A method for detecting Shiga toxin and Shiga-like toxin-I in pure and mixed culture

S. ASHKENAZI and T. G. CLEARY*

Division of Infectious Diseases, Department of Pediatrics, University of Texas Medical School, Houston, USA

Summary. Shiga toxin and Shiga-like toxins (SLTs, syn. Verotoxins) are currently detected by tissue culture assays that are expensive, time-consuming and require specialised facilities and experienced personnel. We have developed a rapid method to detect Shiga toxin and SLT-I (Verotoxin 1) based on their binding to globotriosyl ceramide (Gb₃). Bound toxin was then detected by an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies. The direct detection of cytotoxins from pure culture plates and from a mixed bacterial culture was studied. Using polymyxin extraction (0.1 g/L, 30 min, 37°C) and Gb₃-based ELISA we detected toxin from reference strains *Shigella dysenteriae* 1 strain 60R (Shiga toxin) and *Escherichia coli* O26:H11 strain H30 (SLT-I), and from clinical isolates of *E. coli* O157:H7 and O26:H11 (both SLT-I) from 11 patients with diarrhoea, haemorrhagic colitis or haemolytic uraemic syndrome. Toxin production by these strains was confirmed by a radiolabelled HeLa cell assay and the structural genes were detected by DNA hybridisation. The Gb₃-based ELISA could detect SLT-I in extracts of a mixed culture even when the toxin-positive strains represented only 1% of the mixture. No cross-reactivity was found with bacteria that produce other cytotoxins, such as other *E. coli* and *Shigella*, *Salmonella*, *Aeromonas* and *Campylobacter* spp.

Introduction

Enteric bacteria that produce cytotoxins have been recognised increasingly as important causes of human disease.¹ They can cause diarrhoea or haemorrhagic colitis,^{2,3} and are thought to play a major pathogenic role in the haemolytic-uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura.^{4–6} The cytotoxins implicated in these diseases include Shiga toxin, and the closely related Shiga-like toxins (SLTs), also known as Verotoxins, produced by *Escherichia coli*.^{7–10} At least two immunologically distinct toxins, SLT-I and SLT-II (Verotoxins 1 and 2), have been defined.^{11,12} SLT-I is neutralised by antibodies against Shiga toxin, to which it is virtually identical; there is only a three-nucleotide difference, resulting in a single amino acid difference, between the two toxins.¹³ Although SLT-II is immunologically distinct, this family of toxins is very similar in structure, mechanism of action, binding receptor

and biological activities.^{14–18} A variant of SLT-II, which is produced by *E. coli* strains associated with oedema disease in pigs¹⁹ or human HUS,²⁰ has been defined, cloned and sequenced.¹⁹

Detection of these cytotoxins in the routine clinical laboratory presents considerable difficulty. Toxins are usually detected by their ability to cause cytopathic effects in several cell lines. Radiolabelled DNA probes^{8,21} are still not widely used. Detection of the toxins by enzyme-linked immunosorbent assay (ELISA) has been reported.^{22–24} We have described a rapid and sensitive assay to detect Shiga toxin and SLT-I based on binding to globotriosyl ceramide (Gb₃).²⁵ However, preparation of suitable bacterial specimens required growth of cultures for 48 h and subsequent sonication—a prolonged and cumbersome extraction procedure. Since polymyxin extraction has been shown to be effective in releasing high levels of toxins from the periplasmic space of gram-negative bacteria,^{26,27} it may provide a simpler extraction procedure. Furthermore, the need to examine individual colonies presents problems, because cytotoxin-producing bacteria are typically presented in low numbers in stool samples of patients with diarrhoea and, in particular, HUS. The proportion of cyto-

Received 22 Nov. 1989; accepted 22 Jan. 1990.

* Correspondence should be sent to: Dr T. G. Cleary, Pediatric Infectious Diseases, University of Texas Medical School, 6431 Fannin Street, Room 1.739, Houston, TX 77030, USA.

toxin-producing colonies is often only 5–20%⁴ or even lower.²⁸

The objectives of the present study were to evaluate the efficacy of a Gb₃-based ELISA for detection of Shiga toxin and SLT-I released by polymyxin extraction, both from bacteria grown on various pure culture plates and, most importantly, directly from a mixed bacterial culture. Results of Gb₃ ELISA were compared with the quantitative radiolabelled HeLa cell cytotoxicity assay,^{29,30} and with detection of the structural genes for toxin production by DNA hybridisation.⁸

Materials and methods

Bacteria

Three reference bacterial strains that produce Shiga toxin and SLT-I were used initially for developing the extraction procedure and the assay: *Shigella dysenteriae* serotype 1 strain 60R (Shiga toxin); *E. coli* serotype O26:H11, strain H30 (SLT-I), originally described by J. Konowalchuk,⁹ and *E. coli* serotype O157:H7 (SLT-I), provided by J. Mathewson, who originally obtained the strain from R. Wilson, *E. coli* Reference Center, Pennsylvania State University. For specificity studies, other reference strains were used: *E. coli* K-12, strain C600 (a non-producer of cytotoxin); lysogenic *E. coli* K-12, strain C600 (933w) (SLT-II), kindly provided by A. D. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, MD; and *E. coli* serotype O78:H11, strain H10407 (heat-labile and heat-stable enterotoxin). Other bacteria examined included 40 isolates of *Shigella* spp.,⁷ 56 pathogenic and 10 nonpathogenic *E. coli* strains,²⁹ 52 *Salmonella* isolates,³¹ 10 of *Aeromonas* spp.,³² and 20 of *Campylobacter* spp. The *E. coli* strains included 14 isolates of *E. coli* serotype O157:H7 from patients with haemorrhagic colitis, diarrhoea or HUS and three isolates of *E. coli* serotype O26:H11 from patients with diarrhoea.

Preparation of polymyxin extracts

Bacteria were grown on charcoal-yeast extract (CYE), blood and MacConkey agar plates, incubated overnight at 37°C. Polymyxin B (Sigma) was diluted in phosphate-buffered saline (PBS; 0.2 M sodium phosphate, 0.2 M potassium phosphate) to the desired concentration. To determine the optimal polymyxin B extraction procedure, bacterial cells were incubated with polymyxin B concentrations of 0, 0.01, 0.1, 0.2, 0.5, 1 or 2 g/L for periods of 2, 5, 10, 20, 30 or 60 min. For these experiments, *E. coli* O26:H11 strain H30, grown overnight on CYE, blood or MacConkey agar, was suspended in PBS to obtain a stock suspension of c. 10⁹ cfu/ml and 1-ml volumes were placed in Eppendorf tubes and centrifuged in a microcentrifuge for 5 min. Cell pellets were resuspended in PBS containing the range of concentrations of polymyxin B and incubated in a water bath at 37°C for the test periods.

Samples were then centrifuged (5 min) again and the supernates were sterilised by membrane filtration (pore size, 0.22 µm) and assayed for cytotoxicity. Protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) as a standard. Assays were performed in duplicate and repeated at least three times. Control samples with polymyxin only were treated and assayed simultaneously. After these experiments, a standard extraction procedure was used with incubation for 30 min in polymyxin B 0.1 g/L.

Detection of Shiga toxin and SLT-I in bacteria grown in pure culture

S. dysenteriae 1 strain 60R (Shiga toxin) and *E. coli* O26:H11 strain H30 (SLT-I) were grown overnight on CYE, blood and MacConkey agar plates. A loopful (0.001-ml loop) of confluent bacterial growth was suspended in Eppendorf tubes containing 0.25 ml of polymyxin B 0.1 g/L in PBS. After incubation for 30 min at 37°C, samples were processed as above. To compare the yields obtained by sonic extraction and by polymyxin extraction, 1-ml samples of a stock suspension of *E. coli* H30 (10⁹ cfu/ml in PBS) were extracted by polymyxin (as above) or by sonication,²⁹ and the amounts of cytotoxin obtained were measured. Negative control reference strains were grown, extracted and assayed simultaneously.

Detection of SLT-I from a mixed culture

E. coli O26:H11 strain H30 and *E. coli* K-12 strain C600 were each grown overnight on CYE plates, washed in PBS and resuspended in PBS to a final concentration of 10⁹ cfu/ml. Appropriate volumes of the two suspensions were mixed to obtain mixtures in which the proportion of *E. coli* H30 was 1, 5, 10, 30 or 50%. Controls were pure suspensions of *E. coli* H30 and *E. coli* C600. Each mixture (100 µl) was plated on CYE agar and incubated overnight. A loopful of confluent bacterial growth from each mixture was suspended in an Eppendorf tube containing 0.25 ml of polymyxin B 0.1 g/L in PBS, incubated for 30 min at 37°C and processed as above.

Preparation of purified Shiga toxin

Purified Shiga toxin was used in titration curves to determine the amount of toxin obtained by the extraction procedures. Toxin was purified from a sonic extract of *S. dysenteriae* serotype 1 strain 60R by chromatography with an Affi-Gel Blue column, chromatofocusing, and a hydroxylapatite column, as described previously.³⁰ The purified toxin had a specific activity of 10^{7.3} 50% cytotoxic doses (CD50)/mg of protein.

Preparation of anti-Shiga toxin serum

Rabbit anti-Shiga toxin serum, which also neutralised SLT-I, was obtained by repeated injections of formalde-

hyde-treated purified Shiga toxin (100 µg/dose) as previously described.⁷

Gb₃ ELISA

The assay was performed as described previously.²⁵ Gb₃ (Supelco, Bellefonte, PA) was diluted in chloroform:methanol (2:1) to a concentration of 5 µg/ml and inoculated (100 µl/well) on to polyvinyl chloride microtitre plates (Dynatech Laboratories, Inc., Alexandria, VA). Plates were left uncovered overnight so that the diluent evaporated and Gb₃ attached to the plates. After blocking with BSA (50 g/L, 200 µl/well, 1 h), test samples were added (100 µl/well, 1 h). Bound toxin was detected by monoclonal antibodies and peroxidase-conjugated rabbit anti-mouse antibodies as described previously.²⁵

Reproducibility of the extraction procedure and the assay was determined by duplicate testing. Sensitivity was determined by titration with purified Shiga toxin and comparing the readings with those obtained by polymyxin extraction of reference strains that produce Shiga toxin or SLT-I. Specificity was determined by examining cross-reactivity with polymyxin extracts of well characterised bacterial strains that produce enterotoxins (*E. coli* H10407), SLT-II (*E. coli* C600 [933w]) or were cytotoxin non-producers (*E. coli* C600), and of various other cytotoxin-producing bacterial isolates (table).

Cytotoxicity and neutralisation assays

Quantitative cytotoxic activity was determined by overnight incubation of cell-free toxin preparations with [³H] thymidine-labelled HeLa cells as described previously.²⁹ Radioactivity of attached viable cells was counted and results were expressed as a percentage of HeLa cell survival in comparison with control wells without toxin. The CD₅₀ was calculated from the linear regression of the percentage of HeLa cell survival *versus* the log₁₀ toxin dilution.⁷ Neutralisation was studied by pre-incubation of the toxin for 4 h at 37°C with rabbit anti-Shiga toxin serum or rabbit non-immune serum (control) followed by determination of cytotoxicity with radiolabelled HeLa cells as described above.

DNA hybridisation studies

The DNA probe for SLT-I described by J. W. Newland and R. J. Neill (Walter Reed Army Institute of Research, Washington, DC) was used. The probe is a 1.142-kb *Bam*HI fragment of the recombinant plasmid pJN37-19, cloned from *E. coli* bacteriophage 933J.³⁴ As Shiga toxin and SLT-I differ in only three nucleotides, the SLT-I probe also hybridises with Shiga toxin-producing organisms. The probe was extracted from low temperature gelling agarose and labelled by nick translation with ³²P-labelled 5'-dCTP (Dupont, NEM Research Products, Boston, MA). Bacterial colonies were transferred to Whatman 541 filter papers, lysed as described previously,³⁵ and hybridised under stringent conditions—

formamide 50%, 42°C, 2 × SSC (SSC is 0.5 M NaCl + 0.015 M sodium citrate), 18 h. The following positive and negative control strains were placed on each filter: *S. dysenteriae* serotype 1 strain 60R, *E. coli* O26:H11 strain H30, *E. coli* C600 (933w) and *E. coli* C600.

Results

Polymyxin B extraction

In experiments to optimise the conditions of our novel method of polymyxin extraction, which included direct inoculation from primary plates to polymyxin suspension and detection by Gb₃ ELISA, the amounts of Shiga toxin and SLT-I released increased with increasing concentrations of polymyxin (0–0.1 g/L); a plateau of release was reached at 0.1 g/L. With this concentration of polymyxin, optimal release of cytotoxin was achieved by incubation for 10–60 min. In subsequent experiments, the standard extraction conditions used were polymyxin 0.1 g/L and incubation for 30 min at 37°C.

Detection of cytotoxin from pure cultures

Shiga toxin and SLT-I were detected by standard polymyxin extraction and Gb₃ ELISA from pure cultures on agar media. Positive results were obtained with *S. dysenteriae* 1 strain 60R (Shiga toxin), *E. coli* O26:H11 strain H30 (SLT-I) and *E. coli* O157:H7 (SLT-I). There was no cross-reactivity with *E. coli* O78:H11 H10407 (enterotoxin), *E. coli* C600 (933w) (SLT-II) or *E. coli* C600 control strains examined simultaneously. The range of readings (OD₄₉₀) of these negative-control reference strains was 0.01–0.04 (mean 0.03 SD 0.01). Readings of ≥ 0.1 were considered to be positive results. According to the titration curve, this reading represented 0.2–0.5 ng of purified Shiga toxin per well. Positive results were obtained with 11 clinical isolates of *E. coli* O157:H7 or *E. coli* O26:H11 from patients with haemorrhagic colitis, diarrhoea or HUS. None of the polymyxin extracts of various other cytotoxin-producing bacteria, including other strains of *Shigella*, pathogenic and non-pathogenic *E. coli*, *Salmonella*, *Aeromonas* and *Campylobacter* showed cross-reactivity in the assay (table).

Comparison of polymyxin and sonic extraction

In parallel tests with the same number of bacteria from a stock suspension, the yields of toxin from polymyxin-treated and sonicated cells were com-

Table. Detection of Shiga toxin and SLT-I in polymyxin B extracts of cytotoxin-producing bacteria by Gb₃ ELISA and HeLa cell cytotoxicity assay

Bacterial strains	Results obtained by	
	Gb ₃ ELISA*	Cytotoxicity assay†
Reference strains (toxin)		
<i>S. dysenteriae</i> serotype 1 strain 60R (Shiga toxin)‡	+	+
<i>E. coli</i> serotype O26:H11 strain H30 (SLT-I)‡	+	+
<i>E. coli</i> serotype O157:H7 (SLT-I)‡	+	+
<i>E. coli</i> serotype O78:H11 strain H10407 (enterotoxins)§	—	—
<i>E. coli</i> C600 (933w) (SLT-II)§	—	—
<i>E. coli</i> C600§	—	—
Clinical isolates (number of strains)		
<i>S. dysenteriae</i> 1 (10)	+	+
<i>S. sonnei</i> (13)§	—	—
<i>S. flexneri</i> (13)§	—	—
<i>S. boydii</i> (4)§	—	—
Nonpathogenic <i>E. coli</i> (10)	—	—
Pathogenic <i>E. coli</i>		
EAEC (10)	—	—
EIEC (30)	—	—
EPEC (6)	—	—
ETEC (10)	—	—
<i>E. coli</i> O26:H11 (SLT-I) (3)	+	+
<i>E. coli</i> O157:H7 (SLT-I) (8)	+	+
<i>E. coli</i> O157:H7 (SLT-II) (6)	—	—
<i>Sal. enteritidis</i> (25)§	—	—
<i>Sal. typhi</i> (12)§	—	—
<i>Sal. choleraesuis</i> (15)§	—	—
<i>A. hydrophila</i> (10)	—	—
<i>C. jejuni</i> (20)	—	—

EAEC, entero-adherent *E. coli* (as defined by Mathewson *et al.*,³³) EIEC, entero-invasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*.

* +, OD₄₉₀ ≥ 0.1.

† +, Cytotoxic activity present, neutralised with anti-Shiga toxin serum.

‡ Structural genes for Shiga toxin (or SLT-I) demonstrated by DNA hybridisation.

§ Structural genes for Shiga toxin (or SLT-I) not present.

pared. Polymyxin treatment gave lower OD readings—65–90% of these obtained with sonicated bacteria (fig. 1a). Similar results were obtained with bacteria grown on CYE, blood and MacConkey agars. However, when readings were translated into amounts of toxin (µg)/mg of bacterial protein (specific activity), the amounts of toxin extracted were five-fold higher with polymyxin treatment than with sonication (fig. 1b).

Detection of SLT-I from mixed culture

Gb₃ ELISA was used to detect SLT-I in polymyxin extracts of a mixed culture of SLT-I-positive and -negative strains. Positive results were obtained when the proportion of the SLT-I-positive strain (*E. coli* H30) was as low as 1% (fig. 2). A plateau was reached when the SLT-I-positive strain was

c. 10% of the mixture. Pure growth of the toxin-negative strain (*E. coli* C600) gave negative results under the same experimental conditions.

Correlation with HeLa cell cytotoxicity assay and with DNA hybridisation

The cell-free polymyxin extracts were examined for cytotoxicity. The cut-off point of the assay (OD₄₉₀ 0.1) corresponded to about 10–30 CD50. Extracts of control strains that gave positive results in the Gb₃ ELISA, whether from pure or mixed cultures, were cytotoxic in the radiolabelled HeLa cell assay, and the cytotoxicity was neutralised by rabbit antiserum to Shiga toxin. Extracts of negative control strains were not cytotoxic. In tests with polymyxin extracts of other bacteria, strains that gave positive results for Shiga toxin or SLT-I

Fig. 1. Amounts of SLT-I detected by Gb₃ ELISA in polymyxin ■ and sonic ▨ extracts of *E. coli* O26:H11 strain H30 grown in three media. (A) OD₄₉₀ values in the Gb₃ ELISA. (B) Specific activity (amount of toxin/total bacterial protein, µg/mg) in each extract, determined from titration curve with purified toxin and Bio-Rad assay for total protein. Results are means (SD) of three determinations.

(neutralised by rabbit antiserum to Shiga toxin) in the cytotoxicity assay or hybridised with the DNA probe for SLT-I, also gave positive results in the Gb₃ ELISA. Polymyxin extracts that gave negative results in the cytotoxicity assay (no neutralisation with the anti-Shiga toxin serum) and in DNA hybridisation also gave negative results in the Gb₃ ELISA (table).

Fig. 2. Detection by Gb₃ ELISA of SLT-I from polymyxin extracts of cultures of *E. coli* O26:H11 strain H30 (0–100%) with toxin-negative *E. coli* C600. Results are means (SD) of three determinations.

Discussion

Several approaches have been used for the diagnosis of cytotoxin-related diarrhoeal disease and HUS. Initial attempts have concentrated on isolating *E. coli* serotype O157:H7 by culture of MacConkey-sorbitol agar and on developing rapid diagnostic tests.^{36,37} However, although *E. coli* O157:H7 is the most frequent serotype associated with haemorrhagic colitis and HUS, many other serotypes are involved. Therefore, the diagnostic approach should be directed toward detection of either cytotoxin-producing organisms or free cytotoxin activity. Detection of the genes encoding the relevant toxins in bacterial cells by radiolabelled DNA probes, either restriction fragments^{8,34} or synthetic oligonucleotide probes,²¹ has been used effectively, but concern over handling radioactive materials may make them unsuitable for routine use in a clinical diagnostic laboratory.

Detection of cytotoxins from bacterial isolates or stool extracts is usually based on morphological changes (cytopathic effect) in HeLa or Vero cells. These assays take several days and require facilities for and experience with tissue cultures. Neutralisation studies to identify the toxin are sometimes difficult to interpret due to variable cell growth,²⁴ and because the sensitivity of various cell lines to cytotoxins varies significantly.¹⁸ Simple and rapid assays are needed for routine detection of cytotoxins.

Rapid ELISA methods to detect Shiga toxin and SLTs by binding to monoclonal antibody or to the toxin receptor have been described.^{22–24,38} They

seem to be sensitive and specific and can facilitate cytotoxin detection. However, toxin detection is usually based on examination of individual colonies. This may present a significant problem because the proportion of cytotoxin-producing colonies in stool samples from patients with diarrhoea, and especially with HUS, may be $\leq 5\%$.^{4,28} Therefore many colonies must be screened, which is cumbersome and impractical for clinical laboratories. Downes *et al.* used ELISA to detect cytotoxins in stool extracts, but they were unable to detect SLT-I because it was present in small quantities.²⁴

Polymyxin extraction has been used to release Shiga toxin and SLTs from *Shigella* and *E. coli* strains;^{26,27} the toxin released was similar to that obtained by sonic extraction.²⁶ Polymyxin extraction of colony sweeps has been used successfully to detect cytotoxins from a mixed culture.²⁷ However, although the extraction procedure was rapid and simple, colony sweeps were inoculated into broth and incubated for 5 h, and the cytotoxicity assay was based on morphological changes in Vero cells after 72 h, so that rapid diagnosis was not achieved.

In this study, we used polymyxin extraction, but attempted to detect cytotoxin directly from bacterial growth on readily available culture media by a rapid and simple receptor binding assay (Gb₃ ELISA). The optimal conditions for polymyxin extraction directly from culture plates were similar to those described previously for extraction from bacteria grown in broth^{26,27} and the standard extraction conditions used were polymyxin 0.1 g/L, for 30 min at 37°C.

We have shown that polymyxin extraction of bacterial growth from agar plates and Gb₃ ELISA may be useful in the diagnosis of infections with bacteria that produce Shiga toxin and SLT-I. The sensitivity was surprisingly high; cytotoxin was detected in polymyxin extracts of a mixed culture when the proportion of the cytotoxin-producing strain was as low as 1%. This high sensitivity is probably because of the small volume (0.25 ml) in which bacteria were suspended and optimal condi-

tions for polymyxin extraction. The assay was very specific—no cross-reactivity was found with enterotoxins, SLT-II or other cytotoxin-producing organisms, including other *Shigella* or *E. coli* strains and *Salmonella*, *Campylobacter* or *Aeromonas* strains. These results were in agreement with a radiolabelled cytotoxicity assay with neutralisation by specific antiserum, and with DNA hybridisation tests for the structural genes for toxin production. As Gb₃ is also the binding receptor of SLT-II,³⁹ the latter toxin may be detected also by the same method with appropriate antibodies to SLT-II. It would be very useful if polymyxin extraction and plates pre-coated with Gb₃ could be used to detect both toxins, with specific identification of bound toxin by different antibodies.

The sensitivity of our assay was 0.2 ng of purified toxin per well, similar to that described by Downes *et al.*,²⁴ but lower than that described in two other assays;^{24,38} it correlates with c. 10–30 CD50 in the HeLa cell cytotoxicity assay. Although Gb₃ ELISA is slightly less sensitive than the cytotoxicity assay, it is specific, rapid, and easier to perform and interpret. The slightly lower sensitivity did not prevent detection of toxin from mixed culture, even when the proportion of the toxin-positive strain was only 1%. Low level cytotoxin-producing strains may not be detected by this assay, but their relevance to human disease is questionable.¹

Rapid and simple extraction and detection methods are necessary for routine use in clinical laboratories. They should be useful in large scale epidemiological studies of cytotoxin-related infections. Early diagnosis of these infections may be helpful in controlling person-to-person transmission and evaluating the efficacy of various therapeutic measures on the course of cytotoxin-related diarrhoeal disease and on the development of HUS.

We thank Dr Barbara E. Murray for help with the DNA hybridisation studies, Delores Reich for technical assistance, and Anne Wright for preparing the manuscript. This study was supported by a Public Health Service grant HD-13021 from the National Institutes of Health and by a grant from the Thrasher Research Fund.

REFERENCES

1. Karmali MA. Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev* 1989; 2: 15–38.
2. Riley LW, Remis RS, Helgerson SD *et al.* Hemorrhagic colitis associated with rare *Escherichia coli* serotype. *N Engl J Med* 1983; 308: 681–685.
3. Scotland SM, Willshaw GA, Smith HR, Rowe B. Properties of strains of *Escherichia coli* belonging to serogroup O157 with special reference to production of Vero cytotoxin VT1 and cytotoxin VT2. *Epidemiol Infect* 1987; 99: 613–624.
4. Karmali MA, Petric M, Lim C, Flemming PC, Arbus GS, Lior H. The association between idiopathic hemolytic uremic syndrome and infection by Verotoxin-producing *Escherichia coli*. *J Infect Dis* 1985; 151: 775–782.
5. Neill MA, Tarr PI, Clausen CR, Christie DL, Hickman RO. *Escherichia coli* O157:H7 as the predominant pathogen associated with the hemolytic uremic syndrome: a prospective study in the Pacific Northwest.

- Pediatrics* 1987; **80**: 37-40.
6. Cleary TG. Cytotoxin-producing *Escherichia coli* and hemolytic uremic syndrome. *Pediatr Clin North Am* 1988; **35**: 485-501.
 7. Bartlett AV, Prado D, Cleary TG, Pickering LK. Production of Shiga toxin and other cytotoxins by serogroups of *Shigella*. *J Infect Dis* 1986; **154**: 996-1002.
 8. Newland JW, Neill RJ. DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. *J Clin Microbiol* 1988; **26**: 1292-1297.
 9. Konowalchuk J, Speirs JI, Stavric S. Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun* 1977; **18**: 775-779.
 10. Strockbine NA, Marques LRM, Newland JW, Smith HW, Holmes RK, O'Brien AD. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. *Infect Immun* 1986; **53**: 135-140.
 11. Strockbine NA, Marques LRM, Holmes RK, O'Brien AD. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. *Infect Immun* 1985; **50**: 695-700.
 12. Perera LP, Marques LRM, O'Brien AD. Isolation and characterization of monoclonal antibodies to Shiga-like toxin II of enterohemorrhagic *Escherichia coli* and use of the monoclonal antibodies in a colony enzyme-linked immunosorbent assay. *J Clin Microbiol* 1988; **26**: 2127-2131.
 13. Jackson MP, Newland JW, Holmes RK, O'Brien AD. Nucleotide-sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. *Microb Pathog* 1987; **2**: 147-153.
 14. Fuchs G, Mobassaleh M, Donohue-Rolfe A, Montgomery RK, Grand RJ, Keusch GT. Pathogenesis of shigella diarrhea: rabbit intestinal cell microvillus membrane binding site for shigella toxin. *Infect Immun* 1986; **53**: 372-377.
 15. Jacewicz M, Clausen H, Nudelman E, Donohue-Rolfe A, Kensch GT. Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J Exp Med* 1986; **163**: 1391-1404.
 16. Lindberg AA, Brown JE, Stromberg N *et al*. Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. *J Biol Chem* 1987; **262**: 1779-1785.
 17. Lingwood CA, Law H, Richardson SE *et al*. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin *in vitro*. *J Biol Chem* 1987; **262**: 8834-8839.
 18. O'Brien AD, Holmes RK. Shiga and Shiga-like toxins. *Microbiol Rev* 1987; **51**: 206-220.
 19. Weinstein DL, Jackson MP, Samuel JE, Holmes RK, O'Brien AD. Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. *J Bacteriol* 1988; **170**: 4223-4230.
 20. Oku Y, Yutsudo T, Hirayama T, O'Brien AD, Takeda Y. Purification and some properties of a Vero toxin from a human strain of *Escherichia coli* that is immunologically related to Shiga-like toxin II (VT2). *Microb Pathog* 1989; **6**: 113-122.
 21. Karch H, Meyer T. Evaluation of oligonucleotide probes for identification of Shiga-like-toxin-producing *Escherichia coli*. *J Clin Microbiol* 1989; **27**: 1180-1186.
 22. Donohue-Rolfe A, Kelley MA, Bennish M, Keusch GT. Enzyme-linked immunosorbent assay for shigella toxin. *J Clin Microbiol* 1986; **24**: 65-68.
 23. Kongmuang U, Honda T, Miwatani T. Enzyme-linked immunosorbent assay to detect Shiga toxin of *Shigella dysenteriae* and related toxins. *J Clin Microbiol* 1987; **25**: 115-118.
 24. Downes FP, Green JH, Greene K, Stockbrine W, Wells JG, Wachomülh IK. Development and evaluation of enzyme-linked immunosorbent assays for detection of Shiga-like toxin I and Shiga-like toxin II. *J Clin Microbiol* 1989; **27**: 1292-1297.
 25. Ashkenazi S, Cleary TG. Rapid method to detect Shiga toxin and Shiga-like toxin I based on binding to globotriosyl ceramide (Gb₃), their natural receptor. *J Clin Microbiol* 1989; **27**: 1145-1150.
 26. Griffin DE, Gemski P. Release of Shiga toxin from *Shigella dysenteriae* 1 by polymyxin B. *Infect Immun* 1983; **40**: 425-428.
 27. Karmali MA, Petric M, Lim C, Cheung R, Arbus GS. Sensitive methods for detecting low numbers of verotoxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymyxin extraction of verotoxin. *J Clin Microbiol* 1985; **22**: 614-619.
 28. Scotland SM, Rowe B, Smith HR, Willshaw GA, Gross RJ. Verocytotoxin-producing strains of *Escherichia coli* from children with haemolytic uraemic syndrome and their detection by specific DNA probes. *J Med Microbiol* 1988; **25**: 237-243.
 29. Cleary TG, Mathewson JJ, Faris E, Pickering LK. Shiga-like cytotoxin production by enteropathogenic *Escherichia coli* serogroups. *Infect Immun* 1985; **47**: 335-337.
 30. Prado D, Cleary TC, Pickering LK *et al*. The relation between production of cytotoxin and clinical features of shigellosis. *J Infect Dis* 1986; **154**: 149-155.
 31. Ashkenazi S, Cleary TG, Murray BE, Wanger A, Pickering LK. Quantitative analysis and partial characterization of cytotoxin production by *Salmonella* strains. *Infect Immun* 1988; **56**: 3089-3094.
 32. Kindschuh M, Pickering LK, Cleary TG, Ruis-Palacios G. Clinical and biochemical significance of toxin production by *Aeromonas hydrophila*. *J Clin Microbiol* 1987; **25**: 916-921.
 33. Mathewson JJ, Cravioto A. HEP-2 adherence as an assay for virulence among diarrheagenic *Escherichia coli*. *J Infect Dis* 1989; **159**: 1057-1060.
 34. Newland JW, Strockbine NA, Miller SF, O'Brien AD, Holmes RK. Cloning of Shiga-like toxin structural genes from a toxin converting phage of *Escherichia coli*. *Science* 1985; **230**: 179-181.
 35. Maas R. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. *Plasmid* 1983; **10**: 296-298.
 36. Perry MB, Bundle DR, Gidney MAJ, Lior H. Identification of *Escherichia coli* serotype O157 strains by using a monoclonal antibody. *J Clin Microbiol* 1988; **26**: 2391-2394.
 37. March SB, Ratnam S. Latex agglutination test for detection of *Escherichia coli* serotype O157. *J Clin Microbiol* 1989; **27**: 1675-1677.
 38. Basta M, Karmali M, Lingwood C. Sensitive receptor-specific enzyme-linked immunosorbent assay for *Escherichia coli* verotoxin. *J Clin Microbiol* 1989; **27**: 1617-1622.
 39. Waddell T, Head S, Petric M, Cohen A, Lingwood C. Globotriosyl ceramide is specifically recognized by the *Escherichia coli* Vero cytotoxin 2. *Biochem Biophys Res Commun* 1988; **152**: 674-679.