Detection by ELISA of low numbers of Shiga-like toxin-producing *Escherichia coli* in mixed cultures after growth in the presence of mitomycin C

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Summary. Techniques currently available to detect Shiga-like toxin (SLT)-producing *Escherichia coli* lack sensitivity or require specialised equipment and facilities, and in some cases detect only strains belonging to serotype O157. We have used an ELISA technique, capable of detecting both SLTI and SLTII with crude P1 glycoprotein from hydatid cysts, in combination with enhancement of toxin production by culture with mitomycin C. Supernates of Tryptone Soya Broth cultures containing mitomycin C 200 ng/ml were tested for SLTII. For SLTI, cell lysates pre-treated with polymyxin B were tested. In tests with *E. coli* O157:H7 in mixed culture with *E. coli* strain C600 alone, or with *E. coli* C600, *Proteus mirabilis* and *Enterococcus faecalis*, SLTI could be detected when the proportion of toxigenic organisms represented 1% of the mixture, and SLTII when the proportion was 0.025%. When faecal samples with added *E. coli* O157:H7 were examined in this system, SLTII-producing strains were detected when they comprised <0.1% of the coliform population. This technique is a sensitive and specific assay for detecting low numbers of SLT-producing organisms in mixed culture such as occurs in cases of haemolytic uraemic syndrome and haemorrhagic colitis.

Introduction

Enteric infections complicated by haemorrhagic colitis (HC) or the haemolytic uraemic syndrome (HUS) are commonly associated with *Escherichia coli* strains that produce cytotoxins.^{1–4} These cytotoxins occur in two principal forms—Shiga-like toxin (SLT) I, which is almost identical to the toxin elaborated by *Shigella dysenteriae* type 1, and SLTII which, although immunologically distinct from SLTI, shares approximately 60% amino-acid homology.⁴ The subunit structure, mechanism of action and biological activity (enterotoxicity, neurotoxicity and cytotoxicity) are similar for SLTI and II,⁵ and both toxins bind to globotriaosyl ceramide (Gb3) which may be an important receptor *in vivo*.⁶ In most strains of SLT-producing *E. coli*, the toxin genes are carried by bacteriophage.⁴

The laboratory diagnosis of infection with SLT-producing *E. coli* can be difficult. In many laboratories, diagnosis is made by faecal culture on sorbitol-MacConkey agar to isolate strains of *E. coli* serotype O157 which are sorbitol non-fermenters, followed by agglutination with specific antisera.¹ However, this technique may produce false positive results because

of isolation of non-toxigenic strains of *E. coli* O157 and *E. hermannii*.⁷ Of greater importance is the failure of this technique to detect sorbitol-fermenting SLT-producing strains of *E. coli* of serotypes other than O157, which are frequently implicated as causes of HC and HUS.^{1,8} A recent study in the UK showed that only 72% of infections with cytotoxin-producing *E. coli* were caused by strains belonging to serotype O157;⁸ thus 28% of strains would ferment sorbitol and would not be detected by this method.

Other techniques to detect SLTs are available. In laboratories with tissue-culture facilities, direct inoculation of filtered faecal suspensions on to susceptible cell lines, or examination of *E. coli* culture supernates or cell lysates can be performed.¹ However, the specificity of any cytotoxic activity must be confirmed with appropriate neutralising antibodies. Detection of toxin genes with DNA probes,^{9–11} with or without the polymerase chain reaction (PCR),^{12,13} is an alternative approach.

ELISA techniques have also been developed for detection of Shiga-like toxin with monoclonal antibodies¹⁴ or Gb3^{15,16} to bind toxin. Recently, P1 glycoprotein from hydatid cyst fluid, which contains the same terminal Gal α 1–4 Gal disaccharide as Gb3, has been used to bind SLTI and II for both toxin purification¹⁷ and quantitation by ELISA.¹⁸

Toxin yields of SLTI and II can be markedly

increased by incubation of bacterial cultures with mitomycin C.¹⁹ This technique has been utilised to allow purification of enough SLTII for biochemical characterisation.²⁰

A major problem in the diagnosis of SLT-producing *E. coli* in HUS and HC is that the numbers of toxin-producing organisms decreases as the disease progresses,²¹ such that the number of toxin-producing *E. coli* represents <1% of the total faecal *E. coli* population.^{1,2,8} Therefore, tests aimed at detecting toxin-producing organisms must have adequate sensitivity and be as rapid as possible.

In this study we have used an ELISA, in combination with culture in the presence of mitomycin C, to develop an easily applicable and sensitive assay for the detection of SLTI- and II-producing organisms.

Materials and methods

Bacterial strains

The following strains were used throughout the study. *E. coli* K-12 strain C600 was used as a non-SLT producing control. Strain C600 lysogenised with phage 933J or 933W was used as a producer of either SLTI or SLTII, respectively, and *E. coli* O157:H7 strain 933, which produces both SLTI and II, was used as the toxin-producing strain. All were kindly provided by the late Dr H. Williams-Smith. A clinical isolate of *E. coli* O157:H7 producing SLTII only was used in some experiments.

A strain of *Proteus mirabilis* and a strain of *Enterococcus faecalis* isolated from a faecal sample were used in combination with *E. coli* C600 to simulate mixed faecal flora.

Cultural conditions and preparation of mixed cultures

Bacteria grown in Tryptone Soya Broth (TSB; Difco) overnight at 37°C were used to inoculate TSB or TSB containing mitomycin C (Sigma). Cultures were then incubated at 37°C for 18 h with shaking (110 rpm). Initially, mitomycin C was added at a range of concentrations of 0–1000 ng/ml to determine the optimal concentration required for production of SLT by *E. coli* O157:H7 strain 933.

Mixed cultures were prepared by inoculating 8 µl of dilutions (25–10 000) of a culture of *E. coli* O157:H7 strain 933 and an equal volume of a culture of *E. coli* strain C600 or an equal mixture of *E. coli* C600, *P. mirabilis* and *Ent. faecalis* into TSB. Viable counts (cfu/ml) were performed on CLED Agar (Lab M) on the culture used as the inoculum and on the mixture following overnight incubation.

Preparation of faecal samples

Faeces (c. 4 g) from a patient with diarrhoea was suspended in 10 ml of PBS and the cfu/g of faeces was

determined on CLED agar. The remaining faecal sample was stored at 4°C. Following the determination of the viable count, a freshly prepared suspension of the faecal sample was used to inoculate a series of broths containing mitomycin C 200 ng/ml with c. 10⁶–10⁷ cfu. Dilutions of the *E. coli* O157:H7 strain 933 were then added. Viable counts were performed on the faecal sample and the *E. coli* O157:H7 culture to determine the proportions of faecal: toxigenic organisms in the mixture. A broth inoculated with the faecal suspension alone served as a control. This procedure was repeated with two other faecal samples.

Preparation of culture supernates and cell lysates

After overnight incubation, the cultures were centrifuged for 10 min at 3000 g. For SLTII, supernates were harvested and assayed by ELISA. For SLTI, cell pellets were resuspended in 300 µl of polymyxin B (Sigma) 2 mg/ml in PBS, vortex mixed and incubated at 37°C for 15 min. The lysate was centrifuged in a bench top microfuge for 2 min and the clear supernate was assayed by ELISA. Polymyxin lysis of cell pellets gave at least a five-fold increase in the yield of SLTI.

ELISA

SLTI and II were assayed by an ELISA technique as previously described.¹⁷ Briefly, ELISA plates (Nunc Immunoplates) were coated with crude hydatid cyst material¹⁷ 10 µg/ml in PBS, and then blocked with bovine serum albumin 1%. Supernate or cell lysate preparations were added and bound toxin was detected with rabbit polyclonal antibodies raised against the two toxins separately.¹⁶ A goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) followed by substrate (nitrophenol phosphate; Sigma) was used to detect bound polyclonal antibody. Absorbance was measured at 410 nm. To quantify the amount of toxin in the preparation, Shiga toxin and SLTII, purified as described previously,¹⁶ were diluted in PBS and used as standards in the ELISA.

Results

Experiments to determine the concentration of mitomycin C for the maximal induction of toxin in *E. coli* O157:H7 strain 933 showed that 200 ng/ml was optimal (table I). This concentration was used in subsequent experiments.

The ELISA was able to detect 1.6 ng of purified Shiga toxin (SLTI) and 1.6 ng of SLTII per ml. It was confirmed that *E. coli* strain C600 (933J) produced SLTI only and *E. coli* C600 (933W) produced SLTII only. *E. coli* O157:H7 strain 933 produced both toxins. Comparison of cultures of these three strains grown with and without mitomycin C 200 ng/ml showed that toxin titres were markedly increased in all three strains by growth in the presence of mitomycin C (table II).

Table I. Production of SLTI and II by *E. coli* O157:H7 strain 933 measured by ELISA after growth in TSB containing mitomycin C

Mitomycin C concentration (ng/ml)	Toxin detected (ng/ml) by ELISA	
	SLTI	SLTII
0	10	160
50	400	8000
100	800	10 000
200	1600	32 000
400	900	20 000
800	160	8000
1000	120	5000

Table II. Effect of growth with mitomycin C on production of SLTI and II detected by ELISA in culture supernate (SLTII) and polymyxin-induced cell lysates (SLTI)

Strain	Growth with mitomycin C	Titre in ELISA of	
		SLTI	SLTII
<i>E. coli</i> C600 (933J)	+	400	ND
	—	Neat	ND
<i>E. coli</i> C600 (933W)	+	ND	32 000
	—	ND	400
<i>E. coli</i> O157:H7 (993)	+	400	16 000
	—	Neat	200

ND, not detected.

When *E. coli* O157:H7 strain 933 was co-inoculated at different dilutions with a fixed number (10^7) of *E. coli* C600 into broth containing mitomycin C, it was possible to detect SLTI in cell lysates when the proportion of toxin-producing cells at inoculation was 0.5% (table III). However, detection of SLTII in supernates was possible when the proportion of strains was 0.0125–0.025% (table IV).

Table III. Detection of SLTI, determined by ELISA with cell lysates of *E. coli* O157:H7 strain 933 grown in TSB (with or without mitomycin C 200 ng/ml) in variable proportions with either *E. coli* strain C600 or a mixture of *E. coli* C600, *P. mirabilis* and *Ent. faecalis*

Percentage of <i>E. coli</i> O157:H7 in mixture	ELISA Optical Density (A) at 410 nm (SD) of lysate after culture with		
	<i>E. coli</i> C600 + mitomycin C	mixture + mitomycin C	mixture only*
100	1.67 (0.18)	1.35 (0.7)	0.37
10	NT	NT	0.08
4	1.06 (0.2)	0.71 (0.12)	0.08
2	0.69 (0.15)	0.22 (0.03)	0.06
1	0.32 (0.04)	0.33 (0.06)	0.05
0.5	0.43 (0.10)	0.14 (0.02)	0.06
0.25	0.10 (0)	0.06 (0.01)	0.05
Control	0.06 (0.01)	0.05 (0.02)	0.05

Results are expressed as mean A_{410} (SD) from four separate experiments each performed in duplicate wells.

NT, not tested.

* Mean of duplicate wells from a single experiment.

Similar results were obtained with a mixed culture of *P. mirabilis*, *Ent. faecalis* and *E. coli* strain C600 incubated with *E. coli* O157:H7 strain 933 (tables III and IV). Detection of SLTI in cell lysates was possible when the proportion of toxin producers was 0.5%, and, for SLTII, positive reactions were found when the proportion of toxin producers was 0.0125–0.025% (tables III and IV). When the experiment was conducted in the absence of mitomycin C, SLTI was detected only in pure cultures and SLTII in cultures comprising 0.5% of toxin-producing organisms (tables III and IV).

When a different clinical isolate of *E. coli* O157:H7 which produced SLTI, was co-inoculated with the three-organisms mixture, SLTII production was also detectable when the proportion of the toxin-producer was as low as 0.1%.

With three different faecal samples seeded with *E. coli* O157:H7 strain 933, SLTII was detected when the proportion of toxigenic organisms was <0.1% in all three samples and <0.05% in one case. In each case, SLTII was not detected in any of the faecal samples inoculated into broth without the *E. coli* O157:H7 strain. These samples were not examined for SLTI production.

Discussion

Diagnosis of *E. coli* infections which are associated with HC or HUS requires a rapid, specific and sensitive laboratory test. Existing techniques, such as sorbitol MacConkey agar, lack specificity, others such as tissue culture cytotoxin tests lack speed or are not readily available, e.g., DNA probes and PCR. ELISAs to detect toxins have been developed with various techniques, and are able to detect very small (ng) quantities of either SLTI or SLTII. Thus, although less sensitive than tissue culture, ELISAs do offer

Table IV. Detection of SLTII, determined by ELISA, in supernates of *E. coli* O157:H7 strain 933 grown in TSB (with or without mitomycin C 200 ng/ml) in variable proportions with either *E. coli* strain C600 or a mixture of *E. coli* C600, *P. mirabilis* and *Ent. faecalis*

Percentage of <i>E. coli</i> O157:H7 in mixture	ELISA Optical Density (A) at 410 nm (SD) of supernate after culture with		
	<i>E. coli</i> C600 + mitomycin C	mixture + mitomycin C	mixture only*
1	0.61 (0.06)	0.65 (0.06)	0.44
0.5	0.61 (0.18)	0.54 (0.12)	0.35
0.2	0.38 (0.09)	0.37 (0.10)	0.10
0.1	0.32 (0.04)	0.33 (0.06)	0.05
0.05	0.25 (0.02)	0.23 (0.06)	0.06
0.025	0.20 (0.05)	0.17 (0.05)	0.05
0.0125	0.13 (0.02)	0.09 (0.03)	0.06
Control	0.06 (0.01)	0.06 (0.01)	0.06

Results are expressed as mean A_{410} (SD) from five separate experiments each performed in duplicate wells.

* Mean of duplicate wells from a single experiment.

relative speed, greater availability and potential for automation.

The sensitivity of the current ELISA was 1.6 ng/ml for SLTI and II, which is comparable to the sensitivity of a previously described ELISA with Gb3 as the capture system.²² To increase the sensitivity of our assay, toxin yields of the organisms were increased by growth in broth containing mitomycin C. This enhanced the yield of both SLTI and SLTII from *E. coli* O157:H7 strain 933 >100-fold compared with growth in cultures without mitomycin C.

The mechanism whereby mitomycin C increases toxin yields is not fully understood but may be a consequence of bacteriophage induction (D. W. K. Acheson, unpublished observations). The increase in phage particles and hence viral gene copies brought about by mitomycin C suggests that this compound amplifies the number of toxin genes per bacterial cell and may increase the sensitivity of DNA probes for these toxins. The enhancement of toxin production by mitomycin C also has potential for other methods of detection of SLT-producing *E. coli*.

Currently we have no explanation for the marked differences in the level of SLTI and SLTII produced by *E. coli* O157:H7 strain 933. The lower levels of SLTI prevented detection of small numbers of this organism, methods to improve the yields of SLTI are being evaluated.

When *E. coli* strain C600 alone or mixtures of *E. coli* C600, *P. mirabilis* and *Ent. faecalis* were co-inoculated with varying numbers of *E. coli* O157:H7 strain 933 and grown in broth containing mitomycin C, SLTI was detected when the proportion of the *E. coli* O157:H7 strain was 0.5–1% of the population. Detection of SLTII however was achieved when *E.*

coli O157:H7 represented only 0.0125–0.025% of the total. In tests with faecal samples seeded with *E. coli* O157:H7 strain 933, SLTII was detected when the organism comprised <0.1% of the aerobic flora. This may be important because several studies have shown that, in cases of HUS, the number of cytotoxin-producing organisms was <1%.^{1,8,10} The system described in this study provides a simple means of diagnosing infection with SLT-producing *E. coli* even when the organisms are present in low numbers. The technique must now be evaluated with clinical samples from patients with HC or HUS. The technique is easily applicable in routine diagnostic laboratories without the need for expensive equipment or expertise. However, at the present time antibodies to SLT are not widely available. Commercial development of an SLT assay would be welcomed.

There is growing evidence that SLTII-producing *E. coli* are more important than SLTI-producing *E. coli* in the pathogenesis of HC and HUS.^{23,24} This may reflect the much higher yields of SLTII compared with SLTI, a greater intrinsic toxicity of SLTII compared with SLTI,²³ or a greater prevalence of SLTII-producing strains. A recent survey in the UK showed that 83% of *E. coli* strains from HC or HUS cases produced SLTII only, whereas 5.6% of strains produced SLTI only and 11.3% of strains produced both toxins.⁸ Therefore, an assay that is able to detect low numbers of SLTII-producing strains would be highly advantageous for diagnostic purposes.

DWKA gratefully acknowledge the support of The Wellcome Trust. This work was supported by Grant AI-20253 from the National Institute of Allergy and Infectious Disease. We thank Drs D. W. Denning and M. G. L. Keaney for helpful discussions.

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