A method of enhancing verocytotoxin production by *Escherichia coli*

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1. SUMMARY

The number of verocytotoxin producing *Escherichia coli* (VTEC) present in the faeces during an infection may be very low, making their detection difficult. We report a method for enhancing toxin production by VTEC using mitomycin C as an inducing agent with the aim of improving the detection of VTEC. In pure culture, mitomycin C enhanced toxin production up to 100-fold. When applied to mixed faecal culture, toxin could be detected in mitomycin C treated samples when standard cultures were negative and when substantially fewer verocytotoxin-producing bacteria were present. Use of this method may aid in the detection of VTEC and is appropriate for use in the routine diagnostic laboratory.

2. INTRODUCTION

The proportion of verocytotoxin producing *Escherichia coli* (VTEC) present in the faeces during an infection may be less than 1% [1]. Consequently testing several (individual) colonies for verocytotoxin (VT) is unlikely to detect all cases of VTEC infection. Karmali et al. [2] considered the demonstration of faecal VT a useful indicator of VTEC infection, but the titres detected ranged from as low as 2 to 2048 with a median of 8. However, VT was detected in cases where the VTEC organism could not be isolated, presumably because they were present in low numbers. Using a polymixin B-extraction technique [3] to enhance release of preformed toxin, the maximum titre of VT from colony pools or sweeps of *Escherichia coli* isolates was increased four- to eight-fold compared to culture supernatants. Colony hybridisation using DNA probes for the VT genes allows several hundred colonies from a sample to be tested, thereby increasing the sensitivity [4]. However this technique is not currently available in most routine laboratories. The major-
ity of VTEC reported in the UK belong to serogroup O157 [5] and the use of sorbitol MacConkey agar has been described for the isolation of these organisms. However other serogroups have been reported [6] and this isolation procedure cannot be used for them.

VT production has been shown to be phage-encoded in several strains. Phage- and plasmid-encoded characteristics can be enhanced by the use of non-specific inducing agents such as UV irradiation or mitomycin C [7]. This technique, using mitomycin C, has been used for the enhancement of heat-labile enterotoxin production by E. coli [8]. The aim of this study was to determine whether it was possible to enhance cytotoxin production by VTEC using mitomycin C as the inducing agent, with a view to increasing the likelihood of detecting VT in the filtrates of cultures even when VT producing organisms are present in low numbers.

3. MATERIALS AND METHODS

The reference strain was Escherichia coli E32511 (O157:H-) producing VT2 [9]. Five strains of serotype O157:H7 isolated from stools were also studied, four (66977, 15115, 35879, 35879) were from children under 2 years with haemolytic uraemic syndrome, three of whom were known to have had a prodrome of bloody diarrhoea, and one (strain 38581) was from an adult with bloody diarrhoea. These five isolates produced VT2 as shown by the neutralisation of cytotoxicity by VT2-specific antiserum and by hybridisation with a VT2 DNA probe. The laboratory strain K12 was used as a negative control. All strains were maintained at -20°C in nutrient broth with 10% glycerol added.

The following media were tested to determine which would give the maximal yield of VT: brain heart infusion broth (Difco 0037), proteose peptone broth No. 3 (Difco 0122), trypthone soya broth (Oxoid CM 129) and casamino acid yeast extract (Oxoid LP021). The appropriate sterilised medium was dispensed into 5-ml aliquots and inoculated with a loopful of an overnight nutrient broth culture of the test strain. After incubation at 37°C for 16 h, this starter culture was added to 35 ml of fresh test medium in a 250-ml conical flask and incubated in shaking water bath at 37°C. After 1 h, mitomycin C was added to give a final concentration of 1 µg/ml and then incubated for a further 6 h. Bacteria were harvested by centrifugation for 30 min and the culture supernatant removed for VT testing. In some cases the bacterial pellet was resuspended in 5 ml phosphate buffered saline (PBS) containing polymixin B (0.1 mg/ml) and incubated at 37°C in a water bath for 30 min. After centrifugation for 20 min the supernatants from the polymixin-B extract were filtered through a 0.22-µm millipore filter and tested for VT.

3.1. Mixed faecal culture

Serial dilutions to 10^11 of an overnight culture of E. coli E32511 at 37°C were made in brain heart infusion broth. The concentration of viable bacteria in each dilution was determined by the method of Miles and Misra [10]. 1 ml of each dilution was then added to two separate 5-ml aliquots of a faecal suspension (diluted 1:4 using PBS, pH 7.2), obtained from a single healthy donor and shown to be non-toxigenic. Mitomycin C was then added to one of the duplicate cultures. Fresh BHI broth was added to both cultures to give a final volume of the starter culture of 10 ml. They were then incubated at 37°C for 18 h, after which the titre of VT in the faecal filtrate was determined.

3.2. Detection of verocytotoxin

Verocytotoxin was detected using a HeLa-cell cytotoxicity assay after O'Brien et al. [11]. Serial dilutions of the test samples were made in normal saline to 10^7. 50 µl of each dilution were added to tubes containing HeLa-cell monolayers together with 0.5 ml of Hanks' medium and incubated at 37°C for 72 h. The endpoint was taken as the highest dilution to kill 50% (CD50) of the HeLa cells, determined microscopically. Culture supernatants and filtrates of polymixin-B extracts were also analysed (Dr. S.C. Scotland, P.H.L.S. Colindale) for specific VT2 cytotoxic activity on Vero cells [4].
4. RESULTS

The amount of VT in polymixin-B extracts of *E. coli* E32511 cultured in various media ranged from $10^2$ (tryptone soya broth, casamino acid yeast extract, proteose peptone No. 3) to $10^3$ (brain heart infusion broth) for cultures without added mitomycin C, and from $10^4$ (tryptone soya broth, casamino acid yeast extract, proteose peptone No. 3) to $10^6$ (brain heart infusion broth) for those with added mitomycin C. BHI broth consistently gave the highest yield and was therefore used in all subsequent studies. The results of VT2 cytotoxin production detected in culture supernatants and polymixin-B extracts of cultures with and without added mitomycin C for *E. coli* E32511, the laboratory control strain K12 (VT negative), and for five strains of *E. coli* O157: H7 isolated from patients presenting with bloody diarrhoea (with or without the haemolytic uraemic syndrome) are shown in Table 1. Cytotoxicity testing of the media with added mitomycin C alone was negative. The addition of mitomycin C to the media resulted in at least a 100-fold increase in the amount of cytotoxin detected both in the culture supernatant and the filtrates of the polymixin-B extracts.

On two occasions the culture supernatants and polymixin-B extract filtrates obtained from cultures of E32511 were tested for cytotoxicity against Vero cells. The effect of mitomycin C was confirmed while the cytotoxic effects were neutralised by an antiserum specific for VT2. The titres obtained were higher than those achieved with HeLa cells by up to a factor of 10. In part this may have been due to differences in the sensitivity of Vero and HeLa cells to VT, but also to the use of a longer incubation period (4 days).

Cultures of non-toxigenic stool in BHI broth 'spiked' with the VT2-producer E32511 again demonstrated that the titre of cytotoxin produced was enhanced by the addition of mitomycin C (Table 2). Verocytotoxin was detectable in faecal filtrates with as few as 19 bacteria/10 ml in the

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Number of cultures</th>
<th>Titre of VT Mean (range)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mitomycin C Present A bsent</td>
</tr>
<tr>
<td>E32511</td>
<td>Polymixin extract</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Supernatant from culture</td>
<td>22</td>
</tr>
<tr>
<td>66977</td>
<td>Polymixin extract</td>
<td>3</td>
</tr>
<tr>
<td>15115</td>
<td>Polymixin extract</td>
<td>3</td>
</tr>
<tr>
<td>35879</td>
<td>Polymixin extract</td>
<td>3</td>
</tr>
<tr>
<td>21801</td>
<td>Polymixin extract</td>
<td>3</td>
</tr>
<tr>
<td>38581</td>
<td>Polymixin extract</td>
<td>3</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>Polymixin extract</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Supernatant from culture</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>BHI (Media control)</td>
<td>25</td>
</tr>
</tbody>
</table>

All strains grown in brain heart infusion broth.
Table 2
The smallest number of verocytotoxin-producing bacteria necessary to detect free VT in mixed faecal cultures in the presence and absence of mitomycin C

<table>
<thead>
<tr>
<th>Dilution of growth of E32511</th>
<th>Number of viable bacteria added to 9-ml faecal/media suspension</th>
<th>Titres of VT from cultures with mitomycin added</th>
<th>Titres of VT from cultures without mitomycin added</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5</td>
<td>&gt; 500</td>
<td>10^3</td>
<td>10^3</td>
</tr>
<tr>
<td>10^7</td>
<td>122</td>
<td>10^2</td>
<td>Negative</td>
</tr>
<tr>
<td>10^8</td>
<td>19</td>
<td>10^1</td>
<td>Negative</td>
</tr>
<tr>
<td>10^9</td>
<td>2</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>10^10</td>
<td>0</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Mean of three experiments

culture, whereas without mitomycin C, VT was only detected when more than 500 VTEC bacteria/10 ml were present in the culture.

5. DISCUSSION

Since Konowalchuk et al. [12] first suggested that the production of verocytotoxin by E. coli might be a virulence factor in diarrhoeal disease, evidence has accumulated implicating it in two conditions, namely haemorrhagic colitis (HC) [5,13] and the haemolytic uraemic syndrome (HUS) [2,4]. However, a wide range of clinical symptoms from mild, non-bloody diarrhoea to severe manifestations such as HC or HUS have been reported [14,15]. A significant proportion of stools submitted for microbiological investigation from cases of diarrhoea yield no recognised pathogens using standard methods [16]. E. coli is a normal commensal of the human faecal flora. Nevertheless, colonisation by strains belonging to one of the five currently recognised types of diarrhoeagenic E. coli can result in a diarrhoeal illness [17]. The detection of such pathogenic E. coli currently requires the identification of specific virulence properties or serological markers to differentiate them from the commensal E. coli. The role of verocytotoxin-producing E. coli in community diarrhoea is unknown but their detection is hampered by the inability to readily differentiate them from non-pathogenic E. coli. Two distinct forms of VT exist, VT1 and VT2, both of which are cytotoxic to Vero and HeLa cells but not to Y1 adrenal or Chinese hamster ovary cells.

These data show that the titre of VT2 cytoxotoxin produced by O157 VTEC in vitro can be substantially enhanced by the addition of mitomycin C to the culture medium. This is over and above that which is achieved using polymixin-B extraction alone. Because of the problems in differentiating VT-producing strains from the normal non-pathogenic E. coli on routine selective culture, and the fact that VTEC may be present in low numbers in the faeces, enhancing the production of VT may aid in the detection of cases of VTEC induced disease. The technique of toxin enhancement increases the yield of toxin production by VTEC in pure culture without the need for polymixin-B extraction and is applicable to most routine diagnostic laboratories. This method may aid in the detection of VTEC in mixed faecal culture.

REFERENCES