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SL440

Shiga Toxin-Producing *Escherichia coli*: Detection, Differentiation, and Implications for Food Safety[1]

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Introduction

Shiga toxin is a protein found within the genome of a type of virus called a bacteriophage. These bacteriophages can integrate into the genomes of the bacterium *E. coli*, giving rise to Shiga toxin-producing *E. coli* (STEC). Even though most *E. coli* are benign or even beneficial ("commensal") members of our gut microbial communities, strains of *E. coli* carrying Shiga-toxin encoding genes (as well as other virulence determinants) are highly pathogenic in humans and other animals. When mammals ingest these bacteria, STECs can undergo phage-driven lysis and deliver these toxins to mammalian guts. The Shiga toxin consists of an A subunit and 5 identical B subunits. The B subunits are involved in binding to gut epithelial cells. The A subunit is composed of the catalytically active A1 subunit and the A2 fragment, which stabilizes the AB_5 holotoxin structure. In humans, these toxins can cause hemolytic uremic syndrome (HUS). Such infections involve the production of substances that destroy blood vessels and may cause severe kidney damage. The first documented case of clinical HUS associated with Shiga toxin was in 1983. Since then, STECs have been recognized as a common cause of acute renal failure among children in the United States. In the past, raw or undercooked beef was a common source of STEC contamination. More recently, STEC infections associated with fresh produce have significantly increased, and STEC infections from milk, juice, soft cheeses, and contaminated water have also been reported, thus underscoring the need for the effective detection of the Shiga-toxin-producing pathogens in a variety of food matrices.

There are two types of Shiga toxins—Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Stx1 is composed of several subtypes knows as Stx1a, Stx1c, and Stx1d. Stx2 has seven subtypes: a–g. Strains carrying all of the Stx1 subtypes affect humans, though they are less potent than that of Stx2. Stx2 subtypes a,c, and d are frequently associated with human illness, while the other subtypes affect different animals. Subtypes Stx2b and Stx2e affect neonatal piglets, while target hosts for Stx2f and Stx2g subtypes are not currently known (Fuller et al. 2011). Stx2f was originally isolated from feral pigeons (Schmidt et al. 2000), and Stx2g was isolated from cow feces (Leung and Peiris 2003). Because of the diversity of Shiga toxins in nature and their broad range of hosts, detection and differentiation of these toxins is critical. Researchers have developed numerous methods for detecting and distinguishing between the various types and subtypes of these toxins. One such method involves polymerase chain reaction (PCR) using a multiplex system, which is intended to distinguish between many types and subtypes of Stx at the same time (Scheutz et al. 2012). Another nucleic acid-based method is quantitative real-time PCR (qPCR), which, like PCR, relies on the ability to measure the presence of a given gene in a sample (Bustin et al. 2009). Reverse Transcription qPCR (RT-qPCR) (Bustin et al. 2009), which measures gene expression, can be used in clinical, food testing, and research labs; although, due

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to the instability of RNA, once extracted, RT-qPCR has limited applications in diagnostics and food testing.

A third approach has been developed using monoclonal antibodies that can bind to the toxins and differentiate them based on their immunological properties (Skinner et al. 2015). A fourth approach involves the use of mass spectrometry to detect these toxins (Fagerquist and Zaragoza 2016; Fagerquist et al. 2014). Each approach has benefits and caveats, so it is important to use them in a complimentary fashion for robust detection and differentiation. In fact, the Centers for Disease Control and Prevention (CDC) highlights the need for complementary approaches in the identification of STEC in clinical samples. The CDC suggests a combination of culture-based techniques and a simultaneous assay for the Shiga toxins or the genes encoding these toxins (Gould et al. 2009). Even though this CDC guideline was created for clinical samples, it is prudent to follow similar approaches for environmental and food testing.

This EDIS document was developed for laboratory scientists and technicians working on the detection and identification of STEC in food matrices and environmental samples. This document will also be useful for clients of such laboratories to assist with the determination of which tool set is most appropriate for the detection of STEC. Our goal is to compare and contrast the advantages and disadvantages of common laboratory techniques used for this purpose. Assessment of sample data sets is also included in the document to assist with data interpretation.

Detection of STEC

Culture-Based Detection

O157 STEC can usually be distinguished from most commensal *E. coli* by their inability to ferment sorbitol within 24 hours when plated on a sorbitol-containing agar. To isolate O157 STEC, samples are plated onto a selective and differential media, such as sorbitol-MacConkey agar (SMAC), cefixime tellurite-sorbitol MacConkey agar (CT-SMAC), or CHROMagar O157. After incubation for 16 to 24 hours at 37°C (99°F), the plate should be examined for possible O157 colonies, which are colorless on SMAC or CT-SMAC and are mauve or pink on CHROMagar O157. Both CT-SMAC and CHROMagar O157 are considered to be more selective than SMAC (Church et al. 2007). Non-motile flagella-less (H-) sorbitol-fermenting STEC O157, which are fairly uncommon in the United States and primarily reported in Europe, might not grow on CT-SMAC agar because the bacteria are susceptible to tellurite (Gould et al. 2014).

To isolate non-O157 STEC, the CDC recommends that the Shiga-toxin-positive liquid cultures are streaked to a less selective agar (e.g., MacConkey agar, SMAC, Statens Serum Institut [SSI] enteric medium, or blood agar) (Gould et al. 2014). Traditional enteric media, such as Hektoen agar, xylose-lysine-desoxycholate (XLD) agar, and *Salmonella-Shigella* agar, may inhibit many *E. coli* and are, therefore, less desirable (Blom et al. 1999). Most non-O157 STEC ferment both sorbitol and lactose, although exceptions have been reported (Gould et al. 2014). Representative results of culture-based tests are shown in Figure 1.

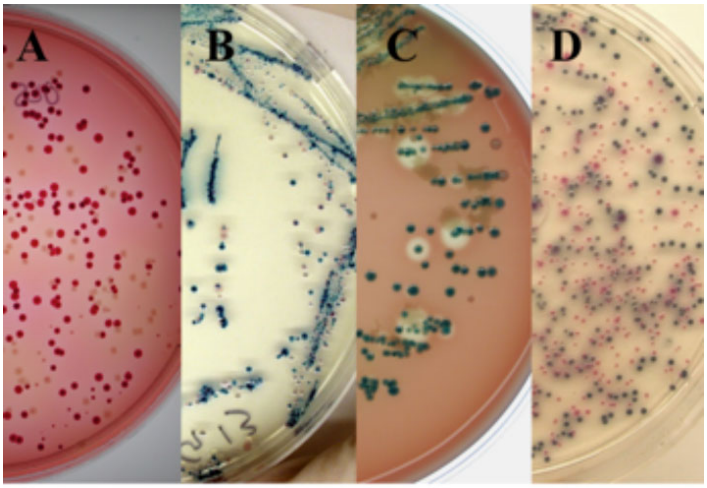

Figure 1. STEC isolation from various selective media. (A) Cells from enrichment broth are plated on CT-SMAC. (B) Suspect colonies appear as pale on CT-SMAC and steel blue on NT-Rainbow Media, and non-O157 STECs appear as pink colonies on NT-Rainbow (C) Suspect STECS expressing b-galactosidase and hemolysin are indicated by blue colonies with a zone of clearing on Sheeps blood agar (D) Typical non-O157 STECs are shown growing on CHROMagar and appear as blue colonies.
Credits: Mike Cooley

Nucleic-Acid-Based Detection

Recently, a PCR-based method has been developed to test samples from various sources for the presence of stx genes (Scheutz et al. 2012). PCR is easy to perform and relatively inexpensive. This protocol involves a multiplex PCR approach that employs multiple primer sets in a single PCR reaction in order to detect different types and subtypes of stx genes in a single sample. This approach was tested by multiple laboratories on various thermocyclers and was found to be robust. However, results indicate that caution must be exercised when testing samples for the presence of stx2 c and d subtypes, because different laboratories reported instances of cross-reactivity between these two subtypes in PCR assays. In situations where cross-reactivity is suspected, researchers may have to repeat the analysis

without multiplexing. If cross-reactivity still occurs (e.g., a PCR product is seen for both stx2c and stx2d subtypes, even when the primers are used in separate reactions, like in Figure 2), researchers may have to verify the result with an orthologous method. In addition to these caveats, RT-qPCR may fail to detect stx mRNA because the genes are not being expressed, which creates the potential for a false negative. Analysts must also be aware of the presence of cryptic bacteriophages. Cryptic bacteriophages are prophages that have become trapped within a bacterial genome as a result of genomic rearrangements or genetic decay. The cryptic phages may be inactive in terms of lysis or phage particle biosynthesis yet still confer benefits to the host such as toxin production or antibiotic resistance (Wang et al. 2010). Shiga-toxin-bearing cryptic phages may still produce toxin and remain a potential threat to human health. Certain environments, such as the human gut, or an exposure to DNA-damaging antibiotics, can induce toxin production. These cryptic phages are typically permanent aspects of a bacterial genome and may harbor *stx* genes that are never or weakly expressed (Wang et al. 2010; Teel et al. 2002).This can make detection by methods that rely on expression of the genes, or production of the toxin, difficult.

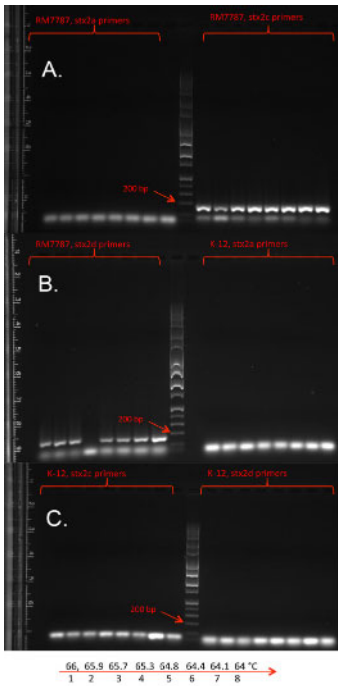

Figure 2. PCR-based detection of STEC. (A) is the gel electrophoresis of a temperature gradient PCR reaction with primers for stx2a and stx2c against the DNA template of an stx2c-containing STEC strain RM7787. (B) Gel electrophoresis of a PCR reaction against the same strain (RM7787) with PCR primers targeting stx2d as well as a control K-12 strain assayed with *stx2a* primers. (C) shows stx2c and stx2d primers against a control K-12 strain. Temperature gradient is listed below all panels. Temperature increases in each consecutive well. Stx2d primers cross-react with the stx2c gene at almost all temperatures in RM7787.

For PCR-based detection, colonies to be tested must be grown on nonselective agars, such as tryptic soy agar (TSA), heart infusion agar (HIA), or blood agar (Gould et al. 2009); tests done on colonies grown on selective media often interfere with the PCR reaction leading to poorly reproducible results. According to the CDC, DNA-based Shiga toxin gene detection is not currently approved for the diagnosis of STEC infection in human clinical samples (Gould et al. 2009). Clinical laboratories do not routinely verify putative positives with PCR-based methods and instead rely on culturing for O157 detection. Currently, FDA-approved, DNA-based tests for subtyping Shiga toxin genes in food only differentiate between stx1 and stx2. In 2012, the CDC began monitoring for non-O157 STEC infections (Stigi et al. 2012). Tests for these STECs have recently been added to surveillance lists with the Food and Drug Administration (FDA)(Feng, Weagant, and Jinneman 2011). Public health laboratories regularly combine culture-based methods with immunological assays, serology, immunoassays, and PCR-based techniques.

Luminex PCR Assay

A multiplex PCR assay using the Luminex system (Luminex Corporation) was approved by the FDA in 2011 for detecting the serotypes of STECS. This approach combines multiple primers into a single reaction, along with hybridization to microbeads, and is able to distinguish between serogroups O26, O45, O103, O111, O121, O145, and O157, O91, O113, and O128 (Clotilde et al. 2015). In addition, this assay also screens samples from various sources for the presence of adherence factor genes *eae* and *aggR* (Feng, Weagant, and Jinneman 2011). Researchers must practice caution when using this technique as authors note that some strains remain untypable by this approach (Clotilde et al. 2015). Additional drawbacks include the high cost of the Luminex platform and reagents.

Detection by Monoclonal Antibodies

The Center for Devices and Radiological Health of the FDA has approved four immunoassays for the detection of Shiga toxin in clinical samples (Gould et al. 2009). The kits are as follows: 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. While these kits can detect Shiga toxin in the enrichment samples, none are able to distinguish between the seven subtypes of Stx2 or the three subtypes of Stx1.

Until recently, differentiation *of all* subtypes of stx2 was impossible by monoclonal antibody (mAB). In 2015, researchers designed sandwich ELISAs capable of detecting and distinguishing between stx2 subtypes a, c, and d(Skinner et al. 2015). These antibodies provide a great way to quickly test samples of various types to obtain putative detection of stx toxins. In addition to these advances, the antibodies have recently been tested in numerous sample types, including pure culture and beef (He et al. 2016). However, care must be exercised when employing these antibodies, especially when differentiating between the stx subtypes a, c, and d. The high levels of homology between these three clinically relevant subtypes of Stx2 make cross-reactivity a potential drawback of this technique. Stx subtypes c and d remain particularly problematic as each subtype has a continuum of variation at the amino acid level, and these antibodies have not been tested against all possible permutations of those subtypes. In particular, only the ELISA for Stx2a has been tested for efficacy in meat (He et al. 2016).

Detection by Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that characterizes atoms and molecules based on their mass. As such, it has a high degree of chemical specificity. Sequence-specific or structural information of a molecule (even a large biomolecule) can also be obtained by tandem mass spectrometry (MS/MS) wherein an analyte molecule is isolated in the gas phase, fragmented, and the resulting fragment ions detected. Recently, a mass spectrometry-based top-down proteomic method was developed for the detection and differentiation of most Stx subtypes in laboratory culture (Fagerquist et al. 2014; Fagerquist et al. 2013). Matrix-assisted laser desorption/ionization (MALDI) time-of-flight-time-of-flight (TOF-TOF)-MS and MS/MS was used to identify the A2 fragment and the B-subunit to distinguish between nearly all subtypes of Stx. This technique relies on the ability of the bacteriophage present in the STEC to be induced by various stressors on laboratory media which means that some *stx*-bearing isolates may not be detected because not all STECs respond to induction in exactly the same way. To date, Stx2 subtypes e and b do not appear to induce (at least in the strains studied thus far) as a result of antibiotic stress by exposure to ciprofloxacin or mitomycin-C. Additionally, this assay requires analysis from cultured bacteria and thus may not work in complex samples such as food, stool or soil. This approach is able to distinguish between Stx1 and Stx2. Within Stx2, the method can distinguish subtypes: a, c, d, f, and g.

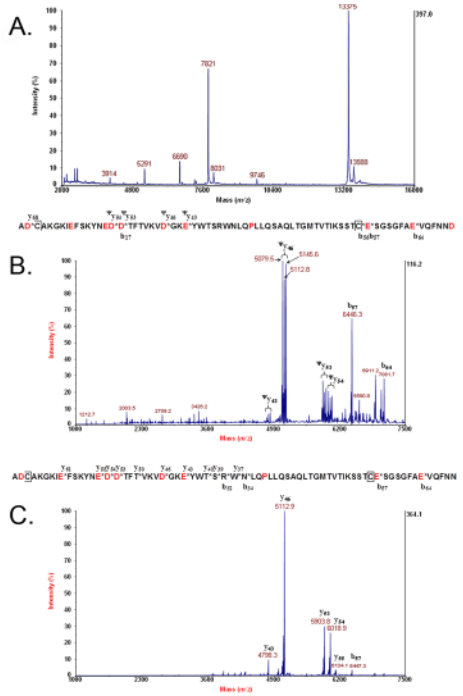

Figure 3. STEC detection with mass spectrometry. (A) MS data for bacterial cell lysate of E. coli O104:H4 (German outbreak strain) grown on LBA with no antibiotic. (B) MSdata for bacterial cell lysate of *E. coli* O104:H4 grown on LBA supplemented with ciprofloxacin. The peak at m/z 7820 is the B subunit of Stx2. (C) MS data for bacterial cell lysate of E. coli O104:H4 grown on LBA supplemented with ciprofloxacin, digested with furin, and disulfide reduced. The peaks at m/z 7821 and m/z 5291 are the B subunit and A2 fragment of Stx2a, respectively. Credits: This figure was originally published in Fagerquist et al. (2014) and is used here with the publisher's permission.

Cell Cytotoxicity Assays

A GFP-expressing line of Vero cells derived from African green monkey kidney and HeLa cells have been developed to detect protein synthesis inhibitors. These cell lines are well-suited to detect Shiga toxin because they are decorated with large amounts of Gb3 and Gb4 receptors, which the toxins use to gain entry into the eukaryotic cell (Quiñones and Swimley 2011). These assays include inoculating cell lines with enrichment cultures or fecal filtrates and observing cytopathic effect. This effect can be measured in a number of ways, including bioluminescence from luciferase or the quenching of fluorescence due to GFP inhibition (Quiñones et al. 2009). This method is highly sensitive, so care must be taken in interpreting results. These cells measure the disruption of protein synthesis, and enrichment cultures, fecal, or blood samples can include many things that disrupt protein synthesis, yielding potential false positives. Orthologous techniques, such as ELISAs or mass spectrometry, may be employed to verify putative positives.

In summary, many powerful techniques have been developed to screen samples for the presence of Shiga toxins.

Each technique has benefits and drawbacks, and analysts must be careful when interpreting results, combining orthologous techniques to verify putative positives. The ability to distinguish between each subtype is critical because not all STECs are clinically relevant.

Shiga Toxin and Food Safety

Shiga toxins of all types have been detected in almost every point of the food production chain. They are commonly associated with the guts of cows, pigs, deer, and poultry. In addition to these sources, the toxins have been found in public waterways and on produce such as alfalfa sprouts (Erickson and Doyle 2011). Given the prevalence of STECs in our food supply, it is critical to have regulations in place in order to protect consumers and the food supply, as well as robust tests that can detect and differentiate between the various Stx types and subtypes. The USDA Food Safety and Inspection Service (FSIS) has instituted regulations for O157:H7 and six non-O157 serotypes of *E. coli* in an effort to ensure the safety of the nation's food supply. These regulations require an infrastructure of laboratories and experts that can test all components that go into food production and report possible contamination events to authorities who can act to prevent outbreaks.

Please keep in mind that the CDC recommends that (1) all O157 STEC isolates be forwarded to state or local public health laboratories for confirmation and additional molecular characterization (i.e., PFGE analysis and virulence gene characterization); (2) detection of STEC or Shiga toxin be reported promptly to the treating physician; (3) isolates be sent to the public health laboratory for confirmation, isolation, and subsequent testing of the organism, and to the appropriate public health authorities for case investigation; (4) samples in which Shiga toxin or STEC are detected but from which O157 STEC are not recovered should be forwarded as soon as possible to a state or local public health (Gould et al. 2009).

Summary

All unprocessed food products typically harbor microorganisms. Some foods and the components that go into food production may contain pathogenic microorganisms such as STECs. When consumed, these STECs can cause serious illness in humans and may even lead to death. In 2011, an outbreak involving an atypical STEC, O104 EaggEC with a genetically divergent lineage from O157, led to more than 800 cases of HUS that spanned multiple continents. Outbreaks like these highlight the importance of developing robust methods to detect and differentiate these toxins in our food supply. In addition to their development, regulations ensuring their strict implementation into the farm-to-fork continuum are critical to ensure food safety and prevent outbreaks.

STEC infections are extremely time-sensitive. Early detection improves outcomes associated with treating patients and controlling outbreaks. Because of this, simultaneous detection of O157 and non-O157 STECs should be standard practice. Detection of STECs within the first 24 hours of infection can greatly improve the clinical outcome for affected patients, reducing the risk for severe disease. Additionally, rapid isolation of the organism assists public health officials in tracking the outbreak and controlling it's spread (Gould et al. 2009).

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