between the two toxins. [7] Shiga toxins act to inhibit protein synthesis within target cells by a mechanism similar to that of ricin toxin produced by *Ricinus communis*[8]. After entering a cell via a macropinosome [9], the protein functions as an N-glycosidase to prevent protein synthesis by cleaving a specific adenine nucleobase from the 28S RNA of the 60S subunit of the ribosome[10]. The most common sources for Shiga toxin are the bacteria *S. dysenteriae* and the Shigatoxigenic group of *Escherichia coli*(STEC) [11][12]. Highly specific receptors on the cells' surface are required for toxin to attach and enter the cell and species including cattle, swine, and deer do not carry these receptors and therefore they harbor toxigenic bacteria without any ill effect. They are shed, however in their feces, from which they may be spread to humans.

STEC are now considered as an important group of bacterial enteropathogens. STEC serotypes are named based on their somatic (O) and flagellar (H) antigens. There are at least 100 serotypes of *E.coli* that are capable of producing Shiga toxins[13].

Most reported STEC infections in the United States are caused by *E. coli O157:H7*, with an estimated 73,000 cases occurring each year [14]. *E. coli O157:H7* was first recognized as a human enteric pathogen following an outbreak of haemorrhagic colitis in the USA in 1982 associated with contaminated ground beef [15]. The earliest possible case of *E. coli O157* infection recorded was in 1975, when the organism was isolated from a patient with an episode of gross bloody diarrhea. Since then, STEC O157 have been implicated in sporadic cases and outbreaks of diarrhea world-wild. *E. coli O157:H7* is a particularly virulent, or infectious, strain of food-borne bacteria. The minimum number of bacteria needed to make someone ill is called the minimum infectious dose (MID). Compared to other bacteria that cause food-borne illnesses, the MID for the O157:H7 serotype is very low[16].

Non-O157 STEC bacteria also are important causes of diarrheal illness. Since 1983, there have been approximately 250 different O serogroups of *E. coli* identified that produce Shiga toxin. About 100 among those 250 subgroups cause diarrhea. Non-O157 strains account for 20-50 % of STEC infections. Non-O157 serotypes are more common in several areas including Argentina, Australia, and Germany[17]. There are six non-O157 serogroups (O26, O45, O103, O111, O121, and O145) account for the majority of reported non-O157 STEC infections in the United States [18]. A 2011 outbreak in Germany was caused by another

STEC,O104:H4. This strain has both enteroaggregative and enterohemorrhagic properties. Both the O145 and O104 strains can cause hemolytic-uremic syndrome(HUS); the former strain shown to account for 2% to 51% of known HUS cases; an estimated 56% of such cases are caused by O145 and 14% by other Enterohemorrhagic *E. coli*(EHEC) strains.

STEC infection Clinical presentation varies from an asymptomatic state to bloody diarrhea and life-threatening complications such as HUS. Compared to healthy adults, senior and young children are most susceptible to STEC infections [13]. Approximately 8% of persons who receive a diagnosis of O157 STEC infection develop HUS. The first description of diarrhea-associated HUS was in 1955 by Gasser [19]. In 1983 HUS was linked with an antecedent STEC enteric infection. Karmali *et al*[20]. HUS is characterized by the abrupt development of haemolytic anaemia, thrombocytopenia, and renal injury[21-23] , with approximately 200 cases reported each year in the US between 2002 and 2006[24]. It typically affects previously healthy children, with a large distribution between 6 months and 5 years of age [25]. Thrombotic thrombocytopenic purpura (TTP), a syndrome with signs and symptoms that are similar to those of HUS, is typically diagnosed in adults. When TTP is diagnosed after a diarrheal illness, the condition is usually caused by infection with O157 STEC or another STEC[26]. Strain virulence and host factors determine the course of the illness and development of HUS [27]. Although many persons with diarrhea-associated HUS have an O157 STEC infection, certain non-O157 STEC strains also can result in HUS [28]. The virulence of non-O157 STEC is partly determined by the toxins they produce; non-O157 STEC strains that produce only Stx2 are more often associated with HUS than strains that

produce only Stx1 or that produce both Stx1 and Stx2 [29]. Although *E. coli O157:H7* has been most commonly identified as the cause of STEC infection, isolation of non-O157 STEC strains from clinical cases, outbreaks and environmental sources has been increasing.

A study at the Centers for Disease Control and Prevention indicated that from 1983-2002 approximately 70% of non-O157 STEC infections in the United States were caused by strains from one of six major serogroups, including O26, O45, O103, O111, O121 and O145 [30]. Virulence factors for non-O157 STEC include, but are not limited to, production of the Shiga-like toxins 1 and/or 2 (Stx1, Stx2) and intimin (eae)[31].

The reservoir of STEC appears to be mainly cattle. In addition, other ruminants such as sheep, goats, deer are considered significant reservoirs, while other mammals (pigs, horses, rabbits, dogs, cats) and birds (chickens, turkeys) have been occasionally found infected. *E. coli O157:H7* is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk. Fecal contamination of water and other foods, as well as cross-contamination during food preparation (with beef and other meat products, contaminated surfaces and kitchen utensils), will also lead to infection. An increasing number of outbreaks are associated with the consumption of fruits and vegetables (sprouts, spinach, lettuce, coleslaw, salad) whereby contamination may be due to contact with feces from domestic or wild animals through cultivation or handling. STEC has also been isolated from bodies of water (ponds, streams), wells and water troughs, and has been found to survive for months in manure and water-trough sediments[32]. Contaminated

drinking-water and recreational waters has also been reported to be the means of waterborne transmission. Person-to-person contact is an important mode of transmission through the oral-fecal route. An asymptomatic carrier state where individuals show no clinical signs of disease but are capable of infecting others, has been also reported. The duration of excretion of STEC is about one week or less in adults, but can be longer in children. Direct contact with farm animals through visiting farms and other venues has also been identified as an important risk factor for STEC infection [22][33-35]. Although outbreaks tend to be sporadic or in small clusters, more reports of outbreaks in produce spanning several states have appeared in the past few years. More recently, there have been unprecedented, large outbreaks of *E. coli O157:H7* infection in Japan and Scotland, and outbreaks due to other STEC in Australia and Europe. There have also been a significant number of outbreaks in the USA associated to the consumption of contaminated vegetable products, such as lettuce and alfalfa sprouts. The largest ever recall of food on record occurred in the USA in 1997, when about 10,000 tons of raw frozen hamburgers were recalled because of suspected contamination by *E. coli O157*. Given the magnitude and severity of recent outbreaks of foodborne diseases caused by *E.coli O157:H7*, there is an urgent need for all sectors in the food chain to work together to reduce or eliminate the health impact of this hazard. The cooperative efforts are needed for public health and environmental health agencies, farmers, animal producers, food processors and caterers, together with research scientists to achieve a significant reduction in the incidence of food borne disease caused by this pathogen.

In the USA, laboratory-based surveillance for E. coli 0157:H7 infections was first

implemented in late 1992, and the results obtained from 2001 to 2011 are reported here. The overall Laboratory-confirmed STEC O157 infections reported to CDC for each year were 2593, 2740, 2222, 2234, 2314, 3014, 2360, 2669, 2215, 2046, and 2366, and the total was 26773. The incidence rate of STEC O157 infection in 2011 is 0.76 per 100,000 population[5]. From 1982 to 2002, 49 states reported 350 outbreaks, representing 8,598 cases, 1,493 (17%) hospitalizations, 354 (4%) HUS cases, and 40 (0.5%) deaths. Transmission route for 183 (52%) was foodborne, 74 (21%) unknown, 50 (14%) person-to-person, 31 (9%) waterborne, 11 (3%) animal contact, and 1 (0.3%) laboratory-related. The food vehicle for 75 (41%) food borne outbreaks was ground beef, and for 38 (21%) outbreaks, produce[36]. Although all STEC infections are reported, for several reasons many cases are likely not recognized[4]. Not all persons ill with STEC infection seek medical care, healthcare providers may not obtain a specimen for laboratory diagnosis, or the clinical diagnostic laboratory may not perform the necessary diagnostic tests. Including under-diagnosis and under-reporting, an estimated 96,534 STEC O157 and 168,698 non-O157 infections occur each year [6].

Appropriate treatment can be implemented promptly if STEC infection is diagnosis at the early stage. Initiation of parenteral volume expansion early in the course of O157 STEC infection might decrease renal damage and improve patient outcome [37]. On the other hand, certain treatments can worsen patient outcomes; for example, antibiotics might increase the risk for HUS in patients infected with O157 STEC, and ant diarrheal medications might worsen the illness[38]. Early diagnosis of STEC infection also might prevent unnecessary procedures or treatments (e.g., surgery or corticosteroids for patients with severe abdominal

pain or bloody diarrhea) [39-41]. Prompt laboratory diagnosis of STEC infection facilitates rapid sub typing of STEC isolates by public health laboratories and submission of PFGE patterns to PulseNet, the national molecular sub typing network for food borne disease surveillance [42]. Rapid laboratory diagnosis and sub typing of STEC isolates leads to prompt detection of outbreaks, timely public health actions, and detection of emerging STEC strains [43,44]. Delayed diagnosis of STEC infections might result in secondary transmission in homes, child-care settings, nursing homes, and food service establishments and might delay detection of multistate outbreaks related to widely distributed foods. Outbreaks caused by STEC with multiple serogroups or PFGE patterns have been documented[45].

In the clinical laboratory, culture and biochemical analysis is the “gold standard” for the identification of STEC. Selective media, such as SMAC and CT-SMAC, may be used to identify O157 STEC due to this serotype’s inability to ferment sorbitol within 24 hours. CT-SMAC or CHROMagar™ O157 for isolation of O157 STEC is suggested since these are more inhibitory for commensally stool flora than SMAC or MAC and have been shown to increase the sensitivity of culture for detection of O157 STEC. To isolate non-O157 STEC from a Shiga toxin-positive specimen, the recommendation is to plate the specimen to a less selective agar such as MAC or washed sheep’s blood agar with calcium chloride (WSBA-Ca) [45]. Followed up by serotyping and testing for Shigatoxin.

However, stool specimens for O157 STEC are not regularly cultured at many laboratories. Furthermore selective and differential media are not available for the culture of non-O157 STEC, these organisms cannot be separated from normal intestinal flora on a normal enteric

isolation media containing lactose. Fewer laboratories culture stool specimens for these bacteria than for *O157 STEC*. The latest approach of using of enzyme immunoassay (ELISA) or polymerase chain reaction (PCR) to detect Shiga toxin or the genes which encode the toxins (Stx1 and Stx2) has advanced the diagnosis of both O157 and *non-O157 STEC* infections.

The Center for Devices and Radiological Health of the Food and Drug Administration has approved 4 immunoassays for the detection of Shiga toxin in human specimens. They are the Premier EHEC (Meridian Diagnostics, Cincinnati, Ohio) and the ProSpecT Shiga Toxin E. coli Microplate Assay (Remel, Lenexa, Kansas) are in a microplate EIA format; the Immunocard STAT! EHEC (Meridian Diagnostics, Cincinnati, Ohio) and the Duopath Verotoxins Gold Labeled Immunosorbent Assay (Merck, Germany) are lateral flow immunoassays[45].

PCR targeting the Shiga toxin genes of *Escherichia coli* is a rapid and sensitive diagnostic tool. It can potentially detect virulent strains that have been separated in culture from patient stool specimens. The target genes of interest are Stx1 and Stx2 and the genes for *eae* and *Ehly* are additional targets to be considered, an organism contain one or more phages on which the stx genes are located. PCR technology is recommended to detect Shiga toxin-encoding genes to guarantee that all STEC will be represented during isolate characterization. It includes the rare sorbitol fermenting *O157 STEC* variants.

Both traditional and real-time PCR methods provide a suitable detection limit to identify

Shiga toxin-producing organisms. A specific methodology will be implemented based on laboratory preference, acceptable timelines, available funding and the laboratory staff experience. The CDC *E. coli* National Reference Laboratory has protocols and expertise to assist laboratories with the implementation of molecular assays. While nonculture tests are useful tools to diagnoses STEC infection, the traditional culture approach should not be replaced. Serotyping and molecular characterization (e.g., pulsed-field gel electrophoresis [PFGE] patterns), need a pure culture of the pathogen obtained by the clinical laboratory or the public health laboratory, which are important for detecting, investigating, and controlling STEC outbreaks.

A real-time culture of stool for *O157 STEC* and EIA testing for Shiga toxin is more effective for identification of STEC infections than the use of either technique alone. Since all *O157 STEC* have the genes for Stx2 (stx2) and intimin (*eae*), which are found in strains that are associated with severe disease, detection of *O157 STEC* should prompt immediate initiation of steps such as parenteral volume expansion to reduce the risk for renal damage in the patient and the spread of infection to others. All of the before mentioned technologies are for identification of current symptomatic infections. Some references indicate a higher rate of infection. Traditionally, examination of antibodies is used to demonstrate past of asymptomatic exposure to the pathogen in question.

There were several studies which have looked at farmers and antibodies to some of the *E.Coli* strains based on the surface antigens. However there are about 200 STEC serotypes that have

been recognized. In addition, from the data collected by National Enteric Disease Surveillance show Idaho was the state that had the highest reported incidence rates of non-O157 STEC infection in 2011 (the rate was 4.3). Our study assay was designed to detect antibodies to Shiga toxins which should provide a more accurate picture of the true infection rate of all strains with Shiga toxin.

Several studies have used ELISA measurement of circulating antibodies against the O157 lipopolysaccharide and Immunoblot assay for the detection of IgG antibody to Stx1 and Stx2, and shown that rural populations have increased exposure to Shiga toxin over urban populations. For example, a study measured circulating antibodies against the O157 lipopolysaccharide in rural Wyoming residents and in blood donors from Casper, Wyoming, and Seattle, Washington, by ELISA was performed. Rural Wyoming residents had higher antibody levels to O157 LPS than did Casper donors, who, in turn, had higher levels than did Seattle donors (respective least squares means: 0.356, 0.328, and 0.310; $p<0.05$, Seattle vs. Casper, $p<0.001$, rural Wyoming vs. either city). Lower age was significantly correlated with EIA scores; gender; and, in rural Wyoming, history of bloody diarrhea, town, duration of residence, and use of non treated water at home were not significantly correlated. These data suggest that rural populations are more exposed to *E. coli O157:H7* than urban populations[47]. In 173 urban residents and 232 rural dairy-farm residents in Southern Ontario, the frequency of anti Stx2 antibodies in urban residents was 46%; in rural residents was 65%, the frequency of anti Stx1 antibodies in urban residents was 12%; in rural residents was 39%.[48] A former Idaho state university student, Achut Raj Poudel, modified the

existing protocols for the detection of antibodies in human serum samples which are produced against the toxin by using an ELISA method[49]. By use of that method he tested 200 human serum samples and had 17% that were positive for Shiga toxin. We used a similar ELISA that is a commercial technique to detect antibody to Shiga Toxin in archived random samples to confirm the modified commercial ELISA method can be used to detect antibodies to toxins directly in serum and provide evidence for estimated STEC infection in Idaho.

Chapter II: Materials and Methods

Serum samples: The test sample consisted of archived deidentified random serum samples obtained from 2005 through 2007 and maintained frozen by Dr. Kathleen Spiegel. Samples used in this study were from Idaho inhabitants and were originally collected for other testing from surveillance hospitals, and routinely discarded by the hospitals after ten days storage at refrigerator temperatures. All patient identification was removed prior to storage. Such samples are commercially available from ARUP and other large laboratories. Samples were handled at all times according to standard (universal) precautions.

Surveillance hospitals: eight regional medical centers in Idaho have participated in this study. These hospitals are St. Luke's Magic Valley Medical Center(South Central region), St. Alphonsus Medical Center(Southwestern region), Portneuf Medical Center(Pocatello, PMC), Eastern Idaho Regional Medical Center (Eastern region), Bingham Memorial Hospital, Harms Memorial Hospital, Oneida County Hospital and Franklin County Medical Center (Preston). The samples are the routine blood sample from these eight hospitals.

Microwell plates: The Microwell plates were part of a commercially kit and were coated with mouse monoclonal antibodies to Shiga toxin 1 and 2, obtained from Premier Shigatoxin Kits. The Microwells were incubated with deactivated Shigatoxin from Premier Shigatoxin Kit overnight at a dilution of 1:1000. Plates were rinsed and dried.

Conjugate: Goat anti Human IgG conjugated to horseradish peroxidase (HRP) in buffered protein solution containing preservative was obtained from Thermo Scientific-Pierce.

(Labeled conjugate). The conjugate is a gentle affinity chromatography system for purifying HRP conjugated antibodies from unreacted enzyme after labeling.

Substrate: 3,3',5,5' - tetramethylbenzidine (TMB) ELISA Substrate for the conjugate was from Thermo Scientifc-Pierce. The TMB Substrates detect HRP activity, yielding a blue color that changes to yellow upon addition of a sulfuric or phosphoric acid stop solution.

Stop Solution : sulfuric acid is for use with the TMB ELISA substrate. Addition of sulfuric acid stop solution changes the blue color to yellow, stabilizing the color development to enable reading.

The positive plate control was positive serum from Achut Poudels experiments which had been stored at -70℃.

The negative plate control was negative serum from Achut Poudels experiments. Previous testing by Dr. Kathleen Spiegel established the concentrations of HRP and substrate which would give a visual color change when all reagents were added together, using sera previously testing positive and against human IgG coated plates.

Antibodies to toxin Testing Methods:

All reagents were allowed to warm to room temperature and mixed gently before use.

A pipette was used for dispensing the reagents. 50 ul of each sample was added to the appropriate microwell.

Apply 1 positive and 1 negative control well per batch. Mix wells by firmly shaking/swirling the plate for 30 seconds. Incubate the plate for 10 minutes at room temperature (22-27 C). Add one drop of enzyme conjugate to each well, incubate for 15 minutes. Shake out the contents of the wells and wash by completely filling each well with distilled water for 5 times. Add 1 drop of substrate to each well, incubate at room temperature for 5 minutes. Add one drop of stop solution to each well.

In a positive reaction, the enzyme bound to the well by toxin converts the substrate to a blue color-yellow after color stabilizer is added to the colored reaction product. Color development can be detected visually. In a negative reaction, there is no antibody to toxin or an insufficient amount of antibody to toxin present to bind the enzyme conjugate to the well and no colored reaction product develops.

Chapter III: Result

We tested 210 serum samples for Shiga toxin antibody reactivity(100 by Xueting Li, 110 by K.Spiegel). 10 samples had been previously examined by Achut Raj Poudel, 200 samples are the random serum collected from 8 surveillance hospitals. 5 batches are used. Each batch had one positive control well and one negative control well. All positive controls yielded a blue color after the substrate is added and changed to yellow upon addition of the stop solution. All negative controls presented as clear. Of these, a total of 25 of 200 serum samples (did not include Achutes 5 positive and 5 negative samples) were positive for Shiga toxin. The positive rate of STEC exposure was 12.5.

Chapter IV: Discussion

10 samples(all that was left 5 positive, 5 negative of those tested by Achut), which had been previously examined by Achut Ra The results matched Acuts previously reported results suggesting this ELISA method can be used to detect antibodies to toxins directly in serum. In addition, by implementing this approach, 200 additional untested archived samples have now been tested, and the similar high rate of Shiga toxin positive serum was detected. The percentage of positives was similar to the research result of Achut Raj Poudel. The positive rate by ELISA detection of antibodies was significantly higher than the incidence rate of laboratory-confirmed human STEC infection reported to CDC(6.6). It suggests that Idaho residents have greater exposure to an antigen or antigens that produce antibodies to the STEC then the CDC reported. As mentioned above, the test samples consisted of deidentified random serum samples which means the samples used in our study have no clinical history. The high sero-positivity maybe because infection associated with STEC, non-O157 STEC or any other Shiga toxin producing organism. It is similar to the above mentioned studies in Wyoming, Seattle and Ontario. The high incidence also could be due to other different causes, for example, the patient is a chronic carrier of the Shiga toxin who is not be reported. And confirmed that the possibility of the STEC, non-O157 STEC or any other Shiga toxin producing organism to infect humans but not cause reportable disease.

There were several studies which looked at farmers and antibodies to some of the *E.Coli* strains rather than detecting antibodies against the toxins. For example, in Haack 's research, they use ELISA to detect antibodies to *E. coli O157* LPS[47]. However there are about 200

STEC other serotypes recognized. In addition, from the data collected by National Enteric Disease Surveillance show that Idaho was the state had the highest reported incidence rates of non-O157 STEC infection in 2011 (the rate was 4.3 per 100,000). As a result, there are numerous pathogens other than *E. coli O157* capable of producing Shiga toxin or Shiga like toxins. In this study, the detection of antibodies to Shiga toxin rather than the organism-specific antigen detects the presence of the STEC or other Stx-producing species regardless of serogroup, which can provide more generalized data to incidence of infection.

Different methods have been used to detect STEC, including tissue culture cytotoxicity Assays, ElISA Assays, Reverse Passive Latex Aggutination, Polymerase Chain Reaction(PCR), Culture for O157 or Non-O157 STEC, for Isolation of STEC and Serological Diagnosis of STEC infection(table 1). Conventional methods for detection STEC is by inoculating stool samples onto Vero cells to observe a cytopathic effect on the cells, and neutralized the toxin by Stx monoclonal antibodies for further confirmation. This method is labor-intensive, time-consuming (48–72h) and requires expensive, dedicated instrumentation, software for analysis, and trained personnel potentially available only in specialized facilities. Compared to the conventional testing and the PCR method, the ELISA Assay used in our study is plate-based assay designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. The ELISA Assay format used in our study is the sandwich assay which is the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody (Figure 1). The ELISA Assay we used can avoid expensive reagents and equipment, provide increased sensitivity and specificity for the

detection of STEC, which is more accessible and inexpensive. And the accuracy and reproducibility were demonstrated with limited internal control samples.

Although, in study assay that detects Shiga toxins antibodies provides increased diagnostic accuracy and precision, the use of goat anti Human IgG conjugated to HRP (Labeled conjugate) is only specific for Human IgG antibody to the Shiga-toxin. As a result, we cannot detect IgM and IgA antibodies in this study. The detection of IgG can provide evidence that person was infected with Shiga toxin at some time during their life but the presence of IgG cannot determine when a person was infected. However, if antibody tests of paired acute phase and convalescent phase serum samples show a fourfold rise in IgG antibody and IgM antibody is present an active infection is indicated. For that reason, in the clinical diagnostic setting, detecting IgM class antibodies appear to be of greatest diagnostic values, and it is recommended that sera from patients with HUS be first tested for IgM or IgM+IgG antibodies. A test such as the one used here, measuring IgG class antibodies is more suitable to epidemiological studies. Also, negative direct Shiga toxin test results can occur if Shiga toxin genes are lost during infection or culture of the isolate.

The duration of IgG antibody level which could be detected may influence the population test results. The shorter period time of detection of IgG antibody in the serum can provide lower reported STEC incidence. Recently, several researchers have used IgG avidity assays to detect recent primary *CMV, rubella, and Toxoplasma gondii* infection in Pregnant Women. IgG avidity assays measure antibody maturity can help discriminate between recently

acquired and distant infection. The further study of IgG avidity assays may provide information about how Shiga toxin IgG antibody can be detected in the serum, and the relationship between the titer of IgG and the second exposure of the antigen.

STEC are found in the intestines and feces of a wide spectrum of healthy animal species, including cattle, sheep, goat, deer, moose, swine, horse, dog, cat, pigeon, chicken, turkey and gull. STEC are transmitted to humans by consumption of contaminated food or water, or through direct contact with infected animals or persons. The samples used in our study were collected from eight regional medical centers in Idaho. In the field of public health, compareing the incidence rate of STEC infection in different areas may provide information about the disease patterns of STEC-associated illness among rural and urban Idaho population, the association between STEC exposure and the livestock(cattle and other ruminants) density, water source contamination and similar potential factors.

An increased incidence of non-O157 STEC infections in the United States have been reported in some studies. The data collected by Idaho Bureau of Laboratories from expanded surveillance study suggest that more than half of Idaho STEC illnesses are resulted from non-O157 serotypes. The Shiga toxin antibodies detected in our study are not further sero-grouped and are presumed to be specific to the toxin which is an improvement over the previous assay which used acrode supernatant of the cultured organisms and may have picked up antibodies to surface antigens. A further advantage of this technique is that the person manufactures the plates and doing the testing is working with non-infectious material,

Another application of this study is to demonstrate the STEC serotypes, can also provide evidence of the incidence of the non-O157 infection which can help to establish STEC detection method and STEC disease control strategy.

Although the samples used in this study were from Idaho indigenous inhabitants, to exclude any selection preference, we may use more specific definition of the Idaho indigenous inhabitants, including the duration of patient's stay in Idaho, patient's travel history during specific period of time, and any diarrhea incurred in the trip. Even though this study provides a considerable number of individuals who may have been unintentionally exposed to the Shiga toxin, it may not conclude that they have been infected with the organism. Therefore, comprehensive research with intensive study is required to examine the accuracy of the data presented in this study. An in-depth research with volume data of human subjects from different parts of Idaho along with their medical history would provide a more accurate conclusion about the individuals who are associated to Shiga toxin or the organism that is causing the disease.

Chapter V: Conclusion

An ELISA method is used to detect antibodies against Shiga toxins in human serum in our study. Our findings confirmed that use of the ELISA technique can provide evidence for anti shigatoxin immunoreaction.

The higher incidence rate of STEC infection of random serum samples suggest that Idaho residents have greater exposure to an antigen or antigens that produce antibodies to the STEC then the CDC reported.

The recommendations for further study would include expanded sample quantity and increased surveillance hospital sites to attain more demographically accurate and precise results. Further study could also follow up serum anti Shiga activity, to determine the duration of detectable anti Shiga toxin IgG antibody.

In summary, our study has confirmed the ELISA method can be used to detect antibodies against Shiga toxin antigen in human serum, and the Idaho residents have greater exposure to an antigen or antigens that produce antibodies to the STEC then the CDC reported.

Figure1

ELASA method for antibody detection against Shiga toxin

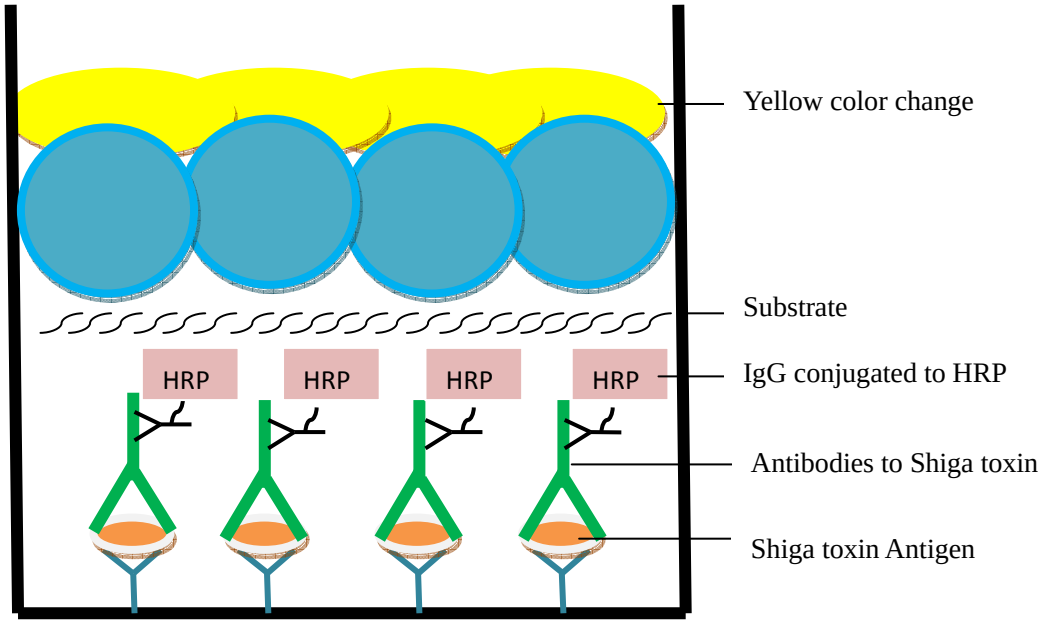

Table 1

Comparison of methods for detection of Shiga toxin–producing *Escherichia coli* (STEC)

Testing method	Principle	Comment
Tissue Culture Cytotoxicity Assays	Vero monolayers are treated with filter-sterilized fecal extracts or fecal culture filtrateds and examined for cytopathic effect after 48 to 72 hour incubation	Labor intensive Time-consuming cumbersome
ELISA Assays	Sandwich technique using immobilized monoclonal antibodies to the toxins as catching ligands	More rapid, detect the presence of STEC regardless of serogroup
RPLA	Incubation of serially diluted polymyxin B extracts of putative STEC cultures, with Stx1 and Stx2 specific antibody-coated latex particals, examining agglutination	Simple, rapid
PCR	Use oligonucleotide primer for amplification of stx genes	Rapid, sensitive, labor intensive, require highly skilled staff
Culture for O157 STEC	Culture on sorbitol-MacConkey agar	18-24 hour incubation Isolates must be tested to confirm Stx production
Culture for Non-O157 STEC	Hemolytic phenotype on washed sheep erythocyte agar	

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Appendix:

Raw data of the result of Shiga toxin antibody test

Random samples

Performed at Meridian

Well location	result	Well location	result	Well location	result	Well location	result
A1	Neg	B1	Neg	C1	Neg	D1	Neg
A2	Neg	B2	Neg	C2	Neg	D2	Pos
A3	Neg	B3	Pos	C3	Neg	D3	Neg
A4	Neg	B4	Neg	C4	Neg	D4	Pos
A5	Neg	B5	Neg	C5	Neg	D5	Pos
A6	Neg	B6	Neg	C6	Neg	D6	Pos
A7	Pos	B7	Neg	C7	Neg	D7	Pos
A8	Neg	B8	Neg	C8	Neg	D8	Pos
A9	Neg	B9	Neg	C9	Neg	D9	Pos
A10	Neg	B10	Neg	C10	Neg	D10	Pos

Raw data of the result of Shiga toxin antibody test

Random samples

Performed at Meridian

Well location	result	Well location	result	Well location	result	Well location	result
E1	Neg	F1	Neg	G1	Neg	H1	Pos
E2	Neg	F2	Neg	G2	Neg	H2	Neg
E3	Neg	F3	Neg	G3	Neg	H3	Neg
E4	Pos	F4	Neg	G4	Pos	H4	Neg
E5	Neg	F5	Neg	G5	Neg	H5	Neg
E6	Pos	F6	Neg	G6	Pos	H6	Neg
E7	Pos	F7	Neg	G7	Neg	H7	Neg
E8	Pos	F8	Neg	G8	Neg	H8	Neg
E9	Pos	F9	Neg	G9	Neg	H9	Neg
E10	Pos	F10	Neg	G10	Neg	H10	Neg

Raw data of the result of Shiga toxin antibody test

Random samples

Performed at Meridian

Well location	result	Well location	result	Well location	result	Well location	result
I1	Neg	J1	Neg	K1		L1	
I2	Neg	J2	Neg	K2		L2	
I3	Pos	J3	Neg	K3		L3	
I4	Neg	J4	Pos	K4		L4	
I5	Neg	J5	Neg	K5		L5	
I6	Neg	J6	Neg	K6		L6	
I7	Neg	J7	Neg	K7		L7	
I8	Neg	J8	Neg	K8		L8	
I9	Neg	J9	Neg	K9		L9	
I10	Neg	J10	Neg	K10		L10	