Diagnosis of infections with Shiga-like toxin-producing *Escherichia coli* by use of enzyme-linked immunosorbent assays for Shiga-like toxins on cultured stool samples

D. LAW, A. A. HAMOUR†, D. W. K. ACHESON‡, H. PANIGRAHI§, LEELA A. GANGULI
and D. W. DENNING*†

Department of Microbiology and * Department of Medicine, Hope Hospital, Eccles Old Road, Salford M6 8HD,
† University of Manchester, Regional Department of Infectious Diseases and Tropical Medicine, Monsall Hospital,
Newton Heath, Manchester M10 8WR, ‡ Department of Medicine, Division of Geographic Medicine and Infectious
Diseases, 750 Washington Street, NEMCH #41, Boston, MA, USA and § Department of Microbiology, Monsall and
North Manchester General Hospitals, Manchester M8 6RB

Summary. Shiga-like toxin-producing (SLT) *Escherichia coli*, particularly those belonging to serogroup O157, are responsible for haemorrhagic colitis, haemolytic uraemic syndrome and some cases of gastro-enteritis. The rapid and reliable diagnosis of all these infections is necessary for correct patient management and for epidemiological reasons, but is rarely possible with present methods. We compared the efficacy of two methods, (i) the culture of faeces in broth that contained mitomycin C followed by enzyme-linked immunosorbent assay (ELISA) for SLTs, and (ii) the culture of faeces on sorbitol MacConkey agar (SMA), in the detection of infections caused by SLT-producing *E. coli*. SLT-producing *E. coli* O157 strains were isolated on SMA from 42 of 475 faecal samples, but SLTs were detected by ELISA in culture supernates or lysates of 54 of 475 samples. SLT-producing *E. coli* strains were isolated subsequently from 11 of 12 ELISA-positive, SMA culture-negative samples by a colony blot technique. In four cases, SLT-producing *E. coli* of serogroups other than O157 were isolated and in seven cases *E. coli* O157 was isolated in small numbers. The ELISA is a rapid and sensitive technique for the diagnosis of SLT-producing *E. coli* infection, especially where low numbers of the organism are present in faeces and when the infection is caused by a serogroup other than O157.

Introduction

Human infections associated with strains of *Escherichia coli* that produce Shiga-like toxin (SLT) or verotoxin present as non-bloody diarrhoea, bloody diarrhoea or haemorrhagic colitis (HC).^{1,2} Haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura are also associated with infection with SLT-producing *E. coli*.^{1,3,4} Although one serotype, O157:H7, predominates in most human infections with SLT-producing *E. coli*, infections can be caused by at least 30 O serogroups of *E. coli*.⁴ The true incidence of SLT-producing *E. coli* infection is probably underestimated because of the limitations of the currently available diagnostic tests. The culture on sorbitol MacConkey agar (SMA) as used by many laboratories only detects *E. coli* strains of serogroup O157 that do not ferment sorbitol, whereas most other *E. coli* serotypes ferment sorbitol.⁵ Moreover, the numbers of SLT-producing *E. coli* in the faeces of patients with HC and HUS decreases markedly as the

diseases progress and this reduces the probability of isolating the infecting organism later in the diseases.⁶

Shiga-like toxins can be detected in faeces and from cultures of *E. coli* with cytotoxicity assays^{5,7} and toxin-neutralising antibodies are required to ensure specificity. DNA probes that hybridise with SLT-I or SLT-II have been developed⁸ and have proved useful in clinical studies.^{9–11} The polymerase chain reaction (PCR) has also been applied to the detection of SLT-producing *E. coli* in clinical samples.¹² At present these techniques are not widely available in routine clinical microbiology laboratories.

Immunological techniques for detecting SLTs, such as enzyme-linked immunosorbent assays (ELISA), have been described.^{13,14} Recently, we developed a diagnostic system based on the culture of faecal samples in broth containing mitomycin C, followed by ELISA for SLT-I and -II.¹⁵ This assay is based on the principle that the toxins produced in most human SLT-producing *E. coli* infections are mediated by bacteriophage and are, therefore, inducible with mitomycin C.¹⁶ This method amplified toxin production and allowed detection of low numbers (< 1 in 1000) of

SLT-II-producing organisms in spiked faecal samples.¹⁵ Because the method detects toxins it is independent of serotype and can also detect infection by serogroups other than O157.

In the present study, standard detection methods for SLT-producing *E. coli* by culture on SMA were compared directly with the ELISA assay in a prospective study, and the results were correlated with the clinical presentation of patients with diarrhoea.

Materials and methods

Clinical definitions

The diagnosis of HUS was made on clinical grounds on the basis of acute renal impairment and microangiopathic anaemia. HC was defined as severe bloody diarrhoea with very little stool content, abdominal pain and little or no fever. Bloody diarrhoea was defined as the passage of frequent bloody stools.

Samples

Samples of faeces were obtained from patients admitted to Hope Hospital, Salford, from the community around the hospital and from patients admitted to the Regional Department of Infectious Diseases and Tropical Medicine at Monsall Hospital, Manchester. Therefore, these samples were from relatively unselected cases of diarrhoea although they tended to have more severe illness. In the latter part of the study, samples were received from other laboratories in the Region, usually when conventional cultures for enteric pathogens were negative or when *E. coli* O157 had been isolated, for confirmation of toxin production by the isolate. After receipt, samples were stored at 4°C before testing.

Faecal samples were allocated to one of four patient groups based upon clinical criteria: group 1 comprised patients with HUS; group 2, patients with HC; group 3, patients with a history of bloody diarrhoea and group 4, patients with non-bloody diarrhoea.

Bacteriological procedures

Samples were examined for bacterial, viral and protozoal pathogens by protocols in place at the referring laboratory. Faecal samples for SLT-producing *E. coli* examination were inoculated on to Sorbitol MacConkey Agar No. 3 plates (SMA; Uni-path) which were examined for the presence of sorbitol non-fermenting (SNF) colonies. Slide agglutination tests on SNF colonies were performed with *E. coli* O157 antiserum (Difco). Organisms that reacted with the antiserum were then tested for toxin production as described below. When SNF colonies were scanty or several colonial types of SNF were observed, each type was inoculated on to SMA and agglutination tests were repeated after overnight incubation. Isolates that reacted with *E. coli* O157 antiserum were then identified

by the API 20E system (bioMérieux, Basingstoke).

Culture conditions

For ELISA, c. 0.1 g of faeces were emulsified in 3 ml of sterile phosphate-buffered saline (PBS), pH 7.4 and 30 µl of the suspension were used to inoculate 3 ml of Tryptone Soy Broth (TSB; Difco) containing mitomycin C (TSB + mit C; Sigma) 200 ng/ml. Broths were incubated for 18 h at 37°C with constant shaking (200 rpm). Cultures were centrifuged and the supernates were used for SLT-II testing. The cell pellet was resuspended in 300 µl of polymyxin B (Sigma) 2 mg/ml in PBS, incubated at 37°C for 10 min and then centrifuged. The clear supernate was removed and used to test for SLT-I.

ELISA procedure

The ELISA was performed as described previously;¹⁵ hydatid cyst material was used to capture toxin. Rabbit anti-SLT-I and anti-SLT-II, prepared as described previously,¹³ and goat anti-rabbit alkaline phosphatase conjugate were used to detect bound toxin. After 100 samples had been processed in this way, the assay for SLT-II was modified in that a monoclonal antibody (MAb), 2B1, specific for SLT-II B subunit,¹⁶ was used to capture this toxin instead of hydatid cyst material, because this technique was shown to be more sensitive. Mouse ascitic fluid (3 µl in 10 ml of PBS) was used to coat the assay plate. To quantify the amount of toxin in the supernates and lysates, Shiga toxin and SLT-II, purified as described previously¹⁷ and diluted in PBS (range 1.6–100 ng/ml) were used as standards in the ELISA. The positive/negative cut-off point for both toxins in culture preparations was 1.6 ng/ml. As a control, *E. coli* O157:H7 (strain 933 provided by the late Dr H. Williams-Smith) which produces SLT-I and II, was inoculated into TSB + mit C and processed at the same time as the cultures.

The ability of *E. coli* colonies to produce SLTs was tested after growth in TSB + mit C. Supernates and cell lysates were prepared and tested as described above. When a sample was ELISA-positive, but SMA-negative, sweeps of colonies were inoculated into TSB + mit C, incubated as described above, and cell lysates and supernates were tested by ELISA. If these preparations gave positive results individual colonies were then tested for their ability to produce toxin.

Colony blot technique

Colony blots were performed on 12 ELISA-positive samples which gave negative results on SMA culture, and on 14 samples from patients with HUS which gave negative results by both ELISA and SMA culture. Tryptone soy agar (TSA) containing mitomycin C 200 ng/ml was prepared. Two 0.45-µm nitrocellulose filters (BioRad, Hemel Hempstead) were placed on top

Table. Results of investigations for Shiga-like toxin-producing *E. coli* on 475 faecal samples

Diagnosis	Number of samples	Toxin detected by ELISA			<i>E. coli</i> O157 isolated on SMA	SLT-producing <i>E. coli</i> isolated colony blot	Number of ELISA-positive, SMA culture-negative samples
		SLT-I	SLT-II	SLT-I and II			
HC	10	0	10	0	8	2	0
HUS	25	0	11	0	8	3	0
Bloody diarrhoea	320	2	18	8	23	4*	1
Non-bloody diarrhoea	120	0	4	1	3	2	0
Total	475	2	43	9	42	11	1

HC, haemorrhagic colitis, HUS, haemolytic uraemic syndrome.

* All four isolates belonged to non-O157 serogroups.

of each other on a TSA plate and 25 µl of faecal suspension (0.5 g in 10 ml of PBS) was spread over the surface of the upper filter. The plate was then incubated at 37°C for 18 h which resulted in *c.* 200–1000 cfu/plate. The lower filter was removed and then blocked with bovine serum albumin 1% in PBS for 1 h at 22°C. The filter was then probed with rabbit antitoxin to SLT-I and -II diluted 1 in 6000 in PBS for 1 h at 22°C. After washing in PBS-Tween 20 0.05% the filters were exposed to goat anti-rabbit IgG phosphatase conjugate diluted 1 in 6000 in PBS for 30 min at 22°C. After washing in PBS-Tween 20, the filter was finally developed with nitroblue tetrazolium-bromo-chloroindoxyl phosphate (BioRad). The presence of purple areas on the filter indicated potential toxin-producing colonies. The filter was then aligned with the top filter and blot-positive colonies were picked off the top filter, subcultured for purity, and tested for toxin production by ELISA. As a control for colony blots a mixture of *E. coli* O157:H7 (strain 933) with *E. coli* C600 was processed as if it were a faecal suspension.

The toxigenic potential of O157 and non-O157 isolates was confirmed by the Division of Enteric Pathogens, Central Public Health Laboratory, London.

Results

Correlation of ELISA with SMA culture

A total of 475 faecal samples from 457 patients was examined in this study. *E. coli* O157 strains were isolated by SMA culture from 42 samples. However, 54 samples gave positive ELISA results (43 SLT-II only, nine SLT-I and -II and two SLT-I only; table). Therefore, the ELISA detected 12 apparent SLT-producing *E. coli* infections that were not detected by SMA culture. SLT-producing *E. coli* were subsequently detected in 11 of 12 ELISA-positive, SMA culture-negative samples by the colony blot technique and by testing colony sweeps. In seven instances, low numbers of *E. coli* O157 were isolated and in four instances SLT-producing *E. coli* of serogroups other than O157 were isolated (two serogroup O128, one

serogroup O103, the one other untyped). *E. coli* colonies from the remaining ELISA-positive, SMA-negative sample gave negative results by colony blot.

In each case of SLT-producing *E. coli* infection in this study, the organism was isolated as the sole recognised pathogen. Thirty-six samples yielded a pathogen other than an SLT-producing *E. coli* and in none of these cases was the SMA culture positive for *E. coli* O157 or the ELISA positive for SLT-I or -II.

Clinical correlation

All of 10 faecal samples from cases of HC, were ELISA-positive for SLT-II after culture, but only eight yielded *E. coli* O157 on SMA. The remaining two samples gave positive results for *E. coli* O157 on colony blot but the organisms were present in very low numbers and were likely to have been overgrown by non-O157 serogroups on SMA. Twenty-five faecal samples were examined from 19 patients with clinically typical HUS. Eleven (44%) samples from nine patients were ELISA-positive but only eight (32%) of these samples yielded *E. coli* O157 directly on SMA. The remaining three samples yielded SLT-II-producing *E. coli* isolates on colony blot which were shown subsequently to be serogroup O157. Fourteen samples from HUS patients, most of which were obtained late in the illness, gave negative results in all tests.

Of 320 samples from patients with a history of bloody diarrhoea, 28 (8.8%) were ELISA-positive and 23 (7.2%) of these yielded *E. coli* O157 on SMA. Four ELISA-positive, SMA culture-negative samples yielded SLT-producing *E. coli* belonging to serogroups other than O157. In one case that was ELISA-positive for SLT-II, no SLT-producing *E. coli* was isolated.

In 120 samples from patients with non-bloody diarrhoea, SLT-producing *E. coli* were detected in five samples (4%) by ELISA but only 3 samples (2.5%) yielded *E. coli* O157 on SMA. In the remaining two cases, *E. coli* O157 was isolated by colony blot.

ELISA performance

The mean A_{405} for the 1.6 ng/ml standards of SLT-I and SLT-II were 0.35 (range 0.30–0.49) and 0.27 (range 0.23–0.35), respectively, with a substrate-con-

taining well as blank. Typical negative samples had A_{405} in the SLT-I and SLT-II ELISA of 0.0–1.2 and 0.0–0.9, respectively. Samples that contained SLT-I typically had A_{405} of 0.5–> 2 and for SLT-II-positive samples the range of A_{405} values was 0.4–> 2. Over 80% of samples containing SLT-producing *E. coli* gave A_{405} values > 2.

If SLT-producing *E. coli* isolation on either SMA or by colony blot was regarded as the definitive test, the ELISA had a sensitivity and specificity of 100% and 99.7%, respectively. Positive and negative predictive values were 98.1% and 100%.

Discussion

The purpose of this study was to compare the efficacy of SLT ELISA on supernates and cell lysates after culture of faeces in broth containing mitomycin C with culture on SMA in the detection of infections caused by SLT-producing *E. coli*. The ELISA method proved superior to SMA culture and detected 11 (20%) of cases of infection with SLT-producing *E. coli* that were not detected by culture on SMA. Low numbers of *E. coli* O157 were detected subsequently in seven of these samples and non-O157 SLT-producing *E. coli* were isolated from four samples. It is not surprising that the SMA method failed to detect infection caused by non-O157 serogroups as these four isolates fermented sorbitol and were indistinguishable from normal flora *E. coli* on SMA. The ability of the ELISA to detect SLT-producing *E. coli* of serogroups other than O157 is a distinct advantage over SMA culture.

The apparent incidence of SLT-producing *E. coli* infection in this study is high (53 of 475 cases) but this is not a reflection of the true incidence of this infection. Eleven isolates were from a single outbreak and 36 faecal samples (including eight from the outbreak) were sent for confirmation of *E. coli* O157 isolation.

Because many laboratories are unable to examine samples for SLT-producing *E. coli* of serogroups other than O157, there is little information concerning the incidence of *E. coli* strains other than O157 in disorders other than HUS, although it is thought to be lower than the incidence of *E. coli* O157.^{18,19} In this study 439 samples formed a blind prospective evaluation of the ELISA method. Seventeen of the samples yielded SLT-producing *E. coli*, of which 13 were *E. coli* O157 and four (24%) were non-O157 SLT-producing *E. coli*. All four non-O157 isolates were from cases of bloody diarrhoea. The high incidence of serogroups other than O157 as a cause of bloody diarrhoea may reflect the use of a sensitive diagnostic test. By means of this method we can now ascertain the

true incidence of non-O157 SLT-producing *E. coli* as a cause of diarrhoea.

There was one apparent false positive reaction in the 475 samples examined by the ELISA method. The culture supernate of this sample contained low (12.5 ng/ml) quantities of SLT-II, but SLT-producing *E. coli* were not isolated from this sample by SMA, colony blot or colony sweeps. The reason for this is not clear but may be due to infection caused by an SLT-producing *E. coli* present in very low numbers.

The diagnosis of HUS is clinical but the detection of SLT-producing *E. coli* can confirm the diagnosis and is of particular value in unusual cases. In this study, the ELISA detected three cases of *E. coli* O157 infection that were not detected by SMA. The low isolation rate of SLT-producing *E. coli* in HUS cases in this study (11 of 25) is similar to that found in other studies and is likely to be due to loss of pathogens as a result of delays in receiving samples. This was illustrated in one case when three samples were received from one child with HUS and were consistently negative by ELISA, SMA culture and colony blot. The samples were 1-week-old before they were examined in this study and *E. coli* O157 had been isolated previously on SMA at the referring hospital. Serological techniques have been employed successfully in diagnosing some infections that are culture negative.²⁰

The main disadvantage of the ELISA method is that, although it detects the presence of toxin-producing organisms in a broth culture, the organism is not available for further characterisation by serotyping, phage typing or toxin studies. If *E. coli* O157 is present in large numbers it will be detected on SMA. However, if low numbers of *E. coli* O157 or a non-O157 SLT-producing *E. coli* are present in the faeces, the ELISA will be positive but no pathogens will be detected on SMA. In such cases we used a colony blot technique and tested colony sweeps to obtain pure cultures of toxin-producing strains for further characterisation. Alternatively, mixed cultures that are ELISA positive could be examined by gene probe techniques.

The culture of faecal samples in broth containing mitomycin C followed by ELISA for SLTs is more sensitive than culture on SMA alone, detects infections in which low numbers of organisms are present and those caused by non-O157 serogroups. We propose that this technique, used routinely in conjunction with culture on SMA, is an economical method of providing a rapid diagnosis of most human gastrointestinal infection caused by SLT-producing *E. coli*.

We thank the Division of Enteric Pathogens, Central Public Health Laboratory, Colindale Avenue, London for serotyping and toxin confirmation tests. D.W.K.A. was supported by a Health Sciences for the Tropics Partnership in Research and Training Grant from the Rockefeller Foundation, New York, USA.

References

1. Kleanthous H, Smith HR, Scotland SM *et al.* Haemolytic uraemic syndromes in the British Isles, 1985–8: association with Verocytotoxin producing *Escherichia coli*. Part 2: microbiological aspects. *Arch Dis Childhood* 1990; **65**: 722–727.
2. Riley LW. The epidemiologic, clinical and microbiologic features of hemorrhagic colitis. *Annu Rev Microbiol* 1987; **41**: 383–407.
3. Karmali MA, Petric M, Lim C, Fleming 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.
4. Karmali MA. Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev* 1989; **2**: 15–38.
5. PHLS *Escherichia coli* O157 Working Group. Laboratory diagnosis of infection caused by verocytotoxin-producing *Escherichia coli* of serogroup O157. *PHLS Microbiology Digest* 1991; **7**: 94–95.
6. Tarr PI, Neill MA, Clausen CR, Watkins SL, Christie DL, Hickman RO. *Escherichia coli* O157:H7 and the hemolytic uremic syndrome: importance of early cultures in establishing the etiology. *J Infect Dis* 1990; **162**: 553–556.
7. Ritchie M, Partington S, Jessop J, Kelly MT. Comparison of a direct fecal shiga-like toxin assay and sorbitol-MacConkey agar culture for laboratory diagnosis of enterohemorrhagic *Escherichia coli* infection. *J Clin Microbiol* 1992; **30**: 461–464.
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. Bettelheim KA, Brown JE, Lolekha S, Echeverria P. Serotypes of *Escherichia coli* that hybridized with DNA probes for genes encoding Shiga-like toxin I, Shiga-like toxin II, and serogroup O157 enterohemorrhagic *E. coli* fimbriae isolated from adults with diarrhea in Thailand. *J Clin Microbiol* 1990; **28**: 293–295.
10. 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.
11. Thomas A, Smith HR, Willshaw GA, Rowe B. Non-radioactively labelled polynucleotide and oligonucleotide DNA probes, for selectively detecting *Escherichia coli* strains producing Vero cytotoxins. VT1, VT2 and VT2 variant. *Mol Cell Probes* 1991; **5**: 129–135.
12. Pollard DR, Johnson WM, Lior H, Tyler SD, Rozee KR. Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *J Clin Microbiol* 1990; **28**: 540–545.
13. Acheson DWK, Keusch GT, Lightowlers M, Donohue-Rolfe A. Enzyme-linked immunosorbent assay for Shiga toxin and Shiga-like toxin II using P₁ glycoprotein from hydatid cysts. *J Infect Dis* 1990; **161**: 134–137.
14. Downes FP, Green JH, Greene K, Strockbine N, Wells JG, Wachsmuth IK. Development and evaluation of enzyme-linked immunosorbent assays for the detection of Shiga-like toxin I and Shiga-like toxin II. *J Clin Microbiol* 1989; **27**: 1292–1297.
15. Law D, Ganguli LA, Donohue-Rolfe A, Acheson DWK. Detection by ELISA of low numbers of Shiga-like toxin-producing *Escherichia coli* in mixed cultures after growth in the presence of mitomycin C. *J Med Microbiol* 1992; **36**: 198–202.
16. Head SC, Petric M, Richardson S, Roscoe M, Karmali MA. Purification and characterization of verocytotoxin 2. *FEMS Microbiol Lett* 1988; **51**: 211–216.
17. Donohue-Rolfe A, Acheson DWK, Kane AV, Keusch GT. Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P₁ glycoprotein and the production of cross-reactive monoclonal antibodies. *Infect Immun* 1989; **57**: 3888–3893.
18. Pai CH, Ahmed N, Lior H, Johnson WM, Sims HV, Woods DE. Epidemiology of sporadic diarrhea due to verocytotoxin-producing *Escherichia coli*: a two-year prospective study. *J Infect Dis* 1988; **157**: 1054–1057.
19. Edelman R, Karmali MA, Fleming PA. Summary of the International Symposium and Workshop on Infections due to Verocytotoxin (Shiga-like toxin)-producing *Escherichia coli*. *J Infect Dis* 1988; **157**: 1102–1104.
20. Chart H, Scotland SM, Rowe B. Serum antibodies to *Escherichia coli* serotype O157:H7 in patients with hemolytic uremic syndrome. *J Clin Microbiol* 1989; **27**: 285–290.