A SIMPLIFIED METHOD FOR DETECTING THE HEAT-LABILE ENTEROTOXIN OF *ESCHERICHIA COLI*

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SUMMARY. A tissue-culture method is described that is suitable for screening large numbers of *Escherichia coli* isolates for heat-labile enterotoxin production. The method uses readily available laboratory media for culture of the *E. coli* and does not require cell-free extracts. Antibiotics are used to increase toxin production to levels that allow cultures from several different colonies to be pooled for testing, thus minimising the number of tissue-culture monolayers required. Y1 mouse adrenal cells are used in microplate cultures. The method is simple, sensitive and economical and is suitable for use in a routine diagnostic microbiology laboratory.

INTRODUCTION

Enterotoxigenic strains of *Escherichia coli* are important causes of diarrhoea in travellers from developed to less developed countries (Rowe, 1979; Gross, Scotland and Rowe, 1979; Gross, 1983). Two different toxins may be produced: (1) the heat-labile enterotoxin (LT), which is similar in action and in biological properties to the enterotoxin of *Vibrio cholerae*, and (2) the unrelated heat-stable toxin (ST). Some strains produce LT only (LT⁺ST⁻), many produce both LT and ST (LT⁺ST⁺) and relatively few produce ST only (LT⁻ST⁺) (Rowe, 1979). Detection of ST is at present possible only by animal inoculation, though immunological methods are being developed (Honda *et al.*, 1981*b*; De Mol *et al.*, 1983). In contrast, LT may be detected by various in-vitro methods, of which tissue-culture assays are the most sensitive (Donta, Moon and Whipp, 1974; Guerrant *et al.*, 1974; Speirs, Stavric and Konowalchuk, 1977).

The aim of this study was to develop a simple, economical tissue-culture method for the detection of LT, suitable for use in a routine diagnostic microbiology laboratory.

MATERIALS AND METHODS

Control organisms. Known LT⁺ and LT⁻ strains of *E. coli* (O148.H28,LT⁺ST⁺ and O1.H7,LT⁻ST⁻) were supplied by the Division of Enteric Pathogens, Central Public Health Laboratory, Colindale, London.

Media tested for ability to support LT production. Each of the control organisms was grown for 24 h at 37°C in five broth media. These were: (1) glucose syncase medium (GS) (Finkelstein *et al.*, 1966); (2) Tryptone Soya Broth (TS) (Oxoid); (3) TS medium with lincomycin 90 µg/ml and

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the addition of polymyxin 100 µg/ml after incubation for 20 h (TSLP); (4) Brain Heart Infusion Broth (Oxoid) with casamino acids (Gibco) 2% (BH); and (5) BH medium with lincomycin and polymyxin as in TSLP (BHLP). Production of LT in each of these media by the control organisms was determined on three or more occasions by the assay method described below in tests with cell-free extracts (produced by centrifugation and membrane filtration of the supernate) and with crude cultures.

Test strains of E. coli. These were obtained by plating directly on to MacConkey agar faecal samples from 150 patients with diarrhoea who had recently returned from abroad. From each culture, four characteristic lactose-fermenting colonies were examined, individually and as a pooled culture seeded with the four colonies, for LT-production. They were grown in BHLP medium, and were examined as cell-free extracts and crude cultures.

Tissue-culture monolayers. Y1 mouse adrenal cells at a concentration of 200 000 cells/ml of growth medium were seeded in 0.1-ml amounts into sterile flat-bottomed microplates (Flow Laboratories Ltd, Irvine, Ayrshire KA12 8NB) with lids and incubated at 37°C in air containing CO₂ 6%. When cells were confluent, growth medium was removed and replaced by maintenance medium and monolayers were used for toxin assay within 5 days.

Y1 cell growth medium. This was Ham's F10 medium with bicarbonate and L-glutamine (Flow Laboratories), supplemented with fetal bovine serum 12%, penicillin 100 units/ml and streptomycin 100 µg/ml.

Y1 cell maintenance medium. This was Ham's F10 medium with bicarbonate (Flow Laboratories), supplemented with fetal bovine serum 1.5%, penicillin 100 units/ml, streptomycin 100 µg/ml and amphotericin B 40 µg/ml.

Toxin assay. The maintenance medium was removed from a fully confluent cell monolayer and replaced with 0.1 ml of *E. coli* BHLP culture (cell-free extract or crude culture; undiluted or diluted), and the plate was left at 37°C for 30 min. The culture was then removed and replaced by maintenance medium. The monolayer was incubated at 37°C for 24 h and examined for the characteristic rounding of cells caused by LT. The highest dilution of a culture that gave such an effect in more than 75% of the cells was recorded as the titre.

FIG.—Production of heat-labile enterotoxin by the LT⁺ strain of *E. coli* in five media. For key to abbreviations see *Methods* section.

RESULTS

The figure shows the toxin titres produced by the LT⁺ control organism in five media, as determined both in cell-free extracts and in crude cultures. Although bacterial growth was apparent, no detectable LT was produced in GS medium. No toxic effect was produced by any of the uninoculated media or by any cultures of the LT⁻ control strain. No differences in toxin titre were noted between cell-free extracts and crude cultures.

We isolated LT-producing *E. coli* from the faecal samples of 7 (4.7%) of the 150 patients; 6 of the 7 patients with positive samples were among the 58 who had returned from outside Europe (incidence 10.3%). The frequencies of LT⁺ colonies among the LT-producing strains were: 4 out of 4, 3 strains; 2 out of 4, 3 strains; 1 out of 4, 1 strain. In all cases the pooled cultures gave a positive result. In all cases identical results were obtained from cell-free extracts and from crude cultures.

Confirmation of positive findings. All isolates that caused toxic effects were checked for identity with the API 10S system (API Laboratory Products Ltd, Basingstoke, Hampshire RG22 6HY). Each culture was then retested in parallel with a portion of the same culture heated to 100°C for 5 min and cooled to 37°C before use, and with another portion that had been neutralised by adding 0.02 ml of a *V. cholerae* antitoxin preparation (kindly supplied by Dr R. Thompson, Wellcome Biotechnology Ltd, Langley Court, Beckenham, Kent BR3 3BS) to 0.1 ml of culture and incubating the mixture at 37°C for 5 min.

The table shows the API 10S profiles and the serogroups of the seven LT-producing strains, and the countries from which the patients had come.

TABLE
Characteristics of LT-positive isolates of E. coli

Isolate no.	API 10S profile	Serogroup	Presumed country of origin
1	7005	O 25	Morocco
2	7305	O 9	Brazil
3	7005	O 25	Egypt
4	7204	O 9	Russia
5	7005	O 6	Tunisia
6	7305	O 6	Spain
7	7005	O 6	Bulgaria

DISCUSSION

Despite the importance of LT-producing strains of *E. coli* as a cause of traveller's diarrhoea (Gross, 1983; Gross *et al.*, 1979; Rowe, 1979) their recognition can be accomplished in only a few diagnostic microbiology laboratories. Possible reasons for this include the drawbacks of currently recommended methods. Animal methods are expensive, time-consuming and difficult to reproduce accurately. Their continued use for the detection of LT would be difficult to justify.

LT is antigenic and various immunological methods for its detection have been developed (Evans and Evans, 1977; Serafim *et al.*, 1979; Rönnerberg and Wadström,

1983). One of the more practicable and more widely appraised is the Biken test (Honda *et al.*, 1981a). Despite the relative ease of these tests they suffer from the serious limitation that all require a specific high-titre antitoxin to highly purified *E. coli* LT. No such antitoxin is available commercially at present, and its production would almost certainly be beyond the scope of many routine diagnostic laboratories.

Tissue-culture methods are simple, can be made economical, and are more sensitive than either animal or immunological methods (Guerrant *et al.*, 1974; Speirs *et al.*, 1977). They do not require the high-titre specific reagents required by the latter. Earlier work on LT demanded the preparation of cell-free extracts of *E. coli* grown in complex chemically defined medium (Finkelstein *et al.*, 1966), a procedure which has since been accepted as standard. Sack *et al.* (1971) found that *E. coli* produced low levels of LT in glucose syncase medium and found it necessary to concentrate the toxin before testing by a lengthy procedure involving high-speed centrifugation, membrane filtration, dialysis and ammonium sulphate precipitation. Although such procedures are necessary if animal inoculation is to be undertaken or purified toxin is to be produced, we have demonstrated that they are unnecessary for routine screening of clinical isolates by a tissue-culture method. Treatment of cultures with polymyxin and inclusion of streptomycin in the cell maintenance medium virtually eliminated problems with contamination which might be expected in work with crude cultures of *E. coli*.

A drawback with any tissue-culture work is cost. Two ways in which this can be reduced considerably are (1) by using fewer cell monolayers, and (2) by using smaller cell monolayers. A practicable means of achieving the first is to screen pooled cultures from several colonies. For this to be a reliable and effective procedure, the amount of toxin produced needs to be maximised. Lincomycin, an inhibitor of protein synthesis, optimally increases production of LT by *E. coli* if included in culture media at a concentration of 90 µg/ml (Levner, Wiener and Rubin, 1977). The subsequent treatment of cultures with polymyxin 100 µg/ml further increases toxin titre (Honda *et al.*, 1981a). Our experiments with the LT⁺ strain showed that when these two antibiotics were used with brain heart infusion broth, marked increases in toxin titre were obtained (see the figure). The most practicable way of reducing the cost is to use tissue-culture monolayers prepared in microplates. Although there are problems with such monolayers in virological use, their convenience and ease of handling make them suitable for toxin assays of this type.

The cytopathic effect produced by LT in Y1 mouse adrenal cells is easily seen, but specific confirmation that this effect is due to LT is desirable. Previous workers (Donta *et al.*, 1974; Sack and Sack, 1975) suggested heat treatment as a sole confirmatory measure. We would suggest that threefold confirmation is required: biochemical identification of the isolate; demonstration of the heat-lability of the toxin; and demonstration of neutralisation of the cytopathic effect by *V. cholerae* antitoxin. As we have shown, such reliable identification of LT-producing *E. coli* is readily achievable in a routine diagnostic laboratory.

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