Enzyme-Linked Immunosorbent Assays for Detecting Antibodies to Shiga-Like Toxin I, Shiga-Like Toxin II, and *Escherichia coli* O157:H7 Lipopolysaccharide in Human Serum

Timothy J. Barrett, James H. Green, Patricia M. Griffin, Andrew T. Pavia, Stephen M. Ostroff, and I. Kaye Wachsmuth

Division of Bacterial and Mycotic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia, USA

Abstract. Shiga-like toxin-producing *Escherichia coli* O157:H7 are important causes of bloody diarrhea and hemolytic uremic syndrome. To facilitate the epidemiologic study of these organisms, we developed enzyme-linked immunosorbent assays (ELISAs) for antibodies to Shiga-like toxin I (SLT I), Shiga-like toxin II (SLT II), and *E. coli* O157 lipopolysaccharide (LPS). We tested serum samples from 83 patients in two outbreaks of *E. coli* O157:H7 diarrhea and from 66 well persons. Forty-three patients (52%) had at least one serum sample positive for anti-O157 LPS antibodies; among 26 culture-confirmed patients, 24 (92%) had at least one positive serum sample. Two (3%) of 66 control sera had positive anti-O157 LPS titers. ELISA results for SLT I and II were compared with those of HeLa cell cytotoxicity neutralization assays on both patient and control sera. Neutralization assays detected anti-SLT I antibodies in at least one serum sample from each of 17 (20%) patients and 7 (10.6%) controls, while 16 (19%) patients and 7 controls had positive titers by anti-SLT I ELISA. Although all serum samples, including control sera, showed nonspecific neutralization of SLT II, no antibody titers to SLT II were detected by either neutralization or ELISA. These results indicate that ELISAs for SLT I and SLT II antibodies are comparable to HeLa cell cytotoxicity neutralization assays. Both the ELISAs and neutralization assays are insensitive in detecting infected patients. However, the ELISA for antibodies to *E. coli* O157 LPS is both sensitive and specific, and may be more useful than assays for antitoxic antibodies in detecting persons with *E. coli* O157:H7 infection.

Shiga-like toxin (SLT)-producing *Escherichia coli* are common etiologic agents of bloody diarrhea and frequently cause a severe form of gastroenteritis called hemorrhagic colitis (HC) [8, 12, 18, 19]. HC is characterized by severe abdominal cramping, bloody diarrhea, and little or no fever [8]. Although usually self-limiting, infection with these organisms may result in serious sequelae such as hemolytic uremic syndrome (HUS) [8, 12, 13, 18] or thrombotic thrombocytopenic purpura (TTP) [2, 8]. Infection with these organisms may also be inapparent or result in only mild diarrhea [18].

At least two types of SLT have been identified. Shiga-like toxin I (SLT I) is neutralized by antiserum to Shiga toxin produced by *Shigella dysenteriae* type 1 and is essentially identical with Shiga toxin by nucleotide or amino acid sequence analysis [11, 15, 25]. Shiga-like toxin II is less closely related to Shiga toxin and is not neutralized by anti-Shiga serum [10, 24]. Because of their toxicity for African green monkey kidney (Vero) cells, these toxins are also known as verotoxins. Verotoxin 1 is identical with SLT I [14, 20], and Verotoxin 2 is similar to, though not identical with, SLT II [9]. Shiga-like toxins are also highly cytotoxic for HeLa cells [24].

SLT-producing strains of *E. coli* serotype O157:H7 were first associated with outbreaks of HC in 1982 [19]. Since that time, several outbreaks of HC have been reported in North America [8, 12, 18] and the United Kingdom [22]. Although other serotypes of SLT-producing *E. coli* have been isolated from patients with both HC [1] and HUS [13], O157:H7 remains the most commonly isolated serotype in North America. Other serotypes have been associated with sporadic disease, but *E. coli* O157:H7 caused all 20 of the outbreaks of hemor-
rhagic colitis described in a recent review of infections owing to SLT-producing E. coli [12].

Isolation of the organism from fecal culture remains the most definitive means of identifying cases of E. coli O157:H7 gastroenteritis. Unfortunately, isolation becomes difficult within a few days after onset of illness [26], and the organism may not be detectable by the time signs of HUS develop. The development of DNA probes for detecting Shiga-like toxin gene sequences has greatly enhanced the ability to screen large numbers of colonies [14, 21]. Studies using these probes have shown that E. coli O157:H7 may represent as little as 1-8% of E. coli isolated during acute illness [21]. Screening of fecal extracts for the presence of free Shiga-like toxin in feces has also proved useful [13]. Although all of these methods are helpful in early diagnosis of E. coli O157:H7 infection, all have limited utility in epidemiologic studies, where investigations are frequently retrospective, and it is important to identify individuals with inapparent infection.

To facilitate epidemiologic studies of this organism, we developed enzyme-linked immunosorbent assays (ELISAs) for detecting antibodies to SLT I, SLT II, and E. coli O157:H7 lipopolysaccharide (LPS). We found that ELISA for antibodies to O157 LPS to be both sensitive and specific, and more useful in identifying cases of E. coli O157 diarrhea than assays for antibodies to SLT I or SLT II. Since all major outbreaks of HC to date have been due to E. coli O157:H7, the detection of antibodies to O157 LPS would be especially useful in outbreak investigations.

Materials and Methods

**Serum specimens.** In total, 128 serum samples were obtained from 84 patients associated with either of two outbreaks of hemorrhagic colitis. Patients' ages ranged from 11 months to >70 years. Forty-eight patients were involved in outbreak 1, in which the causative agent produced both SLT I and SLT II [17]. The remaining 36 patients were associated with outbreak 2, in which the causative agent produced only SLT II [16]. Thirteen patients from outbreak 1 and 14 patients from outbreak 2 had stool cultures positive for E. coli O157:H7; the remainder had diarrhea, but stool cultures were negative or were not performed during the acute illness. All except three patients had bloody diarrhea. One culture-positive patient from outbreak 1 had a serum obtained only on the first day of illness and was excluded from analysis.

**Control serum specimens.** Serum samples were obtained from 66 healthy persons ranging in age from 2 to 74 years living in Wisconsin in 1988. These persons were not associated with an outbreak, and information on recent diarrhea was not obtained.

**HeLa cell cytotoxicity neutralization assay.** HeLa cell cytotoxicity was measured by the 96-well microtiter dilution method of Gentry and Dalrymple [7]. Test sera were diluted in cell culture medium in twofold dilutions beginning with a 1:2 dilution. Partially purified SLT I or SLT II was diluted in cell culture medium to a concentration of approximately 10 times the 50% cytotoxic dose (CD_{50}) and mixed with an equal volume of diluted test serum. This mixture was incubated at 37°C for 1 h, and 0.1 ml was assayed on HeLa cells for residual cytotoxicity.

**Anti-SLT I and SLT II ELISAs.** Immulon 2, flat-bottom microtiter plates (Dynatech Laboratories, Chantilly, Virginia) were coated by overnight incubation at 4°C with 0.1 ml/well of the appropriate monoclonal antibody diluted 1:400 in 0.05 M carbonate buffer, pH 9.6. MAB 13C4 was used for SLT I [23], and BB12 for SLT II [6]. Nonspecific adsorption was blocked by incubation for 1 h at room temperature with 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20, 1%fetal bovine serum (FBS), and 0.5% nonfat dry milk (sample diluent). Diluted toxin preparations (0.1 ml/well diluted polymyxin B extracts of H30 for SLT I and pEB1 [6] for SLT II) were then added and incubated at 37°C for 1 h. After plates were washed three times in PBS with 0.05% Tween and 1% FBS (PBS-T-FBS), 0.1-ml dilutions of test serum were added and incubated for 1 h at 37°C. Test serum was diluted twofold in sample diluent (described above) beginning at 1:40 and ending at 1:1280. Following three washes in PBS-T-FBS, 0.1 ml of alkaline phosphatase-conjugated anti-human IgG or IgM (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland) diluted 1:1000 in sample diluent was added to each well, and the plates were incubated at 37°C for 1 h. Plates were then washed six times in PBS-T-FBS, and alkaline phosphatase substrate (Sigma 104, Sigma Chemical Co., St. Louis, Missouri) was added. Color was developed for 30 min at room temperature, and the optical density (OD) at 405 nm was measured with a Multiscan MCC/340 plate reader (Titer tek, Alexandria, Virginia).

**O157 LPS extraction.** LPS was purified from E. coli O157:H7 strain 933 as follows. Bacterial cells from overnight growth on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Maryland) were harvested and washed once in 0.01 M HEPES buffer, pH 7.4. Cells were then centrifuged and resuspended in lysis buffer (0.01 M Tris-HCl, pH 6.8, containing 4% vol/vol 2-mercaptoethanol) at a concentration of approximately 1 mg protein/ml buffer. The cell suspension was heated to 100°C for 15 min, then allowed to cool to room temperature. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) was then added to a final concentration of 2.5 μg/mg whole cell protein, and the mixture was incubated at 60°C for 1 h. The cell suspension was then reheated to 100°C for 10 min, cooled, and centrifuged at 10,000 g for 30 min. The supernatant was removed and saved for coating ELISA plates.

**Anti-O157 LPS ELISA.** Immulon 2 microtiter plates (Dynatech) were coated with O157 LPS diluted 1:100 in 0.05 M carbonate buffer as described above for SLT I and II ELISAs. Nonspecific reactions were blocked by incubation with sample diluent as described above. Twofold dilutions of serum samples were then made in sample diluent, beginning with a 1:20 dilution and continuing through 1:640. Serum samples reacting at 1:640 were retested to reach an endpoint. After incubating for 1 h at 37°C, plates were washed three times with PBS-T-FBS. Alkaline phosphatase-conjugated anti-human immunoglobulin (IgG, IgM, or IgA; Kirkegaard and Perry) was then added, and the plates were incubated for 1 h at 37°C. Plates were washed six times in PBS-T-FBS, and substrate (Sigma 104) was added. Following incubation at room