Detection of serum and faecal antibodies in haemorrhagic colitis caused by Escherichia coli O157

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Introduction

Strains of Escherichia coli that produce a cytotoxin active against cultured Vero cells are now recognised as important human pathogens. These verocytotoxin-producing E. coli (VTEC) strains have been associated with outbreaks and sporadic cases of haemorrhagic colitis (HC) in North America and in England and Wales, and with sporadic cases of haemolytic-uraemic syndrome (HUS) in Canada and in England and Wales.

Beef, beef products and untreated milk have all been suggested as possible sources of VTEC infection for man. Strains of verocytotoxin-producing (VT+) E. coli O157, the most common serogroup associated with illness in man, have been isolated from cattle arriving for slaughter at two abattoirs in the Sheffield area, although the route of transmission from cattle to man was not determined. Person-to-person transmission of VT+ E. coli O157 has also been recorded.

Strains of VT+ E. coli O157 do not ferment sorbitol, and sorbitol MacConkey medium (SMAC) has been used for their selective culture. Further improvements to the selectivity of SMAC medium have facilitated detection of VT+ E. coli O157 colonies. Genes encoding two distinct verocytotoxins, VT1, and VT2, have been cloned and sequenced and DNA probes and the polymerase chain reaction have been described as sensitive methods for detecting VTEC in faecal samples. Detection of free faecal VT has also been used successfully as a diagnostic method. However, a drawback common to all diagnostic methods based on the detection of the intact organism, gene sequences or toxin in faecal samples is that they are all detectable for only a relatively short time after the onset of symptoms. Serological diagnosis of infection by VTEC, primarily in cases of HUS, by detecting serum VT-neutralising antibodies, agglutinating antibodies to E. coli O157, and antibodies to purified E. coli O157 lipopolysaccharide (LPS) has been described. The secretory immune response to infection by VT+ E. coli O157, and the serological response in HC due to this organism, appear to have been little studied. Therefore, the aim of this study was to compare methods for the detection of serum and faecal antibodies in HC caused by VT+ E. coli O157.

Materials and methods

The study was conducted from 1 April 1989 to 31 March 1992, and the cases investigated were those with culture-confirmed, clinically typical HC caused by VT+ E. coli O157. Three control groups of the same sex, whose ages matched as closely as possible those of the cases, were also selected: (1) those with no known history of recent gastrointestinal illness; (2) those with...
laboratory-confirmed recent infections with recognised intestinal pathogens other than VT* E. coli O157; and (3) those with extra-intestinal infections caused by serogroups of E. coli other than O157. Serum samples were obtained from 28 cases at a mean of 9.8 days after the onset of symptoms and from 34 patients in control group 1, 31 patients in control group 2 and 25 patients in control group 3. Faecal samples were obtained from 93 cases at a mean of 4-2 days after the onset of symptoms, and from 47 patients in control group 2. Further faecal samples, collected at various times after the first, were obtained from five cases. Serum samples were stored at −20°C and faecal samples at −70°C until required.

**Serum VT-neutralising antibodies**

**Cell culture.** Vero cells at a concentration of 200000 cells/ml of growth medium (Eagles MEM with bicarbonate and L-glutamine [Flow Laboratories] supplemented with fetal bovine serum 10%, penicillin 100 U/ml and streptomycin 100 μg/ml) were seeded in 100-μl volumes into sterile flat-bottomed cell-culture grade microtitration plates (Costar, Northumbria Biologicals). Plates were covered and incubated at 37°C in air containing CO₂ 6%. When cells were confluent, growth medium was removed and replaced by maintenance medium (as growth medium but without fetal bovine serum and with amphotericin B 40 μg/ml). Monolayers were used for toxin assay within 48 h.

**Preparation of toxin.** E. coli O26:H11 VT₁ VT₂*, and E. coli O157:H7 VT₁ VT₂ were grown for 20 h at 37°C in 100-ml volumes of a medium previously described for the production of E. coli heat-labile enterotoxin. Polymyxin B was added to a final concentration of 100 μg/ml and incubation was continued for a further 4 h. The cultures were centrifuged at 4500 g for 30 min and the supernate was filtered through a 0.22-μm membrane filter and stored as 2-ml portions at −20°C until required. The dilution of toxin causing cytotoxic destruction of c. 50% of Vero cells (CD50) in an individual monolayer was determined by multiple titrations of toxin in cell maintenance medium, addition of these to monolayers, and microscopic observation after incubation for 72 h.

**Antibody assay.** Sera from cases and control group 1 were examined. A range from 10 to 10240 of two-fold dilutions of sera in 100-μl volumes of maintenance medium was prepared in sterile microtitration plates, and 100 μl of maintenance medium containing 4 CD50 of toxin was added to each; the plates were then shaken and incubated at 37°C for 2 h in air containing CO₂ 6%. Maintenance medium was removed from fully confluent monolayers of Vero cells and replaced by the serum-toxin mixtures. Cells were incubated at 37°C for 72 h in air containing CO₂ 6%, and examined by microscopy. The titre of VT-neutralising antibody was taken as the last dilution showing complete inhibition of the cytotoxic effect.

**Whole-cell agglutinating antibodies**

Only sera from cases and patients in control group 1 were examined. Two-fold dilutions of serum samples from 5 to 640 were prepared in 50-μl volumes in PBS with sodium azide 0.08% w/v (PBSA) in U-welled microtitration plates with one well containing PBSA only as control. E. coli O157.H⁻ strain P737, previously isolated from a case of HC, was grown for 6 h at 37°C in Nutrient Broth (Oxoid; CM67), boiled for 30 min, cooled to ambient temperature and 50 μl was added to each well. Plates were shaken briefly and incubated at ambient temperature overnight. Titres were the highest dilution of serum showing complete agglutination of the organism.

**Antigen preparation for EIA**

The method used for purification of E. coli O157 LPS was modified from those of Westphal and Jann and Cryz et al. Cells from overnight cultures of E. coli O157 strain P737 on Tryptone Soya Agar (Oxoid; CM129) with glucose 1% were harvested into 50 mm Tris-HCl, pH 8.0, with 100 mm NaCl, centrifuged at 3000 g for 10 min, and washed twice in the same buffer. About 4 ml of packed cells were suspended in 35 ml of distilled water pre-heated to 65–68°C; 35 ml of phenol 90% w/v, also pre-heated to the same temperature, was added and the mixture was shaken vigorously at this temperature for 15 min. After rapid cooling on ice to 10°C, the resulting emulsion was centrifuged at 3000 g for 45 min. The upper aqueous layer was removed and stored on ice, the phenol layer was re-extracted as above with 35 ml of pre-heated distilled water, and the aqueous layer was combined with the first extraction. The aqueous phase was dialysed extensively against distilled water and concentrated to a volume of 20–25 ml by dialysis against polyethylene glycol 25% w/v. The LPS was pelleted by centrifugation at 35000 g for 3 h, resuspended in 5 ml of 25 mm Tris-HCl, pH 7.5, 300 mm NaCl, 100 mm MgSO₄ containing RNAase (Sigma) 20 μg/ml and DNAase (Sigma) 20 μg/ml. After incubation at 37°C with gentle agitation for 3 h, proteinase K (Sigma) was added to a concentration of 200 μg/ml and incubation was continued for a further 3 h. The LPS was pelleted as above, resuspended in distilled water and stored at −20°C until required. LPS samples were assayed for 2-keto-3-deoxyoctonate (KDO) as described by Karkhanis et al. and for protein by a standard Coomassie Blue dye binding method. Controls used in the assay for KDO were LPS purified from E. coli O26 (Sigma), KDO (Sigma) and sialic acid (Sigma). Purity of initial samples of LPS was further checked by performing discontinuous SDS-PAGE with a 10% resolving gel, followed by silver staining for LPS and proteins. LPS from E. coli O26 and protein wt standards (Sigma) were used as controls. Thereafter, samples of LPS with the highest achievable ratio of concentrations of KDO:protein were selected for use in the EIA.
The method used for preparation of *E. coli* O157 cells was modified from those of Zadik and Mac-Dougal et al. Cells from overnight cultures of *E. coli* O157 strain P737 on blood agar were harvested into distilled water, formaldehyde was added to c. 1% w/v, and they were incubated overnight at 4°C. Cells were washed three times by centrifuging at 3000 g for 10 min and resuspending in fresh distilled water, before being centrifuged as above and resuspended in 25 mM Tris-HCl, pH 8.0, with 100 mM NaCl containing H2O2 2% v/v. After incubation at ambient temperature for 30 min, cells were washed a further three times in distilled water and suspended in carbonate buffer, pH 9.6.

**EIA procedure**

Polystyrene 96-well EIA plates were used for the LPS EIA; 100 µl of LPS diluted in PBS was placed in each well, with PBS only used for controls. After shaking for 2 min and incubation at ambient temperature for 1 h, plates were incubated overnight at 4°C, and wells were washed five times in PBS with Tween 20 0.5% v/v (PBST). Any uncoated sites were blocked by adding 200 µl of PBST with bovine serum albumin (BSA) 1% w/v, shaking briefly, incubating at ambient temperature for 1 h and washing five times as above. Optimal coating concentration of LPS was determined empirically for each batch by chequerboard titration of dilutions of LPS against dilutions of rabbit antibody raised against *E. coli* O157, followed by development of the assay with horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulins (Dako) used at a dilution recommended by the supplier. Concentrations of LPS used for coating varied slightly from batch to batch, but were usually in the range 10–20 µg/ml.

Coating of polystyrene 96-well EIA plates (Costar) was by the same procedure as for LPS, except that cells were suspended in carbonate buffer, pH 9.6. The optimal coating density was determined empirically as for LPS, and was usually a suspension in carbonate buffer, pH 9.6, that gave an OD400 of c. 0.08.

Only sera from HC cases and those from patients in control group 1 were tested in duplicate at a dilution of 1 in 500 in the LPS-EIA. All sera were tested at a dilution of 1 in 500 in the whole-cell EIA. Sera were examined for IgG antibodies in both assays. Faecal samples were tested in duplicate at a dilution of 1 in 10 in only the whole-cell EIA for IgA antibodies. All dilutions of sera, faeces and conjugates were made in PBST with BSA 1% w/v. The following were added in sequence, plates being shaken briefly, incubated at ambient temperature for 2 h and washed five times in PBST after addition: 100 µl of the dilution of serum or faeces, with control wells having only 100 µl of PBST with BSA 1% w/v; 100 µl of HRP-conjugated rabbit anti-human IgG or IgA (Dako) at dilutions recommended by the supplier. Freshly prepared chromogenic substrate solution (3,3',5,5' tetramethylbenzidine in dimethyl sulphoxide) was added and OD450 readings were recorded as described previously. A corrected OD450 for each sample was calculated by subtracting the mean reading of the negative control wells from the mean reading of the two sample wells.

The specificity of both LPS and whole-cell EIAs was determined with rabbit antisera raised against *E. coli* of serogroups O18ac, O26, O44, O55, O86, O111, O112ac, O114, O119, O124, O125, O127, O128, O142 and O157. The assay was performed as above except...
that a dilution of 1 in 320 of rabbit antiserum was substituted for the human specimen, and detection of this was accomplished with HRP-conjugated swine anti-rabbit immunoglobulins (Dako) used at a dilution recommended by the supplier.

Statistical analysis

Whole-cell agglutinating titres and OD_{450} readings in EIAs for test and control groups of patients were compared by a Wilcoxon two-sample test. A significant difference between the assay results from the two patient groups was accepted if $p$ was $< 0.05$.

Results

Serum VT-neutralising antibodies

Three (10.7%) of 28 sera from HC cases and five (14.7%) of 34 sera from patients in control group 1 contained neutralising antibodies to VT; the cor-
responding figures for VT₁-neutralising antibodies were 25 (89.3%) and 28 (82.4%) respectively. Titres are summarised in fig. 1. The difference between titres in cases and controls was not significant for either VT₁ (p > 0.4) or VT₂ (p > 0.2).

**Whole-cell agglutinating antibodies**

These results are summarised in fig. 2. Sera from all cases and from 32 (94%) of 34 patients in control group 1 had whole-cell agglutinating antibodies. Seventeen (60.7%) cases had an antibody titre ≥ 160, whereas this was true of only nine (26.4%) of 34 control patients (p < 0.05).

**EIA for antibodies against* E. coli* O157**

The EIA to detect antibodies against LPS purified from* E. coli* O157 cross-reacted strongly with rabbit antiserum to* E. coli* O44. No cross-reactions were observed with the whole-cell EIA.

Results obtained with sera from cases and patients in control group 1 in the LPS-EIA and the whole-cell EIA are summarised in fig. 3. With the LPS-EIA,
results obtained from cases and controls were not significantly different (p > 0.1). Differences were significant with the whole-cell EIA: 16 (57%) of 28 sera from cases gave OD_{460} values > 0.15, whereas this was true of only seven (20.5%) of 34 sera from controls (p < 0.005). Therefore, the whole-cell EIA was used for further studies. Results obtained with sera from cases and patients in control groups 2 and 3, summarised in fig. 4, were also significantly different (p < 0.001). Results obtained with faecal samples from cases and patients in control group 2 are compared in fig. 5; 59 (63.4%) of 93 samples from cases produced an OD_{460} value > 0.15 in the whole-cell EIA, whereas this was true of only 10 (21.2%) of 47 samples from control patients (p < 0.001). The results of faecal IgA assays on five cases from whom further faecal samples were obtained are shown in fig. 6. Marked rises in levels of faecal IgA appeared to coincide with cessation of excretion of E. coli O157.

**Discussion**

Although most strains of VTEC causing human illness belong to serogroup O157, VT\(^+\) E. coli of other serogroups are isolated from cases of both HC and HUS. Therefore, detecting antibodies against VT might be a logical approach to the serodiagnosis of VTEC infection. In this study, the detection of serum VT-neutralising antibodies did not prove useful as there was no significant difference between the results obtained from cases and from patients in control group 1. In particular, many control patients had high levels of VT\(_2\)-neutralising antibody, but not antibody to VT\(_1\); this is difficult to explain, but similar findings have been reported by others.\(^6\) It is possible that detection of antibodies to the toxins by EIA tests may be useful, but difficulties in obtaining high yields of pure toxin would make such an assay extremely difficult and prohibitively expensive.

Others have reported detecting antibodies against E. coli O157. Detection of serum agglutinating antibodies was used with some success by Notenboom et al.\(^17\) In our study, 17 (60.7%) of 28 cases had titres of agglutinating antibody \(> 2\) and 16 (57%) of 28 cases had titres of agglutinating antibody \(> 160\), whereas this was true of only nine (26.4%) of 34 control patients; this simple, rapid and economical test may be a useful adjunct to laboratory diagnosis.

Others\(^18,19\) have reported detection of serum IgM antibodies against LPS purified from E. coli O157 as a potentially useful diagnostic test. However, tests requiring lengthy purification of LPS, followed by immunoblotting procedures with reagents labelled with radioactive isotopes, are likely to be beyond the scope of most clinical diagnostic laboratories. Despite optimising the production, purification and assay of LPS from E. coli O157, our results with this as an antigen to detect serum antibodies by EIA were disappointing. Batches of LPS prepared from E. coli O157 consistently cross-reacted with rabbit antisera against E. coli O44; various other cross-reactions of E. coli O157 LPS, usually due to identical repeating carbohydrate structures, have been reported.\(^19\) Titres of IgG antibodies to the LPS of E. coli O157 in cases and controls were not significantly different (p > 0.1); Chart et al.\(^18\) previously reported failure to separate
completely HUS patients and healthy controls on the basis of EIA detection of IgM antibodies against *E. coli* O157 LPS, but did find generally higher titres of antibody in HUS patients. It is difficult to comment on specific differences between the findings reported by Chart *et al.* and those in our present study, as we were examining specimens from cases of HC, rather than HUS.

In a study of the immune response during culture-confirmed dysentery due to *Shigella sonnei*, Winsor *et al.* found that more patients showed rising antibody titres to whole cells rather than to LPS purified from *S. sonnei*; this was true of both serum IgG and faecal IgA. We used whole cells from *E. coli* O157 in an EIA to detect both serum IgG and faecal IgA. Whole cells of *E. coli* O157 did not cross-react with antibodies to *E. coli* O44 as LPS from *E. coli* O157 did, and were quicker, easier and more economical to prepare and standardise than was LPS. When used to detect serum IgG antibodies, statistically significant differences were obtained between samples from cases and each of the control groups 1 (p < 0.005), 2 (p < 0.001) and 3 (p < 0.001). The levels of faecal IgA antibodies detected by whole-cell EIA were markedly different in cases and controls; 59 (63.4%) of 93 samples from cases had an OD value in the whole-cell EIA of > 0.15, whereas this was true of only 10 (21.2%) of 47 samples from control patients (p < 0.001).

Vaccine studies with live attenuated enteric pathogens have demonstrated the independent nature of serum and intestinal antibody responses after challenge, and have shown that intestinal IgA, although appearing sooner after initial challenge than serum IgA, is still detectable after serum levels have disappeared. In the present study, further faecal samples were obtained from five cases. The marked and rapid elevation of faecal IgA levels observed in these cases appeared to coincide with cessation of excretion of the organism, but more work is needed to confirm this observation. Indeed, the rapidity of the intestinal immune response, which has also been noted by others studying naturally acquired infection, may in part explain why detecting faecal IgA antibodies to *E. coli* O157 in HC was the most promising test in our study.

We conclude that EIA tests based on whole *E. coli* O157 cells, and in particular the detection of faecal IgA antibodies, may be useful additions to the tests previously described for the serological diagnosis of infection caused by this organism. However, considering the frequency of cross-reactions of *E. coli* O157 with other organisms and the relatively frequent occurrence of antibodies in control patients, we strongly support the view expressed by Karmali that extreme caution should be exercised in interpreting the results of serological tests in the diagnosis of infection by *E. coli* O157.

References

ANTIBODIES TO _E. COLI_ O157


