Technical method

Simplified procedure for tissue culture in routine detection of cytotoxins

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Owing to increased awareness of the potential problem of infection with *Clostridium difficile*, especially after treatment with antibiotics, and more recently the association of enterotoxigenic strains of *C perfringens* with diarrhoea,¹ clinical microbiologists are increasingly being asked to look for these cytotoxins in faecal specimens. These diagnostic procedures are generally restricted to laboratories with access to facilities for tissue culture. In an attempt to overcome this restriction several alternative methods such as counterimmunoelectrophoresis enzyme linked immunoassays, and latex bead agglutination have been proposed for the detection of *C difficile*.²⁻⁴ These methods, which have recently been reviewed,⁵ have associated problems such as their specificity and sensitivity. The major limitation of all these immunotechniques is that they are monospecific and other cytotoxins, such as *C perfringens* enterotoxin, would not be detected. In addition, these methods would not facilitate the discovery of any new cytotoxins that may be associated with disease.

A simplified procedure for tissue culture that uses cover slip monolayers of cells from the African green monkey kidney (Vero) for the detection of cytotoxins in both stool specimens and laboratory isolates of *C difficile*, as well as other bacteria that produce cytotoxins, is described in this paper. The method requires no facilities for inverted microscopes or specialised experience in techniques for tissue culture and is therefore amenable to introduction into laboratories for routine microbiology.

Material and methods

SOURCE OF SPECIMENS

Stool specimens to be analysed for *C difficile* and its associated cytotoxin were received from various hospitals throughout the United Kingdom.

SOURCE OF BACTERIA

The 27 strains of *C difficile*, the strain of *Bacillus cereus*, and *C sordellii* (PMC-5) were isolated from faecal specimens taken from patients with diarrhoea. The strain of *Escherichia coli* that produces Vero toxin was provided by Dr M Barer (London School of Hygiene and Tropical Medicine), the strain of *Aeromonas hydrophila* by Dr R Gross (Central Public Health Laboratory, Colindale), and *C septicum* (CN 6769 and CN 362) by Dr P Walker (Wellcome Research Laboratories, Beckenham, Kent). The following strains of clostridia from the National Collection of Type Culture were also used: *C septicum* (NCTC 504), *C sordellii* (NCTC 8780), *C sporogenes* (NCTC 532), *C haemolyticum* (NCTC 9693), and *C oedematiens* type A (NCTC 538).

PREPARATION OF BACTERIAL FILTRATES

E coli, *A hydrophila*, and *B cereus* were grown aerobically in brain heart infusion broth (Difco Laboratories, West Molesey, Surrey, England) and incubated for three days at 37°C. The remaining bacteria were inoculated into chopped meat carbohydrate broths (Southern Group Laboratories) and incubated anaerobically for three days at 37°C. After incubation all broths were centrifuged at 8 000 g for 30 minutes at 4°C in an MSE high speed 18 centrifuge (MSE Scientific Instruments, Sussex, England). Supernatant fluids were filtered through 0.2 µm filters (Gelman Sciences Ltd, Northampton, England) and stored at 4°C before testing.

GROWTH AND MAINTENANCE OF VERO CELLS

Stock cultures of Vero cells (Flow Laboratories, Ayrshire, Scotland) were grown and maintained in 500 ml glass medical flat bottles. Growth medium consisted of medium 199 (Flow Laboratories) containing 5% fetal calf serum, 1% glutamine, and 1% of a solution of penicillin and streptomycin to yield a final concentration of 100 units/ml and 100 µg/ml, respectively. The medium was buffered to neutrality with bicarbonate. The maintenance medium was identical but with the fetal calf serum reduced to 1%.

PREPARATION OF COVER SLIPS

Glass coverslips of 13 mm diameter (AR Horwell Ltd, West Hampstead, London) were pretreated to facilitate growth of tissue culture cells by soaking in 5% Decon (BDH Chemicals, Poole, Dorset) for 15 minutes, washed thoroughly with tap and distilled water, and rinsed in diethyl ether before sterilisation with hot air at 160°C for 60 minutes.

PREPARATION AND MAINTENANCE OF COVER SLIP MONOLAYERS

The cell monolayer in the stock bottle was rinsed with about 10 ml of versene and trypsin (phosphate buffered saline - A (PBSA), 2.5% trypsin, and 1% edetic acid, in the ratio of 35:4:1) to remove fetal calf serum. The cells were then dispersed with 2-3 ml of versene and trypsin at 37°C and subsequent repeated pipetting with 10 ml of growth medium. A 1/2 dilution of the cell suspension in 0.1% trypan blue in phosphate buffered saline - A was prepared, the number of viable cells determined using a haemocytometer, and the cell suspension diluted to 2×10^5 cells/ml with growth medium. The sterile cover slips were transferred aseptically to plastic bijoux (Sterilin, Teddington, England), and 1 ml of the cell suspension was added to each. The stock bottle was reseeded with 40 ml of a suspension containing 2×10^4 cells/ml and incubated at 37°C in a carbon dioxide incubator, and after two days' incubation the growth medium was removed and replaced with an equal volume of maintenance medium and held at 33°C until required.

The growth medium from the bijoux was aspirated after incubation overnight at 37°C in a carbon dioxide incubator and replaced with an equal volume of maintenance medium containing penicillin and streptomycin at concentrations of 1000 units/ml and 1 mg/ml, respectively. The prepared bijoux can be reincubated at 37°C in a carbon dioxide incubator until required.

INOCULATION AND EXAMINATION OF TISSUE CULTURE CELLS

Suspensions (1:10) of stool were prepared in conical centrifuge tubes (Sterilin) using phosphate buffered saline - A that contained penicillin and streptomycin at 1000 units/ml and 1 mg/ml respectively as a diluent. They were centrifuged (MSE Minor, MSE Scientific Instruments) at about 2000 g for 15 minutes. Cover slip cell monolayers were inoculated with 0.1 ml of faecal supernatant fluids or sterile culture filtrates in parallel with test tube cell monolayers. The presence of cytotoxin in the chopped meat carbohydrate broth cultures of *C. difficile* was also shown in the method using cover slips by inoculation of 0.1 ml of unsterile broth suspension. A

positive control consisting of 0.1 ml of a broth filtrate from a cytotoxigenic strain of *C. difficile* and an uninoculated negative control were also included. Inoculated cell monolayers were incubated overnight at 37°C, and after aspiration of maintenance medium 2 ml of fixative (industrial methylated spirits: acetic acid, 3:1) was added to each monolayer and kept for 15 minutes at room temperature. To facilitate screening these fixed Vero cell monolayers were stained with 0.1% crystal violet in 20% industrial methylated spirits for 15 minutes, rinsed with distilled water, and mounted with buffered glycerol mountant on glass microscope slides. The cells were examined with a conventional microscope with low power objectives.

The cell monolayers from the test tube were examined unstained using an inverted microscope. Examination of cover slips and test tube monolayers was performed blind by two technicians.

NEUTRALISATION PROCEDURE

All specimens with a cytopathic effect were subjected in duplicate to a neutralisation test to confirm the presence of *C. difficile* cytotoxin. An equal volume of *C. sordellii* antitoxin (Wellcome Research Laboratories), which neutralises *C. difficile* cytotoxin, was added to one set of Vero cell monolayers, and an equal volume of normal horse serum (Wellcome Research Laboratories) was added to the other set as a control for non-specific neutralisation. Both antitoxin and horse serum were diluted 1/50 in phosphate buffered saline - A. Procedures for incubation and visualisation were performed as described above. *C. perfringens* antienterotoxin provided by Dr M Stringer (Central Public Health Laboratory, Colindale) was used to neutralise any cytopathic effects that had not been neutralised by the *C. sordellii* antitoxin. A culture filtrate of a cytotoxigenic strain of *C. difficile* and enterotoxin from a laboratory strain of *C. perfringens* that spontaneously produces this toxin were used as positive controls.

Results

DETECTION OF CYTOTOXIN IN STOOL SPECIMENS

Of 124 faecal specimens examined, 16 were positive by both methods and one was positive by the cover slip method but negative by the tube method. This specimen contained small numbers of toxigenic *C. difficile*. The 108 other specimens were negative. Ten of the 17 positive specimens contained *C. difficile* cytotoxin and seven contained *C. perfringens* enterotoxin.

DETECTION OF CYTOTOXINS PRODUCED BY BACTERIAL CULTURES

Twenty two of the 27 isolates of *C difficile* were cytotoxigenic and five non-cytotoxigenic by both methods. The same results were obtained when aliquots of the unsterilised *C difficile* cultures were applied to cell monolayers. Cytotoxins produced by *B cereus*, *A hydrophila*, *E coli*, *C difficile*, *C oedematiens*, *C haemolyticum*, and *C septicum* (CN 6769 and CN 362) were also detected, as was a cytopathic effect produced by *C sporogenes*. No cytopathic effects were noted for the non-cytotoxigenic strain of *C difficile*, *C septicum* (NCTC 504), or the strains of *C sordellii*. There was full concordance between the results obtained by the cover slip method and those of the conventional test tube method.

Discussion

The main advantages of the system for tissue culture proposed here are that Vero cells are an established cell line that multiplies rapidly, and so stock bottles do not require microscopic examination to check for confluency before preparation of cover slips; the facilities required are readily available in routine microbiology laboratories; and, apart from aseptic technique, specialised skill in techniques for tissue culture is not necessary. The incorporation of high concentrations of antibiotics into both the stool diluent and the cell maintenance medium has overcome problems of bacterial contamination and also removes the requirement for filtration of test material.

Laboratories with access to facilities for tissue culture have found tissue culture assays to be an invaluable diagnostic tool for the detection of *C difficile* cytotoxin. Although several alternative methods have been proposed, none of them is as sensitive as this assay and some of them are not as specific. The enzyme immunoassay developed by Langhan *et al* gave no false positive results but only detected toxin in five of 13 specimens with a cytotoxin titre of 10 and in 20 of 25 specimens with a titre of 100, although it detected cytotoxin in all 41 specimens with titres of 1000 or greater.³ Any system used to detect *C difficile* cytotoxin should ideally be capable of detecting low levels of cytotoxin, as no reliable correlation has yet been shown between cytotoxin titres and severity of disease, and relatively low concentrations of faecal cytotoxin can

be associated with severe disease.⁶

The most commonly used alternative to tissue culture has been counter immunoelectrophoresis. An analysis of all the recently published reports,⁵ however, indicated a tenuous correlation between counter immunoelectrophoresis and tissue culture methods. The problems associated with counter immunoelectrophoresis have also been discussed in other reports.^{7,8}

The recent observation that *C perfringens* enterotoxin is a possible cause of diarrhoea associated with antibiotics,¹ and the finding of toxic effects produced by other bacteria, would indicate that the incorporation of this modified test into the routine examination of all stool specimens in the laboratory may assist in the diagnosis of other diseases. It would also facilitate the chances of detecting new diseases of the gut associated with cytotoxin and aid in the detection and identification of toxigenic micro-organisms.

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