Comparison of five assays for the heat-labile enterotoxin of *Escherichia coli*

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Summary. Enzyme-linked immunosorbent assay (ELISA), DNA–DNA hybridisation, Vero cell assay, the Biken test and a new membrane-filter method were compared in the detection of heat-labile enterotoxin (LT) of *Escherichia coli*. Six subcultures of each of 50 strains of *E. coli* from the Biken collection were evaluated “blind” in the laboratory. The combined results of the most reproducible tests (ELISA and DNA–DNA hybridisation) were used to calculate the sensitivity and specificity of the other assays. The Vero-cell assay had a high sensitivity (98%) but a lower specificity (91%). The Biken and membrane-filter assays had sensitivities of 58–71% and 77–84% respectively, depending on the type of antiserum used. Only one false positive result was obtained with the Biken test; specificity of the membrane-filter assay was 94–95%. The membrane-filter assay, with anti-cholera toxin, is specific and reasonably sensitive. It has particular advantages over DNA–DNA hybridisation and the Biken test, and it may prove suitable for screening large numbers of *E. coli* isolates in epidemiological studies in developing countries.

Introduction

Enterotoxigenic strains of *Escherichia coli* (ETEC) are a major cause of acute diarrhoea in children in developing countries (Ryder *et al.*, 1976; Black *et al.*, 1981), in travellers (Sack *et al.*, 1977; Guerrant *et al.*, 1980) and in sporadic outbreaks amongst children and adults in developed countries (Sack *et al.*, 1975; Rowe *et al.*, 1978; Rosenberg *et al.*, 1978). ETEC produce a non-invasive, secretory diarrhoea by the elaboration of heat-stable enterotoxin (ST) or heat-labile enterotoxin (LT), or both. The LT is a high-mol.-wt immunogenic polypeptide (Clements *et al.*, 1980) and closely resembles the cholera toxin (CT) in structure and activity. It binds to the monosialoganglioside (GM1) residues of the intestinal epithelial cells, causing fluid secretion by stimulating adenylyl cyclase activity (Evans *et al.*, 1972; Gill and Richardson, 1980).

Enterotoxin activity was originally detected by fluid accumulation in ligated ileal loops of rabbits (De and Chatterje, 1953; De *et al.*, 1956). A more convenient way of detecting LT is the use of tissue cultures of Vero cells (Speirs *et al.*, 1977) or Y-1 adrenal cells (Sack and Sack, 1975). The immuno-

genic nature of LT allows its detection in immunological assays; enzyme-linked immunosorbent assay (ELISA) (Sack *et al.*, 1980), agglutination tests (Ronnberg and Wadstrom, 1983), and agar diffusion assays, such as the Biken test (Honda *et al.*, 1981) are used. Additionally, the direct detection by DNA–DNA hybridisation of the genes coding for LT production has been described (Moseley *et al.*, 1980).

These assays detect LT-producing ETEC in different ways and each has advantages and disadvantages in specific applications. We report a laboratory evaluation of the sensitivity, specificity and reproducibility of ELISA, DNA–DNA hybridisation, Vero cell assay, the Biken test and a new membrane-filter method that we have developed.

Materials and methods

Bacterial strains

Fifty *E. coli* strains from the Biken collection were donated by Dr B. Rowe (Division of Enteric Pathogens, Central Public Health Laboratory, Colindale). The collection consists of *E. coli* strains isolated at Osaka Airport Quarantine Station from patients with travellers' diarrhoea (Sutton *et al.*, 1985). Some of these strains produce LT. Each strain was subcultured on to five slopes

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of Nutrient Agar (Oxoid) to produce 50 sets of six cultures (parent strain plus five subcultures). Numbers were allocated in random fashion to the 300 cultures and each was tested twice by the five assay methods. If there was disagreement between the two results, a third test was performed and the majority result accepted. The laboratory workers were unaware of the identity of the subcultures with which they worked. *E. coli* strain 487475 (International Centre for Diarrhoeal Disease Research, Bangladesh) of clinical origin was used as the LT-positive control and *E. coli* strain WF5 (University of Surrey) of environmental origin was used as the LT-negative control. All strains were subcultured and stored as described by Feltham *et al.* (1978).

Preparation of culture supernates

Cell-free culture supernates were prepared according to WHO recommendations (World Health Organization, 1983). Each strain was cultured in 5 ml of Tryptone Soy Broth (Oxoid) containing yeast extract 1.2%. Tubes of broth were incubated in a sloped position and with loose caps to provide maximum aeration. After incubation for 48 h at 37°C, the cultures were centrifuged at 12 000*g* for 15 min and the supernates were removed for use in the Vero cell assay and ELISA. Commercially-produced cholera toxin (Sigma Chemical Co. Ltd, Poole, Dorset) was used, at concentrations of 1–1000 ng/ml, as positive controls in both these assays.

Antisera

The following antisera were used: unpurified anti-cholera toxin (anti-CT, University of Surrey; titre 256 000); unpurified anti-LT (Virion International Distribution Ltd, Gewerbestrasse 10, CH-6330 Cham, Switzerland; titre 32 000); affinity purified anti-LT (Meguro Institute, 7–29 Masumi-cho, Ikeda-shi, Osaka 563, Japan; titre 3200); affinity purified anti-LT (World Health Organization, 1211 Geneva 27, Switzerland; titre 600). Titres were determined by ELISA titration against CT at a concentration of 1000 ng/ml of phosphate-buffered saline.

Vero cell assay

The assay method of Speirs *et al.* (1977), as modified by Giugliano *et al.* (1982), was used. The Vero cells were grown in cell medium 199 (Gibco Ltd, Uxbridge, Middlesex) supplemented with fetal calf serum 1% and a mixture of penicillin 100 IU/ml and streptomycin 100 µg/ml (Gibco) in 96-well Falcon 3072 tissue-culture plates. The cells were then exposed for 18 h to 50 µl of culture supernate. A positive response was recorded when 50% or more of the cells showed morphological changes attributable to the cytopathic effect of the toxin.

ELISA

The ELISA method of Sack *et al.* (1980), modified by Miller *et al.* (1986), was used. Culture supernates (100 µl)

were added to GM1 adsorbed to 96-well Linbro micro-titration plates (Flow Laboratories, Rickmansworth, Hertfordshire). Rabbit anti-LT (100 µl) (WHO, Switzerland), peroxidase labelled swine anti-rabbit immunoglobulin (100 µl) (Dako-Patt, Dako Ltd, High Wycombe, Buckinghamshire) and orthophenylene diamine dihydrochloride (100 µl) (Sigma) were then added sequentially and the reaction was stopped after 20 min by the addition of 2.5M H₂SO₄ (20 µl). The plates were read spectrophotometrically at 492 nm by a Flow Multiskan ELISA reader. From the distribution of optical density values obtained in the wells containing supernates prepared from control negative strain WF5, a negative:positive cut-off point of 0.26 OD unit was calculated, corresponding to a specificity of 97.5%.

DNA–DNA hybridisation assay

Hybridisations were performed with a DNA probe derived from the LT recombinant plasmid pAT153H6. A 0.8-kilobase-pair fragment, comprising the LT sequences, was excised from the purified plasmid DNA by digestion with the restriction endonuclease *Hind* III and separated from the vector by agarose gel electrophoresis. The fragment was then electro-eluted from the gel, purified by DEAE-sephacel chromatography and radio-labelled *in vitro* with ³²P by nick translation (Rigby *et al.*, 1977) to a specific activity of 6 × 10⁷ cpm/µg. Colony hybridisations were performed as described by Grunstein and Hogness (1975) and modified by Moseley *et al.* (1980). Subcultures were spot-inoculated on to nutrient agar, grown at 37°C and transferred to nitrocellulose filters (90 mm diameter BA85; Schleicher and Schuell, Anderman, Kingston, Surrey). Bacterial colonies were lysed with 0.1M HCl and the released DNA was denatured with NaOH. After neutralisation, the single stranded DNA was immobilised on the filters by baking at 80°C *in vacuo* for 1–2 h. Non-specific binding of the probe was blocked by prehybridising the filters with carrier calf thymus DNA. After hybridisation with the radiolabelled LT DNA, the filters were washed and bound probe was detected by autoradiography.

Biken assay

The Biken test was performed as described by Honda *et al.* (1981) except that affinity purified anti-LT, unpurified anti-LT and unpurified anti-CT were used. Two subcultures and two control strains were spot-inoculated on to a Biken agar plate containing the following constituents (/L): casamino acids (Difco) 20 g; yeast extract (Difco) 10 g; NaCl (BDH Chemicals Ltd, Dagenham, Essex) 2.5 g; K₂HPO₄ (BDH) 15 g; glucose (BDH) 5 g; trace salts solution (BDH; MgSO₄ 5%, CoCl₂.6H₂O 2%, FeCl₃ 0.5%) 0.5 ml; purified agar (Oxoid) 15 g. The final pH was adjusted to 7.5. After autoclaving for 15 min at 121°C the medium was cooled to 50°C and lincomycin (Sigma) was added to achieve a final concentration of 90 µg/ml. After incubation at 37°C for 48 h, a well was punched in the agar and a disc

containing polymyxin B 500 IU (Mast Laboratories Ltd, Merseyside) was placed on each colony. The plates were incubated at 37°C for a further 6 h after which 20 µl of antiserum was added to the well. The plates were incubated at 37°C and read for lines of precipitation occurring between the colony and well after 24 h and 48 h.

Membrane-filter assay

Membrane filtration was performed as described by Vadivelu *et al.* (1986). Subcultures were spot-inoculated on to 49 mm cellulose acetate membrane filters (0.45 µm pore size; Gelman Sciences Ltd, Northampton). Four subcultures and two control strains were used per membrane. The membranes were placed on pads soaked in membrane lauryl sulphate broth, containing the following constituents (/L): Tryptone Soy Broth (Oxoid) 30 g; yeast extract (Difco) 12 g; sodium lauryl sulphate (BDH) 1 g; lactose (BDH) 4 g; cresol red (BDH) 0.2 g. The final pH was adjusted to 8.5. After overnight incubation at 44°C, the membranes were transferred on to agar medium containing antiserum (unpurified anti-LT, 2.7%, or unpurified anti-CT, 2%), and incubated at 44°C for a further 48 h. The formulation of the agar medium was as described for Biken agar (Honda *et al.*, 1981) with antiserum added at the appropriate dilution. The optimal dilution of antiserum was determined by testing dilutions of the antiserum in agar against reference ETEC strains known to produce different amounts of enterotoxin as previously determined by ELISA. After incubation at 44°C for 48 h, the membranes were removed and the underlying agar was observed for the presence of zones of precipitation.

Results

Reproducibility

Table I shows the number of sets of six subcultures, out of a total of 50 sets, in which there was perfect agreement in classification as positive or negative for all subcultures derived from the same parent strain. ELISA was most reproducible (perfect agreement for 45 of 50 sets), followed by DNA-DNA hybridisation assay (42 of 50). Inter-assay agreement was also very good for these two tests: 276 of the 300 cultures produced the same result with both tests. The other assays were less reproducible, although the Biken test was better than the Vero-cell or membrane-filter assays.

Sensitivity and specificity

Because it was not known which of the strains produced LT, we used the combined results of the ELISA and DNA-DNA hybridisation assay to provide the "correct" classification for each strain.

Table I. Reproducibility of methods for assay of LT

Assay	Number of sets of strains for which all six cultures gave the same result		
	Positive	Negative	Overall (n=50)
ELISA	22	23	45
DNA-DNA hybridisation	21	21	42
Vero cell	21	10	31
Membrane filter (anti-CT unpurified)	12	18	30
Membrane filter (anti-LT unpurified)	8	16	24
Biken (anti-CT unpurified)	8	28	36
Biken (anti-LT unpurified)	13	27	40
Biken (anti-LT affinity-purified)	11	26	37

There was a total of 12 results for each set of subcultures: six for each of the two assays. A set of six subcultures was defined as "positive" or "negative" if 11 or 12 of these results agreed. This gave rise to 21 positive, 23 negative and six equivocal sets of subcultures. Table II shows the percentage positive results obtained by the Vero-cell, Biken and membrane-filter assays for subcultures classified in this way.

Vero-cell assay showed a very high sensitivity (98.4%) but a specificity of 91.3%. Conversely, the sensitivity of the Biken assay was low—57.9% for anti-CT, 66.7% for affinity-purified anti-LT, 71.4% for unpurified anti-LT—but there was only one false positive result. The sensitivity of the Biken test with anti-CT was significantly lower than with either type of anti-LT ($p < 0.01$ in both cases, McNemar's test). The difference in sensitivity between affinity-purified and unpurified anti-LT was not statistically significant. Table III shows the concordance between results obtained in the Biken test with different antisera. For 262 of the 300 subcultures complete concordance (all tests positive or all negative) was obtained.

The sensitivity and specificity of the membrane-filter assay was intermediate between those of the Vero-cell assay and the Biken test. The sensitivities were 84.1% and 77.0% for anti-CT and anti-LT, respectively; only seven false positives were obtained with anti-CT (specificity 94.9%) and nine false positives with anti-LT (specificity 93.5%). The difference between the sensitivity values for the

Table II. Sensitivity and specificity of the Vero-cell, membrane-filter and Biken assays; subcultures were assigned to "negative", "positive" or "equivocal" categories on the basis of ELISA and DNA-DNA hybridisation results

Assay	Percentage of subcultures in each category that gave positive results in the assay indicated			
	Negative (n = 138)	Positive (n = 126)	Equivocal (n = 36)	Total (n = 300)
Vero cell	8.7	98.4	58.3	52.3
Membrane filter (anti-CT unpurified)	5.1	84.1	61.1	45.0
Membrane filter (anti-LT unpurified)	6.5	77.0	52.8	41.7
Biken (anti-CT unpurified)	0	57.9	41.7	29.3
Biken (anti-LT unpurified)	0.7	71.4	33.3	34.3
Biken (anti-LT affinity-purified)	0	66.7	36.1	32.3

two antisera borders on statistical significance ($p=0.07$). Results obtained with anti-CT and anti-LT agreed for 268 of the 300 subcultures (table IV).

Discussion

To define the sensitivity and specificity of an assay, the correct result for each sample must be known. In the present study, the results of the ELISA and DNA-DNA hybridisation assays were combined in defining the correct result; this is justified by the accuracy and reliability of these assays and by the high levels of reproducibility and agreement achieved in this study. By defining strains for which ELISA and DNA-DNA hybridisation assays disagreed as "equivocal", the possibility of misclassifying strains that code for but do not produce LT (positive by DNA-DNA hybridisation assay, negative by ELISA) and strains that express LT-like molecules that cross-react with antisera to LT (positive by ELISA, negative by DNA-DNA hybridisation assay) was excluded.

Table III. Comparison of different antisera in the Biken test

Result obtained with			Number of sub-cultures
anti-CT unpurified	anti-LT unpurified	anti-LT affinity-purified	
—	—	—	185
—	—	+	5
—	+	—	13
—	+	+	9
+	—	—	1
+	—	+	6
+	+	—	4
+	+	+	77

The terms sensitivity and specificity are used guardedly. First, these are not fixed values; the sensitivity of an assay is related to the degree of positivity of the sample under test, and the specificity depends upon the presence, or otherwise, of possible cross-reacting molecules. It is valid to compare the sensitivities, or specificities, of two tests only when they have been computed from the same set of samples as we have done. Second, it is possible, but unlikely, that ELISA and the DNA-DNA hybridisation assay might both misclassify a subculture. Third, the samples defined as equivocal in table II may represent bacteria that produce LT in small amounts or that produce biochemically-related products (Yolken *et al.*, 1977). If the correct result is "positive" the sensitivity of a test may be lower than estimated here; specificity may be reduced if the correct result is "negative". In table II, the sensitivity of each assay is the figure shown in the "positive" column; the specificity is obtained by subtracting the figure in the "negative" column from 100.

The Vero-cell assay was sensitive, but not very specific. The low specificity can be explained by the cytopathic effects of bacterial products other

Table IV. Comparison of different antisera in the membrane-filter assay

Results obtained with		
anti-CT unpurified	anti-LT unpurified	Number of sub-cultures
—	—	154
—	+	11
+	—	21
+	+	114

than LT (Giugliano *et al.*, 1982), so that it is difficult to obtain reproducibly negative results. The specificity of the assay can be increased by adding a pre-determined dilution of antitoxin to a duplicate series of tests.

The membrane-filter and Biken assays were statistically less sensitive than the other assays. In gel assays precipitation complexes dissolve in the presence of excess antigen or antibody and it is thus difficult to define an optimal concentration of antitoxin that will detect the different levels of enterotoxin produced by different strains of ETEC. In the Biken test, precipitation lines were occasionally hidden under the bacterial colony. Moreover, because these assays rely on release of sufficient enterotoxin, a strain that grows poorly, or one that produces a small amount of enterotoxin may be incorrectly classified as negative. Sensitivity will also be reduced if the agar medium impedes the diffusion of toxin and antitoxin.

The membrane-filter assay proved substantially more sensitive than the Biken test, regardless of antiserum used (table II). The reverse might have been expected because the Biken test can detect both intracellular and extracellular LT by the use of polymyxin B, whereas the membrane-filter assay detects mainly extracellular LT. The specificity of the Biken assay was good; only one "negative" subculture was misclassified. The membrane-filter assay misclassified 5–6% of "negative" subcultures (table II).

The reproducibility of the Biken test was also better than that of the membrane-filter assay (table I). More sets of subcultures showed perfect negative agreement, whereas the number of sets showing perfect positive agreement was similar for the two assays. Further analysis of the data identifies one class of "positive" sets that both assays could identify correctly and consistently, and a second class that they could not. In the second class, the membrane filter assay identified more positive strains than the Biken test, although it seldom achieved consistency over the six related subcultures.

The use of different antisera in the gel diffusion assays gave rise to discordant results with a few subcultures: 32 of 300 in the case of the membrane-

filter assay (table IV), and 38 of 300 in the case of the Biken test (table III). Most discordances occurred with "positive" subcultures and resulted in different sensitivities for tests in which the different antisera were used (table II). Use of anti-CT rather than anti-LT improved the sensitivity of the membrane-filter assay, but reduced the sensitivity of the Biken test. This may be due to the different concentrations at which the antisera were used. Anti-LT was used in the Biken test at the concentration recommended by the commercial suppliers, whereas we determined the optimal concentrations of antisera for all the other assays.

The average number of *E. coli* isolates that can be screened per ml of antiserum varies between assays: 720 isolates with controls can be screened by ELISA per ml of antiserum used at a dilution of 1 in 100 (more with high titre antisera); about 200 isolates can be screened by the Biken test, depending on the number of controls used; between 370 and 500 isolates, including controls, can be tested by the membrane-filter assay, depending on whether the antiserum in the agar is used at a concentration of 2.7% or 2.0%.

In the present study, in which each test was repeated up to three times and the majority result taken as the correct one, the membrane-filter assay with anti-CT had a specificity of 95% and a sensitivity of 84%. A clinical laboratory would usually perform the assay only once and in these circumstances the specificity would be reduced to 93% and the sensitivity to 79% (calculations not shown). However, this sensitivity is still substantially better than those achieved by the Biken test, even with three repetitions (table II).

The membrane-filter assay with anti-CT appears to be worth further development and field testing. It may be especially suitable for laboratories with limited facilities that undertake investigations of ETEC transmission and environmental risk factors.

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