Guidance for Public Health Laboratories

Isolation and Characterization of Shiga toxin-producing *Escherichia coli* (STEC) from Clinical Specimens

This publication was produced jointly by the Centers for Disease Control and Prevention and the Association of Public Health Laboratories
These recommendations are the result of a collaborative effort of public health microbiologists from ten states, the Association of Public Health Laboratories, and the Centers for Disease Control and Prevention.

This publication was supported by Cooperative Agreement Number #1U60HM000803 from CDC. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC.
GUIDANCE FOR PUBLIC HEALTH LABORATORIES
ON THE ISOLATION AND CHARACTERIZATION
OF SHIGA TOXIN-PRODUCING ESCHERICHIA COLI (STEC)
FROM CLINICAL SPECIMENS

April 2012

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INTRODUCTION AND BENEFITS OF THE RECOMMENDATIONS
INTRODUCTION AND BENEFITS OF THE RECOMMENDATIONS

Clinical laboratories are the front line in detecting Shiga toxin-producing *Escherichia coli* (STEC) disease. They are responsible for the initial detection of suspect cases by testing primary specimens. In October 2009, a report entitled “Recommendations for Diagnosis of Shiga toxin-producing *Escherichia coli* Infections by Clinical Laboratories”\(^1\) stated that clinical laboratories should simultaneously test all stools submitted for the routine diagnosis of acute, community-acquired diarrhea for *E. coli* O157:H7 by culture and for non-O157 STEC by an assay that detects Shiga toxins or the genes encoding these toxins regardless of patient age, time of year, or presence or absence of blood in the stool (see below). As part of these recommendations, clinical laboratories should forward, as soon as possible, all confirmed and presumptive O157 STEC isolates and Shiga toxin-positive broths that do not yield O157 STEC to a public health laboratory for organism isolation and characterization. These recommendations are based on evidence which indicates that the use of enzyme immunoassays (EIA) for screening, in conjunction with culture, followed by molecular characterization of isolates at the public health laboratory, provides increased diagnostic sensitivity compared with the use of either method alone.\(^2\)

**Clinical Laboratory Recommendations**

![Flowchart of Clinical Laboratory Recommendations](chart.png)
Public health laboratories have the important role of assessing STEC strains for phenotypic and genotypic characteristics that may link patients to an outbreak or that may indicate an increase in the organism’s virulence. One such assessment tool is pulsed-field gel electrophoresis (PFGE), which allows investigators to compare isolates and provides a critical step in outbreak detection and response. Other assessment tools include lateral flow immunoassays and PCR assays that provide information about the type of Shiga toxin(s) produced and the profile of virulence genes present to help distinguish between strains and assess their pathogenicity.

Ultimately, it is the timely transfer of positive samples from the clinical laboratory to the public health laboratory—along with open communication among clinical microbiologists, public health microbiologists, and foodborne disease epidemiologists—that is vital to the successful characterization of STEC infections and the initiation of outbreak investigations and public health control measures. Public health laboratories are encouraged to partner with the primary testing facilities in their jurisdiction to improve STEC diagnosis and surveillance. Partnership activities include educating professionals in the health care community about appropriate diagnostic testing options, facilitating timely submission of specimens to the public health laboratory, and aiding in the implementation of the clinical best practices at primary testing facilities.

As a companion piece to the clinical laboratory recommendations, this document provides recommendations to public health laboratories for the isolation of STEC from Shiga toxin-positive stools/specimens and for the characterization of isolates including verification, virulence gene detection, and PFGE analysis. These public health recommendations, in conjunction with the recommendations for clinical laboratories, provide a solid framework for the efficient and rapid diagnosis of this important group of pathogens.

If all public health laboratories adopt these recommendations, comparable services will be provided to clinical laboratories throughout the country, which may lead to improved comparability of laboratory findings between jurisdictions for surveillance purposes and outbreak investigations.

These recommendations are the result of a collaborative effort of public health microbiologists from ten states, the Association of Public Health Laboratories, and the Centers for Disease Control and Prevention. The recommendations are a living document, and it is the intention of the Workgroup to modify the recommendations periodically as new information becomes available.
Benefits of the Best Practice Recommendations for Public Health Laboratories

- **Improved service to clinical laboratories and to surveillance programs:** There are no standard algorithms for isolation and characterization of STEC from the types of biological materials typically received in Public Health Laboratories, and there is a general lack of published scientific studies upon which to base specific testing recommendations. These Best Practice Recommendations are based on available information and the collective expertise of the Workgroup members. The recommendations are designed to minimize turnaround times and maximize rates of STEC isolation, with the ultimate goal of improving service both to submitting clinical laboratories and to STEC surveillance programs. Our ability to detect STEC outbreaks and implement appropriate public health interventions is directly dependent on Public Health Laboratory testing practices.

- **Standardization:** These recommendations provide public health laboratories with uniform specimen submission and testing recommendations. Standardization will improve the overall quality of testing, and will also provide surveillance data that can be interpreted easily on a national level. Isolation rates in one state may be directly compared to isolation rates in another if the same methods are used. STEC identification and characterization is a key component of PulseNet, which derives its effectiveness, in part, from standardization.

- **Improvement of testing for HUS:** These recommendations provide guidelines for active follow-up of hemolytic uremic syndrome (HUS) cases and improved recovery of STEC from clinical specimens. These cases serve as sentinels of significant STEC events, and the specific diagnosis of STEC in HUS cases assists with diagnosis and treatment.
BEST PRACTICE RECOMMENDATIONS

All presumptive Shiga toxin-positive enrichment broth cultures and clinical specimens should be cultured for O157 STEC using selective media designed for this serotype.* If negative for O157 STEC, isolation of non-O157 STEC should be attempted by screening individual organisms for Shiga toxin production or the presence of Shiga toxin genes. If STEC is not isolated and the specimen is from a hemolytic uremic syndrome (HUS) patient, immunomagnetic separation methods for O157, O111, O145, O103 and O26 should be attempted or the specimen should be forwarded to a public health laboratory with this testing capability.

All presumptive STEC isolates should be confirmed as _Escherichia coli_, tested for the most prevalent and virulent O serogroup (O157) or the six most common non-O157 serogroups (O26, O45, O103, O111, O121 and O145), subtyped by pulsed-field gel electrophoresis (PFGE), and tested for Shiga toxin production or the presence of Shiga toxin genes.

* If your laboratory chooses to screen the enrichment broth with an enzyme immunoassay or polymerase chain reaction assay, the broth should be cultured per the recommendations, regardless of the screening result. Samples/broths should not be sent to the CDC _E. coli_ National Reference Laboratory unless all recommended testing has been performed on the sample/broth.
RECOMMENDED TESTING ALGORITHMS

For stool specimens or enrichment broths that were positive for Shiga toxin (Stx) or Shiga toxin DNA (stx) at the clinical laboratory or for stool samples submitted from patients with a diagnosis of hemolytic uremic syndrome (HUS)

- Plate stool specimen or broth to a selective and differential medium for O157 STEC (Cefixime-Tellurite Sorbitol MacConkey [CT-SMAC] or CHROMagar™ O157) and a less-selective medium (MacConkey agar [MAC] or washed sheep’s blood agar with calcium chloride [WSBA-Ca]).

- Examine plates for suspect O157 STEC colonies. If suspect O157 colonies are present, select at least 3 colonies for agglutination in O157 latex reagent. If positive for O157 agglutination, refer to page 8, “For characterization of presumptive O157 STEC isolates.”

- If no O157 colonies are identified, test a loopful of growth (sweep) from the less-selective plating medium (MAC or WSBA-Ca) using a methodology that detects Stx, such as EIA or Vero cell culture, or stx genes, such as PCR.

- If the sweep is positive for Stx or stx, test at least five colonies representative of the various colony types on the plate with a methodology that detects Stx or stx such as EIA, Vero cell culture or PCR. If the first five colonies are negative for Stx or stx, an additional five colonies should be selected for testing. Once 10 colonies are tested and found to be negative for Stx or stx, screening may be discontinued.

- If the sweep is negative for Stx or stx, screening may be discontinued and the specimen reported as STEC-negative.

- If there is no growth on the original plating media, screening may be discontinued. Routine submission of such samples to CDC is not recommended unless circumstances of the investigation indicate further testing is needed. Please contact the CDC E. coli National Reference Laboratory for discussion before samples are submitted.

- For specimens from cases of HUS or individuals with special epidemiological significance (such as possible asymptomatic carriage or cases with an epidemiological link to a confirmed case), enhanced testing such as immunomagnetic separation (IMS) (see “Special Considerations” and “Immunomagnetic Separation”) or PCR for additional virulence markers (see “History and Biology”) should be considered.
For characterization of presumptive O157 STEC isolates:

- Confirm as *E. coli* with biochemical analysis
  - Presumptive O157 STEC isolates should be confirmed biochemically as *E. coli* since several other gram-negative enteric species may cross-react with O157 antiserum, such as *Salmonella* O Group N, *Yersinia enterocolitica*, *Citrobacter freundii*, and *Escherichia hermannii*.3

- Confirm O157 serogroup from a pure isolate

- Characterize isolate for stx by PCR for stx1 and stx2 or by an antigen assay that can distinguish between Shiga toxin 1 and 2

- Subtype by pulsed-field gel electrophoresis (PFGE)

For characterization of presumptive non-O157 STEC isolates:

- Confirm as *E. coli* with biochemical analysis

- Characterize isolate for stx by PCR for stx1 and stx2 or by an antigen assay that can distinguish between Shiga toxin 1 and 2

- Determine if the isolate is one of the six most common serogroups (O26, O45, O103, O111, O121, or O145) or O157
  - It is recommended that sorbitol fermenting isolates that produce Stx be screened with O157 in addition to the above serogroups. Sorbitol fermenting O157 STEC have been identified.4
  - Additional O groups may be added based on the prevalence of other O groups within your jurisdiction.

- If the organism’s serogroup is not one of the six mentioned above, the isolate should be sent to the CDC *E. coli* National Reference Laboratory for complete serotyping and virulence gene characterization

- Subtype by pulsed-field gel electrophoresis (PFGE)

Routine testing for the H antigen of any STEC isolates is generally not necessary (See further discussions in "Serotyping of STEC"). H antigen characterization may be required during outbreaks or for publications regarding outbreaks or other special requests from epidemiologists. If needed, H antigen typing on any STEC may be requested from the CDC *E. coli* National Reference Laboratory.
SPECIAL CONSIDERATIONS
SPECIAL CONSIDERATIONS

Follow-up Testing
The public health laboratory may be asked to perform follow-up testing of stool specimens for STEC from convalescent children before they can return to daycare or from adults whose work places them at high risk of transmitting infectious organisms to help assess their suitability to return to work. This typically applies to persons working in food service establishments, in child-care facilities, as caterers, or as healthcare employees with direct patient contact. The criteria for returning to a normal activity schedule should be established in each state jointly by epidemiologists and laboratory scientists in a manner consistent with the best practice presented in this guidance document, current scientific literature, and any applicable state laws or regulations.

Items to consider when drafting such criteria are:

- the clinical laboratory testing practices within your jurisdiction and the performance characteristics of those testing practices regarding their ability to diagnose a case of STEC infection\(^1\);
- the number of negative stool samples that must be documented;
- the timing of stool sample collection for follow-up testing, taking into consideration the patient’s age and the duration of shedding\(^6\);
- the ability of asymptomatic individuals to shed viable organisms\(^6-8\);
- the quality of specimen transport;
- the turnaround time for the follow-up testing method, keeping in mind people’s livelihoods are dependent on the timing and accuracy of the results;
- the virulence potential of the STEC strain.

Outbreak Specimen Testing
The STEC best practice testing algorithm may be altered or deferred in certain extreme outbreak situations. PulseNet Area Laboratories and CDC may be a resource for public health laboratories that are overwhelmed with specimen testing and isolate characterization during an outbreak.

Conversely, enhanced testing beyond these best practice recommendations may be warranted in certain outbreak settings. For example, immunomagnetic separation (IMS) may be useful for identifying subclinical infections in daycare outbreaks.\(^9\) This information may allow for targeted control measures such as cohorting of cases rather than closing the center and possibly spreading disease throughout the community.
Hemolytic Uremic Syndrome Testing

Shiga toxin-induced cases of HUS may be missed entirely unless clinical laboratories are educated about this possibility and are asked to save and submit original stool specimens. Epidemiologists should coordinate with infectious disease physicians, pediatricians, nephrologists or other specialists to ensure that stools are collected and sent to the clinical laboratory. Public health laboratories should coordinate with clinical laboratories to ensure stools are appropriately tested, saved and submitted for further testing when necessary. When submitting specimens from patients with HUS to the public health laboratory, clinical laboratories should clearly label the suspect diagnosis on the requisition slip with the knowledge that specimens are difficult to obtain from HUS patients and that recovery of STEC from HUS specimens may require enhanced detection methods. Public health laboratories should examine stools and EIA broths (possibly even broth samples testing negative for Shiga toxin at the clinical laboratory) from HUS cases using the recommended methods described, with the understanding that enhanced testing may be warranted. Refer to the HUS Section for further explanation of this disease and the importance and availability of immunomagnetic separation (IMS) testing.
CULTURE AND BIOCHEMICAL CHARACTERIZATION

This document recommends* Cefixime-Tellurite Sorbitol MacConkey (CT-SMAC) or CHROMagar™ 0157 for isolation of 0157 STEC since these are more inhibitory for commensal stool flora than Sorbitol MacConkey (SMAC) or MacConkey (MAC) and have been shown to increase the sensitivity of culture for detection of 0157 STEC.10, 11 To isolate non-0157 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)12. Statens Serum Institut enteric medium or blood agar are alternate media but do not offer the differential advantage of MAC for detecting non-0157 STEC or WSBA-Ca for the detection of hemolytic STEC colonies.

All 0157 STEC and 80 percent of non-0157 STEC produce a characteristic enterohemolysin (Ehly). More than 97 percent of 952 isolates in the six most frequently isolated serogroups (O26, O111, O103, O121, O45, O145) received at CDC from 2006 to 2007 possessed the Ehly virulence gene (N. Strockbine, CDC, unpublished data). WSBA-Ca may be used to detect enterohemolytic activity.13 Ehly-producing colonies produce a zone of hemolysis on WSBA-Ca after 18 to 24 hours of incubation. Incorporation of mitomycin C into the WSBA-Ca enhances the appearance of the Ehly hemolysis and increases the proportion of non-0157 STEC that exhibit this activity.14 However, caution should be used, as signs of hemolytic activity do not definitively indicate STEC. Stx screening and biochemical characterization are still warranted. Also, since some STEC strains do not demonstrate the enterohemolytic phenotype and because enterohemolytic nontoxigenic strains have been reported, additional screening methods should be used in conjunction with WSBA-Ca medium.15

*The media, test kits, and manufacturers mentioned are not intended to be all inclusive of what is currently available, nor are they intended to be an endorsement of a particular product.
### Characteristics and properties of the media recommended for isolation of STEC

<table>
<thead>
<tr>
<th>MEDIA</th>
<th>CHARACTERISTICS</th>
<th>PROPERTIES</th>
<th>COLONY MORPHOLOGY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC)</td>
<td>Selective and differential Distinguishes O157 from other fecal <em>E. coli</em></td>
<td>The addition of cefixime and tellurite greatly inhibits <em>Proteus mirabilis</em>, non-O157 STEC, and other sorbitol non-fermenting strains.¹¹</td>
<td>O157 STEC appear Clear Non-O157 STEC appear Pink Other normal enteric flora appear Pink (See Figure 1)</td>
</tr>
<tr>
<td>CHROMagar™ O157</td>
<td>Selective and differential Distinguishes O157 from other fecal <em>E. coli</em></td>
<td>Potassium tellurite, cefixime and cefsulodin reduce the number of bacteria other than <em>E. coli</em> O157:H7 that will grow. The chromogen mix consists of artificial substrates, which release an insoluble colored compound when hydrolyzed by a specific enzyme.¹⁰</td>
<td>O157 STEC appear Mauve Non-O157 STEC appear Steel Blue or Blue Green Other organisms appear Colorless (See Figure 2)</td>
</tr>
<tr>
<td>Rainbow® Agar</td>
<td>Selective and differential Distinguishes O157 from other fecal <em>E. coli</em></td>
<td>Tellurite and novobiocin reduce the number of bacteria other than <em>E. coli</em> O157:H7 that will grow. The chromogen mix consists of artificial substrates, which release an insoluble colored compound when hydrolyzed by a specific enzyme.¹⁶</td>
<td>O157 STEC appear Black/Grey Non-O157 STEC appear Purple or Violet Other organisms appear Pink (See Figure 3)</td>
</tr>
<tr>
<td>Sorbitol MacConkey Agar (SMAC)</td>
<td>Modified MacConkey agar Distinguishes O157 from other fecal <em>E. coli</em></td>
<td>Primary carbon source sorbitol supports growth of non-O157 STEC The bile salts and crystal violet inhibit the growth of gram-positive bacteria.</td>
<td>O157 STEC appear Clear Non-O157 STEC appear Pink Other normal enteric flora appear Pink (See Figure 4)</td>
</tr>
<tr>
<td>Washed Sheep’s Blood Agar¹⁴</td>
<td>Allows observation of enterohemolysin production</td>
<td>Sheep’s blood washed with phosphate buffered saline and enhanced with calcium chloride and mitomycin C.</td>
<td>After 18-24 hours incubation, a zone of hemolysis should be visible surrounding any STEC colonies producing enterohemolysin* (See Figure 5)</td>
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</table>

*Some normal enteric *Escherichia coli* may produce an enterohemolysin after 5hrs of incubation. Any isolates selected from this media should be tested for the presence of Shiga toxin.*
Figure 1:
Left side – Clear colonies of O157 STEC on CT-SMAC plate after incubation for 24 hours
Right side – Pink colonies of non-O157 STEC on CT-SMAC plate

Images courtesy of the New York State Department of Agriculture & Markets Food Laboratory Division

Figure 2:
O157 STEC colonies appear Mauve, and non-O157 STEC colonies appear Steel Blue or Blue Green on a CHROMagar™ O157 plate.

Image courtesy of the New York State Department of Agriculture & Markets Food Laboratory Division
Figure 3:
Rainbow® agar plate with O157 STEC colonies appearing Black/Grey

Image courtesy of the New York State Department of Agriculture & Markets Food Laboratory Division

Figure 4:
Left photo – Pink colonies of non-O157 STEC on SMAC plate
Right photo – Clear colonies of O157 STEC on SMAC plate after incubation for 24 hours

Images courtesy of the New York State Department of Agriculture & Markets Food Laboratory Division

Figure 5:
Washed Sheep’s Blood Agar with STEC after 18-24 hours incubation demonstrating hemolysis

Image courtesy of CDC
Isolation of non-O157 STEC is difficult due to the lack of shared phenotypic traits that can distinguish them from typical *E. coli*. Table 1 shows biochemical reactions of commonly isolated STEC serotypes in comparison to those of “typical” *E. coli* reported by Farmer et al.\(^\text{17}\) and Rice et al.\(^\text{18}\) Reactions that differ by more than 40 percentage points from those listed for “typical” *E. coli* are highlighted. Groups of STEC that share phenotypic traits that differ from those exhibited by “typical” *E. coli* are listed in Table 2. There is no distinguishing trait that is shared by all STEC; however, the development of customized media for isolation of selected STEC serotypes may be possible and may be useful in outbreak settings when a presumptive serotype has been identified.

Presumptive O157 STEC and non-O157 STEC isolates should be confirmed biochemically to be *E. coli*. Commercial manual and automated identification systems are acceptable for identification of *E. coli*.\(^\text{19}\) Typical biochemical reactions for *E. coli* can be found in Biochemical Table 1.
**Biochemical Table 1:**

Biochemical reactions of a typical *E. coli* and of selected serotypes of Shiga toxin-producing *E. coli*.5

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Guidance for Public Health Laboratories on the Isolation and Characterization of Shiga toxin-producing *Escherichia coli* (STEC) from Clinical Specimens
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Selected biochemical reactions of commonly isolated serotypes of Shiga toxin-producing *E. coli*<sup>a</sup>

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Biochemical Table 2: (CONTINUED)

Selected biochemical reactions of commonly isolated serotypes of Shiga toxin-producing *E. coli*\(^a\)

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<td>Sorbitol-negative Biogroup</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>STEC 0157:H7/NM n = 158</td>
<td>99</td>
<td>1</td>
<td>98</td>
<td>100</td>
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<td>91</td>
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<td>5</td>
<td>100</td>
<td>92</td>
<td>14</td>
<td>23</td>
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</tr>
</tbody>
</table>

Footnotes for Biochemical Tables 1 and 2

\(^a\) Values represent percent of positive reactions for each serotype at 48 hours at 35°C unless otherwise specified. Delayed positive reactions are not considered. Tests are performed as described by Ewing and Koneman et al.\(^{20, 21}\)

\(^b\) With the exception of ß-glucuronidase (MUG), the values listed for “typical” *E. coli* are those reported by Farmer et al.\(^{17}\) The number of isolates tested was not stated. Values for ß-glucuronidase were taken from those reported by Rice et al.\(^{18}\) for 620 *E. coli* isolates of human and animal origin.

\(^c\) Serotypes are classified as negative for a particular trait if <10 percent of the members in that group were positive for the trait. Reactions that differ by more than 40 percentage points from those listed for “typical” *E. coli* are highlighted.
SEROTYPING OF STEC
SEROTYPING OF STEC

Somatic and flagellar antigen determinations help to classify organisms into primary phenotypes. Such antigen determination is called serotyping. Serotyping is a subtyping method based on the immunologic characteristics of two surface structures, the lipopolysaccharide (LPS), which contains the O-antigen, and the flagella, which contains the H-antigen. The O-antigen is the outermost component of the LPS, an immunoglycolipid contained in the outer membrane of gram-negative bacteria. O-antigens are composed of multiple repeats of an oligosaccharide unit typically composed of four to six sugars. Variations in the composition, arrangement, and linkages of these sugars all contribute to O-antigen diversity and are the basis for serotype diversity. The H-antigen is a part of the flagellar protein affecting motility. Identification of the H-antigen provides an additional tool for STEC surveillance and organism characterization, but H-antigen determination is not necessary for routine testing.

O-Antigen Determination (Serogrouping)

When testing suspect O157 STEC isolates, the most common method used in clinical laboratories to determine the O-antigen for diagnostic purposes is latex agglutination. Latex particles are coated with antibodies against the O157 antigen. When these particles are mixed with fresh bacterial growth, O157 STEC bacteria will bind to the latex particle to produce visible agglutination indicating a positive reaction. Similar technology is utilized for non-O157 STEC serogroups O26, O91, O103, O111, O128 and O145. Tube agglutination or slide agglutination is another methodology used to determine O-antigen serogroups utilizing sensitized antisera. Sensitized serum is manufactured against a specific antigen for a particular serogroup. When the antiserum is mixed with fresh bacterial growth, an antigen/antibody reaction produces visible agglutination. The Staten Serum Institut (SSI, Miravista, US Distributor) produces OK antisera for slide agglutination for rapid screening.

E. coli OK antiserum is a commercial product for the presumptive identification of E. coli O-antigens. E. coli OK antisera are available as pools of antibodies to screen for several O-antigens simultaneously. OK antisera, which are intended for use in slide agglutination assays, are typically not absorbed and have higher titers to the O-antigens than their corresponding O-specific antisera. The high titers of these reagents allow them to be used for slide agglutinations without coupling to latex particles. The vaccines used to make OK antisera are produced in a way to favor generation of antibodies against the O-antigen; however, antibodies against K-antigens, which are heat-resistant, acidic polysaccharide antigens on the surface of some E. coli cells, and other undefined surface antigens will also be present at levels that can interfere with O-antigen determination. OK antisera should not be used for definitive O group determination, as the K-antigen may cross-react with other antigens present in
the bacterial suspension, contributing to false-positive reactions. In addition, OK antisera should not be used to screen isolates that have not been confirmed as STEC. Routinely screening colonies from Stx/stx-positive specimens with OK antisera to guide Stx/stx testing for isolating STEC is strongly discouraged. Following the recommended testing algorithms will result in the proper use of these antisera.

O-specific antisera are available for definitive serogrouping by tube/microtiter methodology. This is the gold standard assay for O-antigen determination. When O-specific antiserum is mixed with fresh bacterial growth expressing the corresponding antigen, an antigen/antibody reaction results, producing visible agglutination in the form of a thin mat of cells across the bottom of the tube or well of the microtiter plate. In the absence of specific agglutination, a pellet of cells appears in the center of the tube or well. O-specific antisera are typically produced by immunizing rabbits with standardized strains representing each O-antigen and by absorbing the antisera with strains expressing cross-reacting antigens to make the antisera specific for the desired O-antigen. It is important to appreciate that antisera for typing bacteria are developed and validated for use under defined conditions, including the intended species, recommended testing dilutions, and assay conditions and format. Deviation from manufacturers’ recommended protocols can lead to uninterpretable and unreliable results. For additional information on O-antigen determination, refer to references 22, 23, 24.

NOTE: At the time of publication, only the Staten Serum Institut provides O-specific antisera for the non-O157 O-groups O26, O45, O103, O111, O121, and O145.

H-antigen Determination

H7-specific antisera for tube agglutination and latex agglutination are commercially available for O157, but detection of flagellar antigens may be difficult, even after multiple passages in motility medium, and is generally not necessary. H-antigen characterization for non-O157 may be required for outbreaks, publications regarding outbreaks, or other special circumstances. If needed, H-antigen typing on any STEC can be requested from the CDC E. coli National Reference Laboratory.
Table 3:
Serotypes encountered five or more times among 7,125 human Shiga toxin-producing E. coli\(^a\) isolates received between 2000-2010 by the CDC National Escherichia coli Reference Laboratory

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Serotype</th>
<th>Serotype</th>
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</thead>
<tbody>
<tr>
<td>08:H19</td>
<td>0103:NM</td>
<td>0128:H2</td>
</tr>
<tr>
<td>022:H8</td>
<td><strong>0103:H2</strong></td>
<td>0130:H11</td>
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<td>0103:H11</td>
<td><strong>0145:NM</strong></td>
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<tr>
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<td>0103:H25</td>
<td>0145:H16</td>
</tr>
<tr>
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<td><strong>0104:H4</strong></td>
<td>0145:H25</td>
</tr>
<tr>
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<td>0110:H28</td>
<td>0146:H21</td>
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<tr>
<td><strong>045:H2</strong></td>
<td><strong>0111:NM</strong></td>
<td>0153:H2</td>
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<tr>
<td>051:H11</td>
<td><strong>0111:H8</strong></td>
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<td>055:H7</td>
<td><strong>0113:H21</strong></td>
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<td>0181:H49</td>
</tr>
</tbody>
</table>

\(^a\)Outbreak-related serotypes are shown in bold type.

NOTE: NM = nonmotile
MOLECULAR DETECTION
MOLECULAR DETECTION

PCR protocols targeting the Shiga toxin genes of *Escherichia coli* provide a rapid and sensitive diagnostic tool to detect potentially virulent strains that have been isolated in culture from patient stool specimens. The target genes of interest are *stx*1 and *stx*2. The genes for *eae* and *Ehly* are additional targets to be considered (see “History and Biology”). The *stx* genes are located on phages, and an organism may contain one or more of these phages. PCR technology is recommended to detect Shiga toxin-encoding genes to ensure that all STEC will be represented during isolate characterization, including the rare sorbitol fermenting O157 STEC variants. Both conventional and real-time PCR methods provide a satisfactory detection limit for identifying Shiga toxin-producing organisms. The selection of a specific methodology will depend on individual laboratory preference, acceptable timelines, available funding and the experience level of laboratory staff. The CDC *E. coli* National Reference Laboratory has protocols and expertise to assist laboratories with the implementation of molecular assays.

Real-time PCR

Several published studies describe real-time PCR assays and parameters for different real-time PCR instrumentation. These studies list specific primer and probe sequences reported to yield low detection limits. Real-time assay protocols for the testing of food samples for STEC are available to some laboratories through the Food Emergency Response Network (FERN) and the Laboratory Response Network (LRN). Each laboratory must verify and validate the performance of the PCR method selected for use with their particular instrumentation and patient population.

Conventional PCR

Conventional PCR is a molecular testing option for laboratories that do not have real-time PCR instrumentation. This methodology requires the use of a conventional, or block, thermal cycler and resolution of the PCR products using agarose gel electrophoresis. Many primer sequences for the genes that encode for *stx*1 and *stx*2 (as well as *eae* and other virulence genes) have been published. Each laboratory must verify and validate the performance of the PCR method selected for use with their particular instrumentation and patient population.
MOLECULAR SUBTYPING
MOLECULAR SUBTYPING

To rapidly detect foodborne disease outbreaks, state and local public health laboratories participate in PulseNet USA, The National Molecular Subtyping Network for Foodborne Disease Surveillance. PulseNet USA is a national network of public health and food regulatory agency laboratories coordinated by CDC and the Association of Public Health Laboratories (APHL). The network consists of public health laboratories in all 50 states, the District of Columbia and Puerto Rico working in conjunction with federal agencies [CDC, United States Department of Agriculture (USDA), and the Food and Drug Administration (FDA)] to compare molecular fingerprints of specified enteric pathogens that may be foodborne in origin. All isolates of O157 STEC and non-O157 STEC that are submitted to public health laboratories must be subtyped according to PulseNet protocols. The resulting DNA fingerprints must be uploaded to the PulseNet National Databases within four working days of receipt of the pure isolate in the laboratory. The two subtyping methods currently in use by PulseNet laboratories are pulsed-field gel electrophoresis (PFGE) and multi-locus variable number tandem repeat analysis (MLVA).

Pulsed-Field Gel Electrophoresis (PFGE)

- All isolates of O157 STEC should be subtyped by PFGE using the standardized PulseNet protocol for *E. coli* O157:H7.
- All isolates of non-O157 STEC should be subtyped by PFGE using the standardized PulseNet protocol for non-O157 STEC.
- Resulting profiles should be analyzed according to PulseNet guidelines using the BioNumerics software with CDC’s customized scripts.
- Analyzed patterns should be submitted by PulseNet Certified Personnel to the appropriate National Database as soon as possible.
- STEC certification sets are available from CDC for PulseNet participating laboratories.
Multi-locus variable number tandem repeat analysis (MLVA)

While PFGE is the primary method for subtyping isolates for PulseNet, other methods that may further characterize clusters have been validated by CDC and participating laboratories. For example, clusters of common O157 STEC patterns may be further differentiated using MLVA analysis, which involves a multiplex PCR and the separation of DNA fragments based on size using capillary electrophoresis.

- Fragment size data is submitted by PulseNet MLVA certified personnel to the appropriate National Database using the BioNumerics software.
- MLVA typing is available at CDC and at some PulseNet Laboratories.
- Certification sets are available from CDC for those PulseNet participating laboratories that wish to implement this method.

All participating PulseNet USA laboratories maintain local databases of PFGE patterns and have access to the National Databases to assess clusters of matching DNA fingerprints. Laboratorians should work in close collaboration with foodborne disease epidemiologists in their jurisdiction to investigate any local clusters that are identified through PulseNet. Regular collaboration among laboratorians, epidemiologists and environmental health specialists at the local and state levels will facilitate the timely detection of outbreaks and tracing of implicated food products.
IMMUNOMAGNETIC SEPARATION
IMMUNOMAGNETIC SEPARATION

Immunomagnetic separation (IMS) reagents are currently available commercially for *Escherichia coli* serotypes O157, O26, O111, O145 and O103. Protocols for using IMS on stool specimens are described in the package inserts. The IMS concentrate should be plated to appropriate media, as outlined in the Best Practice Recommendations. IMS protocols describe testing of stool and various other sample types, yet there are currently no protocols for using IMS on previously incubated broth cultures, such as those used for EIA testing and forwarded to the public health laboratory. Therefore, stool specimens remain the specimen of choice for IMS enrichment.

Because O157 STEC is currently responsible for the majority of STEC-associated HUS cases,34 O157-IMS followed by non-O157 IMS (if negative for O157 STEC) is a reasonable approach. Alternatively, to minimize specimen handling and reduce turnaround time, simultaneous enrichment may be conducted, especially if automated equipment is available, such as the Dynal BeadRetriever™ (Invitrogen, by Life Technologies). However, the enrichment broths and incubation conditions for non-O157 IMS are different from those for O157 IMS.

Any isolates recovered from the plating of the IMS enrichment broths should be tested by the same biochemical, immunological and molecular tests used to identify and characterize STEC as described in the Best Practice Recommendations.

Immunomagnetic separation (IMS) is available through IMS Area Laboratories or the CDC *E. coli* National Reference Laboratory.

NOTE: While IMS reagents and protocols are available for human specimens, this test is not FDA-approved for use on human samples. The performance of IMS must either be fully validated in each laboratory for use on stool specimens, or reports must contain a disclaimer stating the results obtained using this procedure have not been approved for diagnostic purposes. See validation discussion in the Reporting and Regulatory Considerations section for further information.
REPORTING AND REGULATORY CONSIDERATIONS
REPORTING AND REGULATORY CONSIDERATIONS

Each state or local public health department sets specific reporting guidelines for Shiga toxin-positive stool results, and the wording of reported results should be determined by the public health laboratory director. Please defer to any local requirements when reporting to public health authorities.

The timing and frequency of reporting may also vary per local or state regulations or laboratory protocols. However, if the public health laboratory detects evidence of O157 STEC or an organism that produces Shiga toxin 2, the submitting laboratory should be notified as soon as possible, especially if this information was not supplied to the public health laboratory upon submission of the sample.

Due to the complex multi-laboratory approach for Shiga toxin testing, difficulties may arise in the interpretation of test results when comparing clinical laboratory results with those of the public health laboratory. This may raise questions regarding appropriate public health actions. The primary roles of clinical and public health laboratories are to report the results obtained using their validated testing methodologies. All results must be reviewed by clinicians and/or public health officials in combination with epidemiologic data and the patient’s clinical history. Education of clinical partners about any technical issues regarding the diagnostic tools used to obtain the results is beneficial. Some technical issues to consider when evaluating and comparing test results:

- Differences may exist regarding the manner in which stool specimens or enrichment broths are handled or processed between the laboratories. If the time between the specimen collection date and the date of testing exceeds the manufacturer’s or procedural requirements, the results may not be comparable. In addition, shipping or storage temperatures may affect specimen integrity.

- The sensitivity and specificity of each method used in testing must be considered. The positive predictive value (PPV) of any test is dependent upon the prevalence of the disease in the population being tested. For example, in the case of STEC disease in an area of low prevalence, one should expect to see a higher percentage of false-positive test results for non-culture based assays for Stx.35

- When comparing results from nucleic acid detection methods to those of a protein detection method, the difference in the targets for testing may yield discordant results. For example, if an organism that has the genes encoding for Shiga toxin production is not producing the toxin proteins, the Stx EIA would be negative, but the stx PCR would be positive.
• An organism may stop producing Shiga toxin after multiple passages on plated media or after freezing. These scenarios may contribute to an initial positive EIA result and a positive PCR result, but a subsequent negative EIA result while the PCR result remains positive.

• Each assay’s target and limit of detection must be considered.

Molecular assays, even peer-reviewed published protocols, are considered Laboratory Developed Tests and must meet Clinical Laboratory Improvement Amendment of 1988 (CLIA) standards of test performance. This includes the process of performing validation and verification studies as a laboratory would for any other clinical assay. Documents are available from the Clinical Laboratory Standards Institute (CLSI)\textsuperscript{36} and the College of American Pathologists (CAP), as well as an excellent review published in Clinical Microbiology Newsletter,\textsuperscript{37} to aid in performing these processes correctly. If further clarification regarding any CLIA regulatory issues is necessary, contact the Centers for Medicare and Medicaid Services.

All laboratories that screen clinical samples for the presence of Shiga toxin, or the genes that are responsible for Shiga toxin production, should participate in either an available proficiency testing program or a specimen exchange program with laboratory partners to fulfill CLIA testing requirements.
SHIPPING CONSIDERATIONS
SHIPPING CONSIDERATIONS

As with all reportable infectious diseases, open and direct communication between the submitting clinical laboratory and the receiving public health laboratory regarding Shiga toxin-producing *Escherichia coli* (STEC) specimen submission requirements is essential. Public health laboratories are encouraged to work with the clinical laboratories within their jurisdiction to have them submit positive test materials (e.g., primary specimen, broth, isolate) in order to promote rapid characterization of STEC isolates. Public health laboratories should be prepared to accept isolates (on media slants) and/or broths that have tested as toxin-positive by enzyme immunoassay (EIA). A pure culture of an organism on a heavily-inoculated culture swab may be an acceptable alternative to a slant culture. If a swab is used, the shaft should be truncated to assure a firm fit within the plastic sheath, and the joint secured with a water-tight material, such as Parafilm®, to prevent leakage. Plates are generally acceptable only in the rare instance where patient diagnosis or management would be delayed by subculturing an organism to a slant for transport, and the shipping of plates must be pre-approved by the receiving public health laboratory. Furthermore, some public health laboratories may request or require that the original stool specimen be submitted with the isolate or broth. Public health laboratories should communicate their expectations and any applicable regulations regarding specimen submission to their clinical colleagues. Solutions should be sought to avoid any potential barriers to meeting such requirements. Once received at the public health laboratory, all specimens should be processed expeditiously.

Transport Considerations

When forwarding isolates and broths to CDC or other partner laboratories, public health laboratories must follow all applicable shipping and packaging regulations. At the time of this publication, the United Nations (UN) has classified verotoxigenic *E. coli* (culture only) as a Category A (UN 2814) Infectious Substance. The International Air Transportation Association (IATA) and the Department of Transportation (DOT) have harmonized their shipping guidance and regulations to comply with UN regulations. As defined in these regulations, a culture is any intentional propagation of an infectious agent. Therefore, all suspect or confirmed O157 STEC strains and Shiga toxin-positive EIA broths should be shipped as Category A Infectious Substances. When the infectious substances to be transported are unknown but suspected of meeting the criteria for inclusion in Category A (e.g., a broth culture positive for Shiga toxin or a stool culture from a patient in an O157 STEC outbreak), the above mentioned regulations apply. Both IATA and DOT require that all individuals who package, ship or transport Category A Infectious Substances must have formal, documented training.
Category A agents must be packaged in a watertight primary receptacle. Broths shipped with caps must be secured tightly and wrapped with waterproof material, such as Parafilm®, to prevent leakage. Broths should be shipped with a cold pack to prevent overgrowth of other gram-negative flora. Ambient temperature is generally acceptable for the transport of isolates and pure cultures of organisms on swabs as long as extreme temperatures can be avoided; during periods of high temperatures, a cold pack should be included.

Commercial couriers vary with regard to their acceptance of Category A agents; consult with the preferred commercial courier for current requirements. Shipping Category A specimens via commercial couriers usually incurs a surcharge, in addition to normal shipping fees. Category A Infectious Substances are not accepted by the US Postal Service at the time of this publication.\textsuperscript{42}

Shipping via a private (non-commercial) courier that is dedicated to transport of clinical samples does not exempt specimens from DOT or IATA regulations; Category A specimens must be packaged according to the Division 6.2 regulations with appropriate documentation, even if not being transported by a commercial carrier.\textsuperscript{38, 43}

Regardless of the transport arrangements implemented by a laboratory, a UN-approved Category A shipping container must be used for cultures or specimens known to contain Shiga toxin, and specimens must be packaged and documented according to the DOT/IATA regulations. Public health laboratories should communicate with clinical laboratories in their jurisdiction about these regulations, as well as any region-specific mechanisms in place to transport positive test materials (e.g., primary specimen, broth or isolate). The goal of both the submitter and the recipient is to promote rapid recognition of STEC infections and outbreaks of disease. All packaging and shipping procedures developed by a laboratory should be written into a standard operating procedure and followed consistently.
Members of the pathotype of *Escherichia coli* referred to as Shiga toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC) were first described by Konowalchuk and coworkers in 1977. To identify virulence traits that might play a role in the pathogenesis of diarrheal disease caused by *E. coli*, these authors tested culture filtrates from a collection of *E. coli* strains from humans, animals and foods associated with diarrheal disease for their effects on Vero cells *in vitro*. Ten of the 136 strains studied produced a distinctive heat-labile toxin that killed Vero cells and was antigenically distinct from the heat-labile toxin of enterotoxigenic *E. coli* (ETEC). Purified cytotoxin from strain H30 (serotype O26:H11) was also reported to cause fluid accumulation (enterotoxic activity) in ligated rabbit ileal loops. Konowalchuk and colleagues designated the cytotoxin they discovered as VT (also referred to as verocytotoxin or verotoxin) for its cytotoxic effect on Vero cells, and its production by *E. coli* associated with diarrheal disease was soon confirmed by other investigators.

While Konowalchuk and colleagues were studying potential virulence factors of diarrheagenic *E. coli*, others were re-examining the mechanism by which *Shigella* cause diarrhea due to reports that Shiga toxin from *Shigella dysenteriae* type 1 possessed enterotoxic activity.  O’Brien and coworkers described a cytotoxin in some *E. coli* strains from persons with diarrhea that had biologic activities similar to those produced by Shiga toxin from *S. dysenteriae* type 1 and was neutralizable by antibodies against purified Shiga toxin. O’Brien et al. designated the toxin produced by these *E. coli* as Shiga-like toxin (SLT) in recognition of its shared biologic and antigenic properties with Shiga toxin.

In 1983, O’Brien and colleagues established the connection between VT and SLT by showing that the purified cytotoxin from *E. coli* strain H30 had the same subunit structure, isoelectric point, and range of biologic activities as purified Shiga toxin. In that same year, O’Brien and colleagues also demonstrated that the Vero cytotoxic activity of bacterial lysates from strains of *E. coli* serotype O157:H7 from an outbreak of hemorrhagic colitis in the United States in 1982 was neutralizable by antibodies against purified Shiga toxin. Following the 1982 outbreak of *E. coli* O157:H7 in the United States, numerous studies identified *E. coli* O157:H7 as a cause of outbreaks and sporadic diarrheal disease.

After the identification of SLT (VT) as a virulence factor in *E. coli* O157:H7 and other serotypes of *E. coli*, there was a rapid expansion of research into the biology, genetics, pathogenesis, and epidemiology of the toxins and organisms producing them. Subsequent genetic studies showed that the genes encoding the Shiga-like toxins were encoded on lysogenic bacteriophages and findings from biologic and genetic studies on lysogens created with certain Shiga toxin-converting phages demonstrated antigenic variation within the Shiga-like toxins and revealed a second distinct group of toxins that
were not neutralizable with antibodies against purified Shiga toxin, yet shared the same biologic properties, protein structure, gene organization and substantial DNA sequence homology. The second group of toxins was designated SLT-II or VT2. Findings from the cloning and sequencing of Shiga toxin from \textit{S. dysenteriae} type 1 and the biologic and genetic characterization of a growing number of variants within each group of toxins affirmed the overall relatedness of these toxins to Shiga toxin and the concept that the toxins constitute a family of toxins. This body of evidence led Calderwood et al.\textsuperscript{63} to propose a revised system of nomenclature based on the Shiga toxin name which omits the word “like;” however, arguments in support of the verotoxin nomenclature were quickly put forward by Karmali and colleagues. Because compelling cases in favor of both the verotoxin/VT and Shiga toxin/Stx nomenclatures systems can be made, researchers will customarily acknowledge in their publications the existence of the two parallel nomenclatures which can be used completely interchangeably. This document uses the Shiga toxin nomenclature and refers to \textit{E. coli} producing these toxins as STEC.

**STEC Nomenclature**

No formal system of nomenclature exists for naming bacterial toxins, and as a result, parallel nomenclatures frequently arise for the same toxins. This has been the case for the potent cytotoxins produced by \textit{S. dysenteriae} type 1 and certain strains of \textit{E. coli}. The cytotoxins produced by these bacteria may be referred to interchangeably as “Shiga toxins” or “verocytotoxins.” The Shiga toxin nomenclature recognizes Kiyoshi Shiga, the Japanese bacteriologist who first discovered \textit{S. dysenteriae} type 1 and the toxin it produces in 1898, and the verocytotoxin nomenclature recognizes the ability of the cytotoxins produced by \textit{E. coli} to kill Vero cells in cell culture. In this document, we will use the Shiga toxin nomenclature. The classification scheme and basis for designating toxin types was last specifically addressed in 1996.\textsuperscript{63, 64} In this proposal, the family of Shiga toxins was defined as toxins that shared the following properties with Shiga toxin, the prototype toxin: (i) DNA sequence homology and operon structure, with the A subunit gene immediately upstream of the gene for the B subunit; (ii) stoichiometry of five B subunits to one A subunit in the mature holotoxin; (iii) identical enzymatic activity of the A subunit, i.e., the removal of a specific adenine residue in 28S rRNA in the 60S ribosomal subunit; (iv) binding to specific eukaryotic cell membrane glycosphingolipid receptors containing a galactose-\(\alpha1\rightarrow4\) galactosyl disaccharide moiety; and (v) biologic properties, including enterotoxicity in rabbit ileal loops, neurotoxicity in mice and cytotoxicity to a number of receptor-expressing tissue culture cell lines, including Vero, HeLa, and many others.
Toxins meeting the above criteria can be differentiated into two main types, designated Stx1 and Stx2, by the following additional characteristics: (i) lack of cross-neutralization by homologous polyclonal antisera and (ii) lack of DNA-DNA cross-hybridization of their genes under conditions of high stringency. By these criteria, Shiga toxin from *S. dysenteriae* type 1 and Stx1 from *E. coli* would be regarded as the same toxin type. Because Shiga toxin from *S. dysenteriae* type 1 serves as the prototype toxin for the Shiga toxin family, the Stx designation for this toxin is retained and may be used when referring to the prototype toxin or to the entire family of toxins. Toxins within these two types may be further differentiated into subtypes based on significant differences in biological activity, serological reactivity, and/or receptor binding. Separation of toxins into subtypes based on biological properties, however, has proven problematic to broadly implement because the biological assays are labor-intensive, require specialized knowledge, and can be difficult to interpret.

To simplify classification and make it more accessible and reproducible, the WHO Collaborating Center for *Escherichia* and *Klebsiella* in Copenhagen, Denmark, has developed and steadily built consensus for a translated DNA sequence-based system for classifying the Shiga toxins as initially proposed by Whittam in 1998. Phylogenetic algorithms are used to assign a new allele to a toxin type (Stx1 or Stx2) and subtype (e.g., Stx1a, Stx1b, Stx2a, Stx2b). Toxin subtypes are further subdivided into variants; variants are designated if the translated amino acid sequence differs by one or more amino acids from a previously described variant. Table 4 lists the toxin types, toxin subtypes, and the number of toxin variants currently recognized and catalogued by the WHO Collaborating Center for *Escherichia* and *Klebsiella*. The first validly published sequence represents each specific variant which is designated according to the following three components separated by hyphens: toxin subtype—the first published O group for *E. coli* (or the species name)—strain number. For example, the format for a toxin variant of subtype Stx1a would be Stx1a-O157-EDL933.

For patient diagnosis and epidemiologic surveillance, it is desirable for public health laboratories to minimally type and preferably subtype the Shiga toxin genes. There is growing evidence that strains producing certain subtypes of Stx2 are associated with increased risk of causing severe infections.
## Table 4:
Shiga toxin nomenclature: adapted and revised from references 24 and 69

<table>
<thead>
<tr>
<th>Toxin type</th>
<th>Toxin subtype</th>
<th>Number of recognized toxin variants</th>
<th>Reference toxin variant designation</th>
<th>Reference prototype organisms</th>
<th>Serotype of reference prototype organism</th>
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<td>S1a-0157-EDL933</td>
<td>EDL933</td>
<td>O157:H7</td>
<td>M19473</td>
<td>59, 71</td>
</tr>
<tr>
<td></td>
<td>Stx1c</td>
<td>6</td>
<td>Sx1c-0174-DG131-3</td>
<td>DG131/3</td>
<td>O174:H8</td>
<td>Z36901</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Stx1d</td>
<td>1</td>
<td>Stx1d-08-MHII13</td>
<td>MHII13</td>
<td>O8:K85ab:HNT</td>
<td>AY170851</td>
<td>74</td>
</tr>
<tr>
<td>Stx2</td>
<td>Stx2a</td>
<td>21</td>
<td>S2a-0157-EDL933</td>
<td>EDL933</td>
<td>O157:H7</td>
<td>X07865</td>
<td>59, 71</td>
</tr>
<tr>
<td></td>
<td>Stx2b</td>
<td>16</td>
<td>S2b-0174-031</td>
<td>031</td>
<td>O174:H21</td>
<td>X65949</td>
<td>73, 75</td>
</tr>
<tr>
<td></td>
<td>Stx2c</td>
<td>18</td>
<td>S2c-0174-031</td>
<td>031</td>
<td>O174:H21</td>
<td>L11079</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Stx2d</td>
<td>18</td>
<td>S2d-091-B2F1</td>
<td>B2F1</td>
<td>O91:H21</td>
<td>AF479828</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Stx2d</td>
<td>18</td>
<td>S2d-073-C165-02</td>
<td>C165-02</td>
<td>O73:H1</td>
<td>D0059012</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Stx2e</td>
<td>14</td>
<td>S2e-0139-S1191</td>
<td>S1191</td>
<td>O139:K12:H1</td>
<td>M21534</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Stx2f</td>
<td>3</td>
<td>S2f-0128-T4-97</td>
<td>T4/97</td>
<td>O128ac:[H2]</td>
<td>AJ010730</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Stx2g</td>
<td>4</td>
<td>S2g-02-7v</td>
<td>7v</td>
<td>O2:H25</td>
<td>AY286000</td>
<td>81</td>
</tr>
</tbody>
</table>

a. Toxin type refers to the two major branches of the Shiga toxin family that share structure and function but are not cross-neutralized with heterologous antibodies. The two branches include Stx/Stx1 and Stx2.

b. Toxin subtypes have traditionally been defined according to antigenic variability, differences in toxicity for tissue culture cells or in animals, their capacity to be activated by mouse elastase and differences in DNA or amino acid sequences. The subtypes in this table are defined by phylogenetic analyses as proposed by Flemming Scheutz and colleagues at the 4th Annual Workshop of the EU Reference Laboratories for *E. coli*, Rome 30 October 2009, http://www.iss.it/binary/vtec/cont/8.pdf (manuscript in preparation).

c. For historical reasons, the Stx/stx nomenclature (no Arabic number) is used to refer to Shiga toxin and the genes encoding it when they occur in *Shigella* spp. Designations for Shiga toxins or the genes encoding these toxins when they occur in *E. coli* and other bacteria include an Arabic number after “Stx” or “stx.”

d. There are several toxins suffixed by “d” in the literature: The Stx2d/VT2d toxins of O91:H2182, the VT2d (= stx2-0group/ strain designation and/or year) variants by Paton et al.73, 83, 84 and the
SLT-IId/VT2d (in current scheme classified as Stx2f/VT2f) toxin produced by strain H.I.8 (serotype O128:H2) as proposed by Gyles. Toxins subtypes classified with the “d” designation are activatable Stx2/VT2 toxins as proposed by Melton-Celsa et al. or have a predicted amino acid structure that has the potential to be activated.

e. The nucleotide sequence of the former stx2ev/vtx2ev of strain H.I.8 (re-serotyped as O89:H2) is nearly identical to the recently published stx2f/vtx2f sequence found in strain T4/97 (serotype O128:H2) from feral pigeons. H antigens in brackets were determined with molecular instead of serologic methods.

The genes for the Shiga toxins are organized in an operon consisting of an A subunit gene adjacent to a B subunit gene. These genes typically reside within the chromosome on functional or sometimes defective prophages which play an important role in facilitating recombination between the toxin genes and disseminating them to other bacteria. The holotoxin is comprised of an A subunit polypeptide (32 kDa) that is non-covalently associated with five B subunit polypeptides (7.7 kDa). The A subunit contains the enzymatically active part of the toxin and the B subunits mediate binding of the toxin to the eukaryotic cell surface via glycolipid receptors, typically globotriaosylceramide (Gb3) or globotetrasylceramide (Gb4). Following binding, the toxin is endocytosed and transported in retrograde via endosomes and the Golgi apparatus to the endoplasmic reticulum, from where it is translocated to the cytosol. In the cytosol, the A subunit is cleaved by the cellular protease furin into the enzymatically active A1 fragment (27.5 kDa; N-glycosidase), which blocks protein synthesis by removing one adenine residue from the 28S ribosomal RNA of the 60S ribosomal subunit and an A2 (4.5 kDa) fragment (for a review see references 91 and 92).

Like Shiga toxin produced by S. dysenteriae type 1, designated Stx, the related toxins produced by E. coli, designated Stx1 and Stx2, share the following biological properties which define the Shiga toxin family: cytotoxicity for Vero, HeLa and human vascular endothelial cells; enterotoxicity for ligated rabbit intestinal segments; and lethality for animals. They differ sufficiently from each other at the nucleotide and amino acid sequence level (approximately 55 percent homology at the amino acid level) that there is no cross hybridization between the two under conditions of high stringency and no cross-neutralization of cytotoxic activity with antisera against each toxin, traits which define the two toxin types (groups) within the Shiga toxin family. Stx2 is more toxic for human renal microvascular endothelial cells and mice than is Stx. Unique biological features of the most common Stx2 subtypes are summarized in Table 5.
Table 5:
Biological characteristics of common Stx2 subtypes

<table>
<thead>
<tr>
<th>Stx2 Subtype</th>
<th>A subunit</th>
<th>B subunit</th>
<th>Glycolipid receptor</th>
<th>Activated by intestinal mucus</th>
<th>LD₅₀ (ng) for mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx2a</td>
<td>100</td>
<td>100</td>
<td>Gb₃</td>
<td>No</td>
<td>6.5</td>
</tr>
<tr>
<td>Stx2c</td>
<td>100</td>
<td>97</td>
<td>Gb₃</td>
<td>No</td>
<td>1,000</td>
</tr>
<tr>
<td>Stx2d</td>
<td>99</td>
<td>97</td>
<td>Gb₄</td>
<td>Yes</td>
<td>2.4</td>
</tr>
<tr>
<td>Stx2e</td>
<td>93</td>
<td>84</td>
<td>Gb₃</td>
<td>No</td>
<td>NT</td>
</tr>
</tbody>
</table>

a. Adapted from reference 94.
b. Values taken from 95.

Intimin and enterohemolysin genes

The genes for intimin (eae) and enterohemolysin (ehx or Ehly) were among the first virulence genes to be identified in STEC. The eae gene encodes an outer membrane protein (intimin) which interacts with other bacterially encoded products to mediate intimate adherence of the bacteria to host intestinal epithelial cells and form attaching and effacing lesions, a pathology also seen in enteropathogenic *E. coli* (EPEC) infections. The eae gene is located within a 35 Kb region of the chromosome in a region referred to as the locus for enterocyte effacement (LEE). The enterohemolysin genes (operon of four genes ehxA-D) reside on a large plasmid referred to commonly as the EHEC plasmid or as pO157 in *E. coli* O157:H7. Enterohemolysin, which may play a role in the disease process by inducing cytokine production, is a potent cytotoxin that causes lysis of washed sheep erythrocytes in the presence of calcium ions. This phenotype is exploited for isolating STEC with Washed Sheep Blood Agar with Calcium (WSBA-Ca).

To provide virulence profiling and subtyping information, many investigators began including the eae and ehxA genes along with the stx1 and stx2 genes in their PCR or DNA hybridization assays. As researchers repeatedly subcultured STEC isolates, they quickly realized that the phages carrying the Shiga toxin genes, as well as the other target genes, could be lost. This observation lead to an appreciation for the vulnerability of diagnostic algorithms that target traits encoded on mobile genetic elements.

In the case of STEC diagnosis, the impact of stx gene loss was documented to be problematic by Bielazewska and colleagues. In their studies, they used a PCR assay for the stx and eae genes and demonstrated variable stability within the stx genes maintained in different STEC lineages. Interestingly, the loss of stx genes was significantly
Guidance for Public Health Laboratories on the Isolation and Characterization of Shiga toxin-producing *Escherichia coli* (STEC) from Clinical Specimens

more frequent among isolates from persons with bloody diarrhea (15 percent) compared with those from persons with non-bloody diarrhea (1 percent; P <0.001). In this study, *stx*-negative, *eae*-positive *E. coli* strains were isolated from approximately 1.5 percent of patients and were hypothesized to have arisen during the course of infection. Specimens that yielded these *stx*-negative, *eae*-positive strains were collected 4-13 days (median 8 days) after the onset of diarrhea and no other pathogens were detected in these patients.

The frequency of STEC isolation in the above study of 10,668 patients with bloody and non-bloody diarrhea was not stated; however, among 787 epidemiologically unrelated HUS patients who were cultured 8-9 days after onset of diarrhea, *stx*-negative, *eae*-positive *E. coli* strains were isolated from 43 patients, while 440 HUS cases were STEC positive.\(^98\) Among the 787 HUS patients, 61 percent (483/787) had evidence of an STEC or putative STEC infection; and in 9 percent (43/483) of these, the total possible STEC cases were positive only for *stx*-negative, *eae*-positive *E. coli*. These findings highlight the value of using additional virulence genes (*eae* and *ehxA*) to retain the ability to detect putative STEC when the *stx* genes are lost, as well as the importance of collecting and testing specimens as close to the onset of symptoms as possible to minimize the loss of important target genes. Another benefit of including the additional virulence genes in STEC diagnostic assays is that strains with greater pathogenic potential (e.g., infections with strains positive for *stx2* and *eae* that have been significantly associated with cases of hemorrhagic colitis and HUS\(^99,\,100\)) can be readily identified and reported to physicians and public health officials.
EPIDEMIOLOGY AND CLINICAL LABORATORY DIAGNOSIS
Guidance for Public Health Laboratories on the Isolation and Characterization of Shiga toxin-producing Escherichia coli (STEC) from Clinical Specimens

EPIDEMIOLOGY AND CLINICAL LABORATORY DIAGNOSIS

Epidemiology

Estimates published in 2011 indicate that 31 pathogens on which surveillance data is available cause 9.4 million US cases of foodborne illnesses every year. Of these illnesses, 3.6 million are attributable to bacterial pathogens, with O157 STEC accounting for approximately 63,150 illnesses and non-O157 STEC accounting for approximately 112,750 illnesses every year.\(^{101}\)

STEC infection causes a wide spectrum of clinical illness, including non-bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS).\(^{102}\) Approximately 6-8% of persons (15% of children <5 years old) who seek medical attention for O157 STEC infection develop HUS, a life-threatening condition characterized by thrombocytopenia, hemolytic anemia and acute renal failure.\(^{102}\) Many non-O157 STEC strains may also cause HUS; however, the vast majority of diarrhea-associated HUS cases in the US are caused by infection with O157 STEC.\(^{103}\) Infections are most common in young children (<5 years), and the risk of HUS is highest in this group; however, STEC infections and HUS can occur in persons of all ages.\(^{104}\)

STEC are found in the intestines and feces of healthy animals (especially cattle and other ruminants) and are transmitted to humans by consumption of contaminated food or water, or through direct contact with infected animals or persons.\(^{105}\) Co-infection with multiple serotypes of STEC is possible.\(^{28}\) Undercooked beef, particularly ground beef, has been implicated in many O157 STEC outbreaks; however, other foods including unpasteurized juice, raw milk and raw produce (e.g., lettuce, spinach) have also been implicated in outbreaks.\(^{106}\) Non-O157 STEC outbreaks have been attributed most commonly to daycare exposure, yet a large variety of food items have also been implicated (see Table 6). The following is a list of possible exposures associated with STEC infection:

- visit to a farm, petting zoo or other venue where animals were present
- consumption of raw or undercooked meat
- consumption of raw produce
- contact with a person with STEC infection or linked to an outbreak of STEC infections
- bathing in, swimming in, or drinking recreational fresh water
- drinking water from a private well or non-chlorinated source
### Table 6:
Outbreaks of non-O157 STEC infection through 2008, United States (preliminary data, subject to change)

<table>
<thead>
<tr>
<th>YEAR</th>
<th>SEROGROUP</th>
<th>EXPOSURE/VEHICLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990</td>
<td>0111</td>
<td>Unknown</td>
</tr>
<tr>
<td>1994</td>
<td>0104</td>
<td>Pasteurized Milk</td>
</tr>
<tr>
<td>1998</td>
<td>0121</td>
<td>Unknown</td>
</tr>
<tr>
<td>1999</td>
<td>0121</td>
<td>Lake water</td>
</tr>
<tr>
<td>1999</td>
<td>0111</td>
<td>Salad bar/ice from barrel</td>
</tr>
<tr>
<td>1999</td>
<td>0145</td>
<td>Day care</td>
</tr>
<tr>
<td>2000</td>
<td>0103</td>
<td>Punch</td>
</tr>
<tr>
<td>2001</td>
<td>0111</td>
<td>Day care</td>
</tr>
<tr>
<td>2001</td>
<td>026</td>
<td>Lake water</td>
</tr>
<tr>
<td>2004</td>
<td>0111</td>
<td>Apple cider - Also included cases of Cryptosporidium</td>
</tr>
<tr>
<td>2005</td>
<td>045</td>
<td>Food handler</td>
</tr>
<tr>
<td>2005</td>
<td>026</td>
<td>Day care</td>
</tr>
<tr>
<td>2006</td>
<td>045</td>
<td>Animal contact - Goats</td>
</tr>
<tr>
<td>2006</td>
<td>0121</td>
<td>Day care</td>
</tr>
<tr>
<td>2006</td>
<td>0121</td>
<td>Salad</td>
</tr>
<tr>
<td>2006</td>
<td>026</td>
<td>Strawberries, blueberries, or both. Also 3 of 4 cases with farm exposure reported petting goats at petting zoo on farm</td>
</tr>
<tr>
<td>2007</td>
<td>0111</td>
<td>Person-to-person</td>
</tr>
<tr>
<td>2007</td>
<td>0111</td>
<td>Ground beef</td>
</tr>
<tr>
<td>2007</td>
<td>026, 0121, 084</td>
<td>Ill food worker(s)</td>
</tr>
<tr>
<td>2007</td>
<td>045</td>
<td>Petting zoo</td>
</tr>
<tr>
<td>2008</td>
<td>0111</td>
<td>Restaurant/ill food worker(s)</td>
</tr>
<tr>
<td>2008</td>
<td>0111</td>
<td>Day care</td>
</tr>
</tbody>
</table>

Most reported STEC infections in the United States are caused by O157 STEC; however, more than 100 STEC serotypes have been associated with sporadic human illness and outbreaks. The most common non-O157 serogroups are O26, O45, O103, O111, O121 and O145, based on human isolates submitted by state public health laboratories to the CDC *E. coli* National Reference Laboratory between 2003 and 2008 (Strockbine, unpublished data).

In 2010, the Foodborne Diseases Active Surveillance Network (FoodNet) reported 442 (0.9 per 100,000 population) O157 STEC infections and 451 (1.0 per 100,000) non-O157 STEC infections. In FoodNet sites, non-O157 STECs were the fourth most common reported bacterial enteric pathogen after *Salmonella*, *Shigella*, and *Campylobacter*. The incidence of O157 STEC infections was reduced to reach the 2010 national health
target of less than 1.0 per 100,000 population. This reduction may be explained, in part, by improvements in epidemiological investigations of STEC infections and changes in testing practices among clinical and public health laboratories.

Clinical Laboratory Diagnosis

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 because of this serotype’s inability to ferment sorbitol within 24 hours. Another characteristic biochemical reaction for the identification of O157 STEC is the lack of production of the enzyme ß-D-glucuronidase (see Table 7). The substrate 4-methylumbelliferyl-ß-D-glucuronide (MUG) is incorporated into some selective media for O157 STEC, usually in addition to sorbitol. Unfortunately, selective media for non-O157 STEC serogroups are not available at this time; these organisms cannot be distinguished from normal intestinal flora on a normal enteric isolation media containing lactose.

In order to aid in the detection and identification of non-O157 STEC, enzyme immunoassays (EIA) to detect Shiga toxin directly in a stool specimen or from an overnight enrichment culture of the stool were licensed starting in the mid-1990s. Second generation Shiga toxin tests can differentiate Stx1 and Stx2. These assays may improve clinical management of STEC cases since production of Stx2 is associated with more severe disease and outcomes than Stx1. It has recently been shown that ancillary virulence genes on serogroup-designating O-islands enhance virulence.
<table>
<thead>
<tr>
<th>TEST</th>
<th>COMPANY</th>
<th>FORMAT</th>
<th>TARGET</th>
<th>TIME</th>
<th>SPECIMEN*†</th>
<th>COMMENTS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BloStar OIA ® SHIGATOX</td>
<td>Inverness Medical Professional Diagnostics, Inc (Boston, MA)</td>
<td>Optical immunoassay</td>
<td>Shiga toxins, cannot differentiate</td>
<td>15 min</td>
<td>S, E, I, P</td>
<td>Withdrawn from market in 2009</td>
<td>112</td>
</tr>
<tr>
<td>Duopath® Verotox-Ins Gold Labeled Immunosorbent Assay</td>
<td>Merck (Germany)</td>
<td>Lateral flow immunoassay</td>
<td>Shiga toxins, can differentiate between 1 and 2</td>
<td>20 min</td>
<td>I</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>Immunocard STAT® EHEC</td>
<td>Meridian Diagnostics, Inc (Cincinnati, OH)</td>
<td>Lateral flow immunoassay</td>
<td>Shiga toxins, can differentiate between 1 and 2</td>
<td>20 min</td>
<td>E, I</td>
<td></td>
<td>114</td>
</tr>
<tr>
<td>Premier EHEC®</td>
<td>Meridian Diagnostics, Inc (Cincinnati, OH)</td>
<td>Microplate EIA</td>
<td>Shiga toxins, cannot differentiate</td>
<td>~ 3.5 hr</td>
<td>S, E, I</td>
<td>Testing after overnight broth enrichment is recommended; manufacturer’s insert for direct testing on stools says relative sensitivity is 73%</td>
<td>115</td>
</tr>
<tr>
<td>ProSpecT® Shiga Toxin E. coli (STEC) Microplate Assay</td>
<td>Remel (Lenexa, KS)</td>
<td>Microplate EIA</td>
<td>Shiga toxins, cannot differentiate</td>
<td>~ 3 hr</td>
<td>S, E, P</td>
<td>Testing after overnight broth enrichment is recommended; manufacturer’s insert for direct testing on stools says relative sensitivity is 97%</td>
<td>116</td>
</tr>
<tr>
<td>VTEC Screen “Salmon”/Denka Seiken RPLA</td>
<td>Denka Seiken (Japan)</td>
<td>Reversed passive latex agglutination (RPLA)</td>
<td>Shiga toxins, can differentiate between 1 and 2</td>
<td>4 hr</td>
<td>I</td>
<td>Not available in the United States</td>
<td>117-119</td>
</tr>
</tbody>
</table>
Appropriate specimen for testing based on manufacturer’s recommendations: S, direct stool; E, enrichment broth; I, isolate; P, stool in transport medium (Cary-Blair).

EIA testing of broth cultures or growth from primary isolation plate, rather than directly on stool specimens, is recommended because the amount of free fecal Stx in stools is often low\textsuperscript{120} and because the manufacturers’ inserts report increased sensitivity and specificity when testing on broth cultures. Adapted from reference 1.
HEMOLYTIC UREMIC SYNDROME TESTING

Hemolytic uremic syndrome (HUS) is a serious complication of Shiga toxin-producing *Escherichia coli* (STEC) infection, with approximately 200 cases reported each year in the US between 2002 and 2006. Prompted by the finding of an STEC in the stool of a patient who died from hemolytic uremic syndrome, Karmali et al. examined the stools of sporadic cases of HUS and found direct or indirect evidence of STEC infection in 11 of 15 cases. Karmali and colleagues subsequently confirmed this landmark observation in a prospective controlled study that linked cases of HUS with isolation from the stool of STEC belonging to at least six different O serogroups (O26, O111, O113, O121, O145 and O157).

However, detection and isolation of STEC is often problematic in these patients, possibly due to a decreased pathogen load in the patient’s stool specimen since HUS typically develops one week or more after diarrhea begins. The use of immunomagnetic separation (IMS) to enhance standard culture techniques has been shown to dramatically increase isolation of O157 STEC in stools from patients with HUS. Karch et al detected *E. coli* O157:H7 using standard direct plating methods in 7 of 20 (35 percent) patients with HUS who had serological evidence of O157 STEC infection. Using IMS enrichment, O157 STEC was detected in 18 of 20 (90 percent) patients. IMS reagents for some non-O157 STEC have recently become available. Comparative studies, similar to the one mentioned above, have not been conducted for non-O157 disease detection in humans, yet a similar increase in sensitivity is assumed.

Establishment of a specific bacterial etiology in cases of post-diarrheal HUS is important for public health prevention and control activities, and provides useful prognostic information for clinicians. Shiga toxin-induced cases of HUS may be missed entirely unless clinical laboratories are asked to save and submit original stool specimens and any EIA broth subcultures from HUS cases, even if negative for STEC initially. Epidemiologists should coordinate with infectious disease physicians, pediatricians, nephrologists or other specialists to ensure that stools are collected and sent to the clinical laboratory. Public health laboratories should coordinate with clinical laboratories to ensure stools are appropriately tested, saved and submitted, along with EIA broths, for further testing when necessary. When submitting specimens from patients with HUS to the public health laboratory, clinical laboratories should clearly label the suspect diagnosis on the requisition slip with the knowledge that specimens are difficult to obtain from HUS patients and that recovery of STEC from HUS specimens may require enhanced detection methods by the public health laboratory.
Public health laboratories should examine stools and EIA broths (even broth samples testing negative for Shiga toxin at the clinical laboratory) from HUS cases using the recommended methods described, and conduct further testing as appropriate. Also, serologic diagnostic testing on serum from persons whose illness meets the clinical case definition of HUS but whose stool specimens have not yielded an STEC isolate may aid in case classification (see “Serodiagnosis”).
SERODIAGNOSIS

When enhanced culture results are STEC negative, serology may be used to suggest the etiology of post-diarrheal HUS in patients. By determining an etiologic agent for a patient’s illness, epidemiological linkages can be confirmed or established, thus improving the quality of surveillance data. By the time HUS develops, approximately one week after onset of diarrhea, antibodies to the LPS are usually detectable in patient sera.

Serologic testing for IgM and IgG antibodies to the lipopolysaccharide antigen of O157 and O111 is available at CDC by request to the Immunodiagnostic Laboratory within the Enteric Diseases Laboratory Branch. The test is also available for other serogroups but has not been validated and may be useful when infection with a specific serogroup suspected (e.g., during an outbreak). Ideally, at least one serum specimen should be collected within one or two weeks after diarrhea began, and convalescent specimens collected about 14-28 days later. If a serum collected in the first two weeks of diarrheal illness is not available, the earliest possible serum specimen should be tested and a second specimen collected within the next 14-28 days. To aid in the interpretation of serologic test results, information including date of collection, dates of red cell transfusions, dates of plasmapheresis, any history of immunosuppressive conditions, and medications should be submitted with the serum sample.
CONCLUDING REMARKS
CONCLUDING REMARKS

These recommendations are based on current knowledge and available literature regarding STEC diagnostic realities. However, our knowledge about the epidemiology and the pathogenicity of STEC is rapidly evolving. Research is ongoing to determine which virulence factors and combinations of virulence factors and other markers may predict the severity and long-term prognosis of an STEC infection. This information will lead to a paradigm shift in the way STEC is diagnosed as this new detailed information is utilized to guide patient therapy.

Diagnostic methods are already rapidly evolving to reflect developments in molecular array and sequencing technologies. However, public health resources are dwindling, and there may not be sufficient resources available to culture-confirm all positive results of new and existing diagnostic tests. Since our current surveillance systems are built upon the characterization of bacterial isolates, the lack of culture confirmation will affect our surveillance data, and the detection and investigation of outbreaks will suffer. It is therefore critical that the diagnostic methods of tomorrow both fulfill the needs of clinicians and those of public health. Thus, the new clinical diagnostic methods must combine the clinically actionable information of the serotype and virulence profile of the infecting strain with the subtyping information necessary for public health action. Assays which provide this information on pure STEC cultures are available or are in development. In the future, one can expect these will be implemented routinely into national surveillance programs.
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