Culture negative cytotoxin positive stools in community acquired diarrhoea

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Abstract

In a significant proportion of cases of community acquired diarrhoea no recognised pathogen is detected. Verocytotoxin producing *Escherichia coli* (VTEC) are associated with haemorrhagic colitis and the haemolytic uraemic syndrome but their role in community acquired diarrhoea is not fully understood. Using a method of toxin enhancement in mixed faecal culture, 175 stools negative on culture for recognised pathogens were tested for the presence of cytotoxin and 28% were found to be positive. Nine were neutralised by anti-VT1, 29 by anti-VT2, and eight by neither. No control stool samples yielded any cytotoxin. VTEC should be considered as a causative agent in sporadic community acquired diarrhoea.

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A significant proportion of stool samples submitted for analysis from cases of community acquired diarrhoea do not contain pathogens.¹ While a proportion of these patients may have a non-infective cause, such as drugs or functional bowel disorders, for their diarrhoea, it is probable that current routine microbiological techniques do not recognise or are not sensitive enough to identify all infective causes. Indeed many organisms suspected of having a pathogenic role are not routinely cultured for.

Verocytotoxin (VT) producing *Escherichia coli* (VTEC) are known to be associated with the haemolytic uraemic syndrome^{2,3} and haemorrhagic colitis^{4,5} but their role in community acquired diarrhoea is not fully understood. Detection of VTEC in the faeces is difficult as they may be present only in very low numbers.⁵ Detection of faecal cytotoxin, however, has been proposed as an alternative method of identifying cases of haemolytic uraemic syndrome or haemorrhagic colitis,³ and may be positive even when the VT producing organism can not be isolated. Such an indirect approach to diagnosing an infective aetiology in diarrhoea has been applied to other organisms such as *Clostridium difficile* and enterotoxigenic *E coli* (ETEC). Using a method⁶ shown to significantly enhance VT production in mixed faecal culture, we have undertaken a preliminary study of the role of potentially cytotoxic organisms in community acquired diarrhoea, negative on culture for recognised pathogens.

Patients and methods

Stool samples submitted to the Newcastle General Hospital Public Health Laboratory from patients in the community aged more than 10

years who were suspected of having infective diarrhoea or a contact of a diarrhoeal case in whom infection with parasites, *Salmonella sp*, *Shigella sp*, *Campylobacter sp*, or *Clostridium difficile* had been excluded were studied. Stools from normal controls were also included. Clinical details were obtained from the request card and by postal questionnaire to the referring general practitioner to determine age, sex, length of history, contact with other cases or animals, any underlying illness, history of foreign travel, drugs and whether there had been nausea, vomiting, pain, fever, blood or mucus in the diarrhoea during the illness. The nature of the stool sample received, whether liquid, semisolid, or solid was noted.

DETECTION OF CYTOTOXIN

The stools were cultured as described previously⁶ without knowledge of their source, the nature of the case (that is suspected infective diarrhoea or contact) or the area of residence. Portions of stool were emulsified in phosphate buffered saline (PBS) 1:4. Five ml of brain-heart infusion broth were inoculated with 1 ml of stool suspension and incubated overnight at 37°C. This starter culture was then added to 35 ml of brain-heart infusion broth in a 250 ml conical flask and cultured for one hour at 37°C after which mitomycin C was added to give a final concentration of 1 µg per ml and cultured for a further 6 hours at 37°C. At the end of this period the culture was centrifuged at 3000 rpm for 30 minutes and the supernatant filtered through a 0.22 µm milipore filter and dilutions of the filtrate tested for cytotoxicity against Hela cells. A positive result was indicated by killing of at least 50% of the Hela cells (CD50) within 72 hours. In a proportion of cases the culture filtrates were also assayed for cytotoxicity against Vero cells.

NEUTRALISATION STUDIES

Antisera to the two verocytotoxins VT1 and VT2 were raised in rabbits⁷ by inoculating purified filtrates of cultures of *E coli* E3787 and E32511,⁸ recognised producers of VT1 and VT2 respectively (kindly provided by Dr B Rowe, Public Health Laboratory, Colindale).

In cases where a positive cytotoxic effect for Hela cells was detected, neutralisation by either VT1 or VT2 antisera was determined, specificity of the antisera having been checked against filtrates of pure cultures of the control strains. In the cases where neutralisation by either VT1 or VT2 or the combined antisera was negative, neutralisation by *C sordellii* antiserum was also tested.

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Results

A total of 175 culture negative samples were studied together with 25 control samples from normal adults. Altogether 162 of the samples were from suspected cases of infective diarrhoea and 13 from contacts of diarrhoeal patients. The age of these cases ranged from 12 to 90 with a mean of 40 years. Eighty one per cent of the general practitioners' postal questionnaires were returned completed. Forty six (28%) of the suspected cases showed a cytotoxic effect (cytotoxin titre >1:10 in their stool culture), nine were neutralised by anti-VT1, 29 by anti-VT2, and eight by neither. No samples were neutralised by *C sordellii* antitoxin. Five of the samples from contacts showed a cytotoxin in their stool culture, four of which could be neutralised by anti-VT2 and one by anti-VT1. Forty samples were assessed in the Vero cell assay. Of these, six samples were cytotoxin positive in the Hela cell assay and five were also positive with Vero cells. One case negative in the Hela cell assay showed a positive cytotoxic effect against Vero cells. No control samples from healthy adults yielded any cytotoxin. Forty two (36.5%) of the stools submitted were liquid, 101 (62.4%) were semi-solid, and 18 (11%) were solid. No liquid stools were submitted by contacts.

There were no significant differences between the cytotoxic and non-cytotoxic groups in terms of their age, length of history, nature of stool, symptoms of nausea, vomiting, abdominal pain, blood in the diarrhoea, history of travel, or contact. We could not identify any clustering of cases in relation to time or area of residence other than from contacts of an index case.

Discussion

The present results suggest that VT producing *E. coli*, or other organisms producing a VT like cytotoxin that were not detected by conventional means play an important role in the causation of community acquired diarrhoea.

VT producing *E. coli* are known to be associated with sporadic cases of the haemolytic uraemic syndrome and epidemics of haemorrhagic colitis. Because these organisms rapidly fall to very low numbers in faeces their detection may be difficult. DNA probes for VT1 and VT2 can detect the presence of the genes encoding cytotoxin production and enables a large number of colonies to be tested simultaneously² but this technique is not routinely available in most diagnostic laboratories and does not give information concerning whether the gene is expressed. The detection of free faecal VT has been proposed as an alternative. The culture method employed here of mixed faecal cultures with added mitomycin C as an inducing agent has been shown to enhance appreciably the amount of VT produced, even

when low numbers of organisms are present in the stool⁶ thus improving the ability to detect these organisms. This technique may also enhance the production of other toxins as has been shown for labile toxin of *E. coli*,⁹ and indeed in 5% of suspected cases an unidentified cytotoxin was detected. Using the technique of enhancing cytotoxin production we cannot firmly ascribe a pathogenic role to VTEC in the causation of these cases but it does indicate that in a significant proportion of cases an organism with the potential to produce Vero cytotoxin and other unidentified cytotoxin is present. Indeed the finding of VT in asymptomatic contacts of index cases suggests that the carriage of VTEC does not always result in disease. Salmon *et al.*¹⁰ in their study of a point source outbreak of haemorrhagic colitis found four VT positive cases who were asymptomatic.

These data indicate the potential reservoir of cytotoxin producing organisms in the community, suggest that VTEC should be considered as a possible cause of sporadic community acquired diarrhoea, and indicate the need for further epidemiological studies. Enhancement of toxin production in mixed faecal culture seems to be a practical means of improving the detection of cytotoxin producing infective agents in diarrhoeal illness.

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