Preface

The study of the pathogenesis of Shiga toxin-producing *Escherichia coli* (STEC) infections encompasses many different disciplines, including clinical microbiology, diagnostics, animal ecology, and food safety, as well as the cellular microbiology of both bacterial pathogenesis and the mechanisms of toxin action. *E. coli: Shiga Toxin Methods and Protocols* aims to bring together a number of experts from each of these varied fields in order to outline some of the basic protocols for the diagnosis and study of STEC pathogenesis. We hope that our book will prove a valuable resource for the clinical microbiologist as well as the cellular microbiologist.

For the clinical microbiologist, our aim is to detail a number of current protocols for the detection of STEC in patient samples, each of which have their own advantages. Chapter 1 provides an introduction into the medical significance of STEC infections. Chapters 2–7 follow with protocols for the diagnosis and detection of STEC bacteria in patient and animal samples.

For the cellular microbiologist, we have brought together a number of experts from basic microbiologists to cell biologists to provide different protocols useful in studying the varied aspects of STEC pathogenesis. Chapters 8–13 concentrate on the cellular microbiology of STEC infections, describing protocols to study host–pathogen interactions as well as studies on the hemolysin of STEC. In Chapters 14–22, various protocols are described for studying the details of Shiga toxin (Stx) biology, from the purification of the toxin to studies of the effects of Stx on various host cell functions. Finally Chapters 23–25 provide detailed protocols for the study of STEC-mediated disease in various animal models.

The format of the chapters will be familiar to those who have used other volumes in the Methods in Molecular Medicine series. The Notes section at the end of each chapter pays particular attention to detailing the potential problems that may be encountered, as well as providing alternate methods for the protocols described.

Finally, we hope *E. coli: Shiga Toxin Methods and Protocols* will benefit those interested in both the clinical and pathological aspects of STEC infections, as well as provide a number of valuable protocols for those

researchers studying host–pathogen interactions. We would like to thank the contributing authors as well as John Walker and the staff at Humana Press for their assistance in putting this volume together.

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Methods for Detection of STEC in Humans

An Overview

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1. Introduction

Timely and accurate diagnosis of Shiga toxigenic *Escherichia coli* (STEC) disease in humans is extremely important from both a public health and a clinical management perspective. In the outbreak setting, rapid diagnosis of cases and immediate notification of health authorities is essential for effective epidemiological intervention. Early diagnosis also creates a window of opportunity for therapeutic intervention. Agents capable of adsorbing and neutralizing free Shiga toxin (Stx) in the gut lumen have been described *(1,2)*, and these are likely to be most effective when adminstered early in the course of disease, before serious systemic sequelae develop. Also, the clinical presentation of STEC disease can sometimes be confused with other bowel conditions; thus, early definitive diagnosis may prevent unnecessary invasive and expensive surgical and investigative procedures or administation of antibiotic therapy, which may be contraindicated *(3)*. However, detection of STEC is fraught with difficulty, particularly for strains belonging to serogroups other than O157. In the early stages of infection, there may be very high numbers of STEC in feces (the STEC may constitute >90% of aerobic flora), but as disease progresses, the numbers may drop dramatically. In cases of hemolytic uraemic syndrome (HUS), the typical clinical signs may become apparent as much as 2 wk after the onset of gastrointestinal symptoms, by which time the numbers of the causative STEC may be very low indeed. Also, in some cases, diarrhea is no longer present and only a rectal swab may be available at the time of admission to the

hospital, limiting the amount of specimen available for analysis. For these reasons, STEC detection methods need to be very sensitive and require minimal specimen volumes.

Shiga toxigenic *E. coli* diagnostic methods are based on the detection of the presence of either Stx or *stx* genes in fecal extracts or fecal cultures, and/or isolation of the STEC (or other Stx-producing organism) itself (reviewed in **refs. 4–7**). These procedures differ in complexity, speed, sensitivity, specificity, and cost, and so diagnostic strategies need to be tailored to the clinical circumstances and the resources available.

2. Detection of Stx

2.1. Tissue Culture Cytotoxicity Assays

Cytotoxicity for Vero (African green monkey kidney) cells remains the "gold standard" for the demonstration of the presence of Stx-related toxins in a fecal sample. Vero cells have a high concentration of Gb_3 receptors in their plasma membranes as well as Gb_4 (the preferred receptor for Stx2e) and thus are highly sensitive to all known Stx variants. In a typical assay, Vero monolayers (usually in 96-well trays) are treated with filter-sterilized fecal extracts or fecal culture filtrates and examined for cytopathic effect after 48 to 72 h incubation. Historically, this assay has played an important role in establishing a diagnosis of STEC infection, particularly where subsequent isolation of the causative organism has proven to be difficult *(4)*. The sensitivity is influenced by the abundance of STEC in the fecal sample, as well as the total amount and potency of the Stx produced by the organism itself, and the degree to which the particular Stx is released from the bacterial cells. Karmali et al. *(8)* found that treating mixed fecal cultures with polymyxin B to release cell-associated Stx improved the sensitivity of the Vero cell assay, such that it could reliably detect STEC when present at a frequency of 1 CFU (Colony-forming unit) per 100. Clearly, some STEC produce very high levels of toxin and these can be detected at even lower frequencies; however, the converse also applies.

Although detection of Stx by tissue culture cytotoxicity is a valuable diagnostic method, it is labor intensive, time-consuming and cumbersome. Not all microbiological diagnostic laboratories are appropriately set up for tissue culture work, with Vero cell monolayers available on demand. Moreover, speed of diagnosis is important and the results of cytotoxicity assays are generally not available for 48–72 h. Also, the presence of cytoxicity in a crude filtrate could be the result of the effects of other bacterial products or toxins; thus, positive samples should always be confirmed (and typed) by testing for neutralization of cytotoxicity by specific (preferably monoclonal) antibodies to Stx1 or Stx2.

2.2. ELISA Assays for the Direct Detection of Stx

A number of enzyme-linked immunosorbent assays (ELISA) have been developed for direct detection of Stx1 and Stx2 in fecal cultures and extracts. Like Vero cytotoxicity, these have a potentially important role in diagnosis, because they are capable of detecting the presence of STEC (or other Stx-producing species) regardless of serogroup. However, ELISA assays are more rapid, permitting a result within 1 d. Most of the published ELISA methods involve a sandwich technique using immobilized monoclonal or affinity-purified polyclonal antibodies to the toxins as catching ligands. Purified Stx receptor (Gb_3) or hydatid cyst fluid (containing P_1 glycoprotein, which also binds Stx) have also been used to coat the solid phase. After incubation with cultures (or direct fecal extracts), bound toxin is detected using a second Stx-specific antibody followed by an appropriate anti-immunoglobulin–enzyme conjugate. Some assays employ a Stx detection antibody directly conjugated to the enzyme or a biotinylated detection antibody that is used with a streptavidin–enzyme conjugate *(5)*.

Importantly, Stx ELISA assays are now commercially available in kit form (e.g., Premier EHEC from Meridian Diagnostics; LMD from LMD Laboratories, Carlsbad, CA). Reported specificities for both the in-house and commercial ELISA assays, determined by testing reference isolates and by comparing ELISA results for fecal extracts with culture and Vero cytotoxicity, have generally been very high. The sensitivity of the various ELISA assays is affected by a number of variables, including the avidity of the antibodies employed as well as the type and amount of Stx produced by a given strain. Early in-house ELISAs were generally less sensitive than the Vero cytotoxicity assay and sensitivity was inadequate to reliably detect low levels of Stx found in direct fecal extracts. However, the amount of free Stx present in primary fecal cultures is generally higher, particularly when broths are supplemented with polymyxin B and/or mitomycin C to enhance the production and release of Stx. Under such circumstances, ELISAs have been reported to be capable of detecting the presence of STEC comprising as little as 0.1% of total flora *(9,10)*. Moreover, in two large studies, the Premier EHEC ELISA has been shown to be at least as sensitive as Vero cytotoxicity for detection of STEC in fecal culture extracts *(11,12)*. Such assays will be of considerable utility for routine clinical laboratories without access to more specialized diagnostic procedures, particularly for detection of non-O157 STEC.

2.3. Reverse Passive Latex Agglutination

A reverse passive latex agglutination (RPLA) assay for detection of Stx production is also commercially available in kit form (VTEC-RPLA from Oxoid,

Unipath Limited, Basingstoke, UK; Verotox-F from Denka Seiken, Tokyo, Japan). This test involves incubation of serially diluted polymyxin B extracts of putative STEC cultures, or culture filtrates, with Stx1- and Stx2-specific antibody-coated latex particles and examining agglutination after 24 h. Beutin et al. *(13)* detected toxin production (of the appropriate type) in strains containing stx_1, stx_2, and stx_2c, but it did not detect toxin produced by the strains carrying stx_2e. However, a number of Stx2 and Stx2c producers gave positive reactions only when undiluted extracts were tested, which suggested that sensitivity might be inadequate for testing primary fecal culture extracts. More promising results have since been reported by Karmali et al. *(14)*, who demonstrated 100% sensitivity and specificity with respect to the Vero cytotoxicity assay when testing culture filtrates of reference STEC isolates, as did the previous study. However, analysis of dilutions of purified toxins demonstated that the end-point sensitivity of Verotox-F was comparable to Vero cytotoxicity. Although data on the performance of these assays using mixed fecal culture extracts are not yet available, it appears that they are simple, rapid, and accurate and may enable widespread screening for STEC by clinical laboratories.

3. Detection of *stx* Genes

3.1. Hybridization with DNA and Oligonucleotide Probes

Access to cloned stx_1 and stx_2 genes and their respective nucleotide sequences enabled the development of DNA and oligonucleotide probes for the detection of STEC (reviewed in **ref. 5**). The introduction of non-radioactive labels such as digoxigenin (DIG) and biotin has overcome many of the disadvantages associated with ^{32}P- or ^{35}S- labeled probes, which were used in earlier studies. Typically, these probes have been used for testing large numbers of fecal *E. coli* isolates, or for the direct screening of colonies on primary isolation plates for the presence of *stx* genes by colony hybridization *(15)*. These procedures are both sensitive and specific, and when stringent washing conditions are used, strains carrying stx_1, stx_2, or both can be differentiated. Although hybridization with DNA or oligonucleotide probes is not a particularly sensitive means for screening broth cultures or fecal extracts for the presence of STEC, it is a powerful tool for distinguishing colonies containing *stx* genes from commensal organisms, as discussed later.

3.2. Polymerase Chain Reaction

Access to sequence data for the various *stx* genes has permitted design of a variety of oligonucleotide primer sets for amplification of *stx* genes using polymerase chain reaction (PCR) (reviewed in **ref. 5**). Crude lysates or DNA extracts from single colonies, mixed broth cultures, colony sweeps, or even

direct extracts of feces or foods can be used as templates for PCR. *Stx*-specific PCR products are usually detected by ethidium bromide staining after separation of the reaction mix by agarose gel electrophoresis. Some of the *stx* PCR assays described to date combine different primer pairs for stx_1 and stx_2, and, in some cases, stx_2 variants, in the same reaction. These direct the amplification of fragments which differ in size for each gene type *(16–19)*. Other *stx* PCR assays use single pairs of primers based on consensus sequences, which are capable of amplifying all *stx* genes, with subsequent identification of gene type requiring a second round of PCR, or hybridization with labeled oligonucleotides directed against type-specific sequences within the amplified fragment *(20,21)*. Apart from the added sensitivity, secondary hybridization steps act as independent confirmation of identity of the amplified product. Restriction analysis of amplified portions of stx_2 genes has also been used to discriminate between stx_2 and stx_2 variants *(22–24)*.

Polymerase chain reaction technology is ideally suited to the detection of *stx* genes in microbiologically complex samples such as feces and foodstuffs, and it is potentially extremely sensitive. However, such samples may contain inhibitors of *Taq* polymerase, and sensitivity is often suboptimal when direct extracts are used as template. For both feces and food samples, the sensitivity of PCR assays is vastly increased if template DNA is extracted from broth cultures *(18,21)*. Broth enrichment (which can involve as little as 4 h incubation) serves two purposes. Inhibitors in the sample are diluted and bacterial growth increases the number of copies of the target sequence. Optimization of sensitivity is of paramount importance, because the numbers of STEC in the feces of patients with serious Stx-related diseases or in suspected contaminated foodstuffs may be very low indeed. Another consideration that may impact upon performance of some *stx*-specific PCR assays is the DNA sequence polymorphisms that are known to exist. This is particularly so for stx_2-related genes, for which significant variation has been reported (reviewed in **ref. 5**). Sequence divergence between the primer and its target (particularly at the 3' end of the primer) will significantly reduce the efficiency of annealing with potentially dramatic effects on sensitivity of the PCR reaction. When selecting or designing primers, care must be taken to avoid regions where sequence heterogeneity has already been reported. PCR assays that use a single primer pair to amplify both stx_1 and stx_2 may be less susceptible to this potential complication. Target sequences that are conserved between otherwise widely divergent genes are likely to encode structurally important domains; thus, random mutations will be strongly selected against.

Speed of diagnosis of STEC infection is also an important consideration in the clinical setting. The precise time required for a PCR assay varies with the amplification protocol itself (number of cycles and incubation times at each

temperature), the method used for DNA extraction, and the procedure for detection of the PCR products. The minimum time required for direct PCR analysis of an unenriched fecal sample analyzed by agarose gel electrophoresis could be as little as 4 h. Inclusion of a broth enrichment step and use of a more sophisticated DNA purification procedure would increase this time to 12–24 h, whereas hybridization of PCR products with *stx* probes could add a further day. The cumulative increase in sensitivity resulting from each additional step needs to be balanced against the increase in time, and this equation will vary in accordance with the particular clinical or epidemiological context.

It has often been argued that PCR is a technique that should be confined to reference laboratories, because it is labor intensive and requires highly skilled staff. However, an increasing number of clinical laboratories are now routinely using PCR for a range of applications. Unlike the Stx-specific antibodies and other specialized reagents needed for ELISA assays, custom-made oligonucleotide primers are inexpensive and universally available and have a very long shelf life. Modern versatile PCR thermal cyclers are no more expensive than ELISA plate readers and can handle assays in the 96-well format for laboratories that have a high specimen throughput. Moreover, a variety of alternatives to agarose gel electrophoresis have been developed for high-volume, sensitive, semiautomatable detection of PCR products (e.g., the TaqMan and AmpliSensor fluorogenic PCR assay systems) *(25,26)*.

3.3. PCR for Detection of Other STEC Markers

Polymerase chain reaction has also been used for the detection of genes encoding accessory virulence factors of STEC, such as *eae*, a component of the locus of enterocyte effacement (LEE) pathogenicity island, which encodes the capacity to form attaching/effacing lesions on enterocytes, and EHEC-*hlyA*, which encodes an enterohemolysin and is located on a large (approx 60 MDa) plasmid present in may STEC strains *(27,28)*. This information may be of clinical significance, because there appears to be a link between the presence of these genes and the capacity of an STEC isolate to cause serious human disease *(29,30)*. PCR assays exploiting sequence variation in the 3' portion of the *eae* gene have been used as a basis for distinguishing O157 STEC strains from certain other common serogroups *(27,31)*. However, availability of sequence data for the genetic loci (*rfb* regions) encoding O-antigen biosynthesis in *E. coli* serogroups such as O157, O111, and O113 *(32,33)* have enabled development of more reliable serogroup-specific PCR assays. Two other genetic markers associated with O157:H7 STEC strains have also been used as the basis of PCR assays. These are the *fliCh$_7$* gene, which encodes the H7 antigen *(34)*, and a single base mutation in the *uidA* gene (detected by mismatch amplification mutation assay), which is responsible for the β-glucoronidase-negative phenotype of O157:H7 strains *(35)*.

Polymerase chain reaction primers specific for the various STEC markers referred to here are typically deployed as components of multiplex PCR assays, which also detect *stx* genes, enabling simultaneous detection and partial genetic characterization of STEC in a sample. However, the increased complexity of these assays renders them less suitable for routine, high-volume screening of fecal samples or foods. In our laboratory, we have adopted a two-tiered approach in which fecal culture extracts are initially screened using a *stx* PCR assay yielding a single PCR product for all *stx* types *(21)*. Any positive samples are then subjected to multiplex PCR analysis using two primer sets. The first utilizes four primer pairs and detects the presence of *stx$_1$*, *stx$_2$* (including variants of *stx$_2$*), *eae*, and EHEC-*hlyA* *(32)*. The second assay uses three primer pairs directed against serogroup-specific sequences in the *rfb* regions of *E. coli* O157, O111, and O113 *(33)*. These two multiplex assays provide independent confirmation of the initial *stx* screening assay, as well as information on the serogroup and virulence traits of the STEC strain or strains present in a sample. Details of these assays are provided in a later chapter in this volume.

4. Isolation of STEC

Although a substantial amount of information on the causative STEC can be obtained by molecular analysis of mixed cultures, isolation of the STEC itself must be considered as the definitive diagnostic procedure. Apart from confirming the molecular data, isolation permits additional characterization of the STEC by a variety of methods, including O:H serotyping, phage typing, restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), amplification-based DNA typing, and so forth. Although such characterization may have limited clinical application, it is of great importance from an epidemiological point of view, particularly in the outbreak setting, and methods for this are presented in a later chapter in this volume.

4.1. Culture for O157 STEC

Culture on sorbitol–MacConkey agar (SMAC) has been the most commonly used method for isolation of O157 STEC. This is because unlike the majority of fecal *E. coli* strains, most O157:H7 and O157:H⁻ STEC, which are the most common causes of human STEC disease in many parts of the world, are unable to ferment sorbitol *(36)*. SMAC plates are inoculated with the fecal specimen and examined after 18–24 h incubation for the presence of colorless, sorbitol-negative colonies. Individual colonies can then be tested by slide or tube agglutination with (commercially available) O157- and H7-specific antisera or latex reagents. It should, of course, be remembered that not all O157 *E. coli* produce Stx, thus toxigenicity needs to be confirmed by tissue culture, ELISA, or RPLA, as discussed earlier.

The sensitivity of SMAC is limited by the capacity to recognize nonfermenting colonies against the background of other organisms on the plate, and this is difficult when the O157 strain comprises less than 1% of the flora. Isolation rates can be improved by incorporation of cefixime to inhibit *Proteus* sp. and rhamnose, which is fermented by most sorbitol-negative non-O157 *E. coli* (O157 strains generally do not ferment rhamnose) *(37)*, or cefixime and potassium tellurite (CT-SMAC) *(38)*. Although screening fecal cultures on SMAC and its variants is inexpensive and involves minimal labor and equipment, it will principally detect STEC belonging to serogroup O157. Serious STEC disease has been associated with many other serogroups, and although some of these can also be sorbitol-negative, the majority are sorbitol-positive *(4)*. Furthermore, Stx2-producing, sorbitol-positive *E. coli* O157:H⁻ isolates have been associated with cases of HUS in Germany and the Czeck Republic *(39,40)*. These strains were also very sensitive to tellurite, which mitigates against the use of CT-SMAC for isolation of STEC in these regions.

E. coli O157:H7 can also be distinguished from other *E. coli* strains by failure to produce β-D-glucuronidase *(41)*, an enzyme that can be readily detected fluorigenically using the substrate 4-methylumbelliferyl-β-D-glucuronide or colorimetrically on plates supplemented with 5-bromo-6-chloro-3-indolyl-β-D-glucuronide *(42)*. Again, this criterion is not useful for the detection of non-O157 STEC or the sorbitol-positive O157 STEC isolates referred to earlier, as these are generally glucuronidase-positive.

Various specialized commercial agar media for isolation of O157 STEC are now available. Rainbow Agar O157 (Biolog Inc., Hayward, CA), for example, contains selective agents for *E. coli* and chromogenic substrates for β-D-glucuronidase and β-galactosidase. Glucuronidase-negative, galactosidase-positive O157 strains appear as black or gray colonies on this medium, whereas commensal *E. coli* strains are pink. It has also been claimed that some non-O157 STEC strains overproduce β-galactosidase relative to β-D-glucuronidase on this medium, giving the colonies a distinctive intermediate color. To date, analyses of the efficacy of this medium for detection of either O157 or non-O157 STEC in fecal samples are limited, but at least one study has shown that Rainbow Agar O157 is clearly superior to SMAC *(43)*. CHROMagar O157 (Becton Dickinson Microbiology Systems) also distinguishes O157 on the basis color; O157 colonies are mauve, and other bacteria are either blue or colorless. For both of these media, the manufacturers suggest incorporation of additional selective agents (novobiocin and tellurite, respectively) to improve isolation rates. Again, it should be emphasized that isolation of a putative O157 strain from either of these chromogenic selective media is not a definitive diagnosis in itself, and as for SMAC, isolates must be tested to confirm Stx production.

4.1.1. Direct Detection of O157 Antigen in Fecal Samples

Direct immunofluorescent staining of fecal specimens using polyclonal anti-O157-FITC is a potential alternative to SMAC for detection of *E. coli* O157 involving only about a 2-h turn-around time *(44)*. Commercial ELISAs for rapid (less than 1 h) detection of the presence of O157 antigen in fecal specimens are also available (LMD from LMD Laboratories, Carlsbad, CA; Premier *E. coli* O157 from Meridian Diagnostics, Inc., Cincinnati, OH). Both the immunofluorescence and ELISA tests have similar or superior sensitivity to SMAC *(12,44,45)*, and, importantly, are capable of detecting sorbitol-fermenting O157 STEC, should they be present. A number of other O157 immunoassay detection kits are commercially available (e.g., Ampcor *E. coli* O157:H7 [Ampcor]; Tecra *E. coli* O157 [Tecra]; EHEC-TEK [Organon Teknika]), but data on their utility for detection of *E. coli* O157 in human fecal cultures or extracts are not available. Again, all of these assays require confirmation either by culture or by demonstration of Stx in the sample.

4.2. Culturing for Non-O157 STEC

The high dependence of most clinical laboratories on SMAC culture for screening fecal samples from patients with suspected STEC infection has undoubtedly led to an over-estimation of the relative importance of O157 STEC as a cause of human disease. However, it has been known for many years that *E. coli* strains belonging to a large range of serotypes as well as certain strains of other bacterial species are capable of producing Stx and causing serious disease in humans *(4)*. Regrettably, there is no definitive biochemical characteristic that distinguishes STEC belonging to serogroups other than O157 from commensal fecal *E. coli* strains, a fact that significantly complicates isolation of such organisms. However, nearly all O157 STEC, as well as a significant proportion of non-O157 STEC strains, produce the plasmid-encoded enterohemolysin EHEC-Hly. Such strains are not hemolytic on standard blood agar, but produce small, turbid hemolytic zones on washed sheep erythrocyte agar (supplemented with Ca^{2+}) after 18–24 h incubation at 37°C. Production of EHEC-Hly is highly indicative that a given isolate is an STEC, but the predictive value of a negative result is low *(30,46)*. As a consequence, hemolytic phenotype on washed sheep erythrocyte agar is a useful means of identifying colonies for further analysis, but nonhemolytic colonies should also be tested.

The only comprehensive means of isolating STEC or other Stx-producing organisms involves direct analysis of colonies on nonselective agar plates using either *stx*-specific nucleic acid probes or antibodies to Stx, and a variety of protocols for this purpose have been described (reviewed in **ref. 5**). This is a labor-intensive process and can only be justified for specimens that have tested

positive in screens for Stx (by cytotoxicity or ELISA) or for *stx* (by PCR). Colonies from agar plates can be directly blotted onto a suitable membrane (e.g., nitrocellulose or polyvinylidene difluoride [PVDF] for immunoblots, or positively charged nylon for hybridization). A carefully aligned replicate of the filter must be made and then it can be processed and reacted with antibody or nucleic acid probe by standard procedures. Theoretically, up to several hundred discrete colonies can be tested on a single filter, although this may require dilution and replating of primary cultures. Alternatively, colonies from primary isolation plates can be picked off and inoculated into 96-well microtiter trays containing broth. This is a time-consuming step (15–20 min per tray), but the 96-well format enables the subsequent use of semiautomated machinery to make replicate copies of trays and, after incubation, to transfer aliquots onto appropriate filters; the trays are also convenient for preservation of the isolates at –80°C. Comparisons of the sensitivity and specificity of immunoblotting and DNA probing for the detection of STEC colonies indicate that the latter is probably a more reliable method. Immunoblot techniques have the further disadvantage of having to grow colonies on special media in order to optimize production and/or release of Stx *(47)*.

4.3. Immunomagnetic Separation for Isolation of STEC

Immunomagnetic separation (IMS) is a potentially powerful enrichment technique for the isolation of STEC from low-abundance specimens. The procedure involves coating magnetic beads with anti-LPS (lipopolysaccharide) and mixing these with broth cultures or suspensions of feces or suspect food homogenates. Beads and bound bacteria are then trapped in a magnetic field, the unbound suspension is decanted, and the beads are washed. After additional binding/washing cycles, the beads are plated and resultant colonies tested for reactivity with the appropriate O-antiserum and more importantly for Stx production. The principal drawback of IMS is, of course, its serogroup specificity, and, at present, only O157-specific magnetic beads are available commercially (Dynabeads anti-*E. coli* O157 from Dynal, Oslo; Captivate O157 from Lab M). Notwithstanding this, it is an extremely valuable enrichment technique in circumstances where deliberate and exclusive targeting of this serogroup is justifiable (e.g., analysis of samples epidemiologically linked to proven cases of O157 STEC disease). Several studies have shown that IMS enrichment using the commercial O157-specific beads prior to plating on CT-SMAC significantly increases the isolation rate of *E. coli* O157 from fecal samples *(48,49)*. Also, during the investigation of an outbreak of HUS caused by an O111:H– STEC strain, enrichment using an in-house O111-specific IMS reagent enabled isolation of O111 STEC from a suspected food source after direct plating and colony hybridization had yielded negative results *(50)*.

5. Serological Diagnosis of STEC Infection

Diagnosis of STEC-related disease can be particularly problematic when patients present late in the course of disease, because the numbers of STEC in feces may be extremely low and hence undetectable even by PCR analysis of enrichment broths. However, STEC infection often elicits humoral antibody responses to a range of bacterial products, which may permit elucidation of the etiology of infection by serological means, as discussed in a subsequent chapter in this volume.

Several previous studies have examined immune responses of patients with STEC disease to Stx1, Stx2, and LPS (reviewed in **ref. 5**) and, more recently, to products of the LEE locus such as intimin, Tir, EspA and EspB *(51)*. Theoretically, Stx should be the preferred target because all STEC, by definition, produce Stx1 and/or an Stx2-related toxin. However, previous studies have shown that only a minority of patients with proven STEC disease mount detectable serum antibody responses to the respective toxin type, as judged by either ELISA, cytotoxicity neutralization, or Western blotting *(52–55)*. Moreover, an appreciable proportion of healthy individuals may have detectable serum antibodies to Stx1, particularly in rural populations *(54)*. This would complicate interpretation of results obtained using single serum specimens unless geographically- and age-matched baseline data for the healthy population were available. Ideally, acute and convalescent sera should be tested for rising or falling antibody titres.

More encouraging results have been obtained by testing for antibodies to LPS, although this diagnostic approach suffers from the disadvantage of being able to target only specified serogroups. Not surprisingly, the majority of these studies have focused on serodiagnosis of O157 STEC infections. A high proportion of patients infected with this STEC serogroup have elevated acute-phase serum antibody levels to O157 LPS, as measured by ELISA or passive hemagglutination assay, and the background seropositivity rate in healthy controls is generally low *(52,56–60)*. In several of these studies, anti-LPS titers fell rapidly during the immediate post-acute phase, and so elevated titers in a single specimen may, indeed, be a reliable indicator of current or very recent infection. Clinical laboratory testing, at least for O157 antibodies, is also facilitated by the availability of a commercial latex agglutination test kit, which has been shown to be both sensitive and specific *(61)*. Although data on serological responses to infections caused by other STEC-associated serogroups are more limited, such analyses have been shown to be helpful in determing the etiology in a number of sporadic cases of HUS *(62,63)* and in the investigation of at least three outbreaks of HUS caused by non-O157 STEC strains *(50,64,65)*.

Diagnosis of STEC infection on the basis of serological responses to LEE-encoded proteins has also been advocated. This has the advantage of targeting

a wider range of STEC types, although not all strains associated with serious human disease are LEE-positive. Antibody responses to intimin (the *eae* gene product) were more frequent among HUS patients than responses to other LEE proteins, but the frequency of intimin seroconversion was lower than for O157 LPS *(51)*. It should also be remembered that other enterobacterial pathogens, including enteropathogenic *E. coli*, are LEE-positive and so would be expected to elicit anti-intimin responses in humans. Problems of interpretation may also arise with anti-LPS responses, as even for O157, the association between Stx-production and serogroup is not absolute, and for all serogroups, highly purified LPS antigens are required to minimize crossreactions. Thus, caution should be exercised when interpreting serological data, particularly in the absence of coroborating evidence (e.g., Stx production or the presence of *stx* genes in fecal cultures).

6. Strategies for STEC Detection

Selection of the most appropriate methodology for detection of STEC will involve striking a balance among speed, specificity, sensitivity, and cost of the alternatives. Ideally, clinical microbiology laboratories should screen all fecal samples from patients with acute diarrhea (not just those that are bloody) for the presence of STEC, using methods which are not serogroup restricted. PCR analysis of primary fecal cultures is probably the most sensitive and specific means of screening for the presence of STEC. However, for those laboratories that lack PCR capability, direct screening of fecal cultures for the presence of Stx using one of the commercially available ELISA (or possibly RPLA) kits is recommended. Verocytotoxicity, although slower, is a highly satisfactory alternative. Methods targeted specifically at O157 STEC (e.g., CT-SMAC culture, O157 antigen detection, etc.) are suboptimal stand-alone primary screens, but if comprehensive screening is not possible, it is better to use these methods than not to screen at all. It would be prudent, however, for such laboratories to refer negative specimens from cases of severe bloody diarrhea or suspected HUS to a reference laboratory.

All samples and cultures that test positive after screening should be sent to a reference laboratory for confirmation and attempted isolation of STEC if adequate resources are not available on site. Given the widespread instability of *stx* genes during subculture *(66)*, it is important that initial samples and primary cultures are referred in addition to putative STEC isolates. It is at the isolation stage where the specialized plate media referred to earlier may save time by directing attention to suspect colonies, particularly where they are in low abundance. However, if using such media rather than nonselective plates, it is essential to test a range of colony types and not just those with the STEC-associated phenotype. Given the sensitivity of PCR screens, a proportion of genuine STEC-positive specimens may not yield an isolate even after heroic

attempts. It may still be possible to obtain meaningful additional information about the causative organism in such circumstances. PCR analysis will indicate toxin type and whether virulence-related genes, or genes associated with important serogroups are also present in the sample. However, the interpretation of this information is complicated by the possibility that the composite genotypic profile may represent the sum of genotypes of more than one STEC organism. At least in cases of HUS, information on the likely infecting serogroup can also be obtained by serological tests for anti-LPS.

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