Comparison of methods in the detection of enterotoxigenic *escherichia coli* in a Malaysian laboratory

* Y.M. Cheong, MBBS, MSc, MRCPath
Head, Bacteriology Division

* M. Jegathesan, MBBS, FRCPath, FCAP, FRCPA
Director

** A. Ansary, BSc, PhD
Lecturer

** M. Othman, BSc
Research Assistant

* *Institute for Medical Research, Kuala Lumpur*
** *University of Malaya, Kuala Lumpur*

Summary

The prevalence of Enterotoxigenic *Escherichia coli* (ETEC) in 433 stool samples from diarrhoeal cases of all ages was studied using two commercially available test kits for the detection of heat labile toxin (LT) and the infant mouse assay for the heat stable toxin (ST). 16 samples (3.7%) were positive for ETEC, of which nine were producing ST alone, six LT alone and only one was producing both LT and ST. Although the percentage of isolation rate was low, its occurrence was almost as common as the *Shigella spp* and *Salmonella spp* in the same study. Of the two test kits examined, the Phadebact ETEC-LT Test 50 (Pharmacia Diagnostics, Uppsala, Sweden) was found to be more suitable for use in a routine diagnostic laboratory. Ten out of 12 (83%) of the strains tested were resistant to one or more antibiotics.

Key words: Enterotoxigenic *Escherichia coli,* Comparison of methods.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) has been implicated as a major cause of infant diarrhoea in less developed countries[1] as well as the agent most frequently responsible for traveller's diarrhoea.[2] This group of organisms have come into prominence in the early 1970s due to the work carried out by Gorbach and Sack.[3,4] It causes diarrhoea by elaborating one or two toxins, the heat-labile (LT) and the heat-stable (ST) enterotoxins. Identification of such strains is by the detection of these toxins.

In Malaysia, enteropathogenic *E.coli* (EPEC), the first class of *E.coli* described to be implicated in diarrhoea outbreaks[5] are now routinely examined for in children after a study conducted by Jegathesan and co-workers showed that 9% of the diarrhoeal cases in children under ten years of age was due to EPECs.[6] However, for ETEC, no data on its prevalence is available yet in this country. The reason is probably due to the non-availability of a simple and suitable commercial test kit for its detection. The classical methods of using rabbit ileal loop[7] or tissue culture techniques for LT[8,9] and the infant mouse assay for ST[10] are not suitable for a routine diagnostic or public health laboratory. Although immunological methods for LT detection have been described like the radioimmunoassay (RIA)[11] and enzyme linked immunosorbent assay (ELISA)[12], the reagents are not easily available. Another simple immunological test, the Biken

Test[13] requires at least four days and large amount of antisera to perform the test, and therefore is not practical for diagnostic purposes.

Recently, two immunoassay tests have been made available commercially here to detect LT. They are the Phadebact ETEC – LT Test 50 (Pharmacia Diagnostics, Uppsala, Sweden) and the Vibrio Cholerae Enterotoxin – Escherichia coli Heat Labile Enterotoxin Detection Kit (VET – RPLA) (Denka Seiken Co., Ltd. Tokyo, Japan). Both are agglutination tests, the first one is a co-agglutination test using protein A rich staphylococci coated with antibodies against highly purified LT enterotoxin raised in rabbits while the second is a reverse passive latex agglutination test using latex particles sensitized with purified antibodies against *Vibrio cholerae* enterotoxin. With the availability of these two test kits we decided to study the prevalence of ETEC amongst our diarrhoeal cases and to see whether they contribute to a sufficiently high percentage of bacterial pathogens to warrant routine testing. At the same time we also evaluated the suitability of the test for routine usage.

Material and Methods

All stool specimens taken from diarrhoeal cases that were sent to our laboratory for culture, from mid July 1986 to mid May 1987 were examined for the common bacterial enteropathogens as well as *E.coli.* Most of the specimens came in Cary Blair Transport Media and were from a few surrounding district hospitals and private practitioners' clinics. Cultures for *Salmonella spp, Shigella spp, Vibrio spp, Aeromonas spp, Yersinia enterocolitica* and *Campylobacter jejuni,* follow standard procedures of direct plating onto Mac Conkey, Deoxycholate Citrate, Xylose-Lysine Deaminase, Monsurs and Blaser Wang Agar Media plus enrichment in alkaline peptone water and selenite medium.

For the examination of *E.coli,* at least ten lactose fermenting colonies from the Mac Conkey Agar plate were picked onto Triple Sugars Iron Agar and subequently identified by the typical biochemical reaction of Indole positive, Motility positive, Voges-Proskauer negative and Citrate negative tests (I.e IMViC). These strains were then pooled onto one Nutrient Agar slope per patient and tested for EPEC and ETEC strains.

EPEC strains: These were first screened by the slide agglutination test with the *E.coli* polyvalent two, three and four antisera (Wellcome Diagnostics) and then confirmed with the specific antisera by the tube agglutination test.

ETEC strains: For the detection of ETEC-LT strains the VET-RPLA and Phadebact ETEC-LT test kits were used, while the ETEC-ST strains were tested for by the infant mouse assay technique.[10]

VET-RPLA Test: This method utilizes latex particles sensitised with purified antibodies against *Vibrio cholerae.* The *E.coli* strains were cultured onto CAYE medium with 90 ug/ml of Lincomycin at 37°C for 18–20 hours for toxin production. Then they were suspended in 1 ml of physiological saline with 10,000 units/ml of polymyxin B. The suspension was shaken at 37°C for 30 minutes and centrifuged at 3000 rpm for 20 minutes. The supernatant was used as the test specimen. For screening purposes, the supernatant were tested at neat and 1:2 dilution and only the positive ones were repeated at the various dilutions. The agglutination test was done according to the instructions in the pamphlet using V-bottommed well microtiter plates. The results were read after storing the plate at room temperature for 20–24 hours.

Phadebact ETEC-LT Test: This method is based on the coagglutination technique, whereby antibodies raised in rabbits against highly purified LT enterotoxin is coupled to the Protein A layer of non-viable staphylococci. A loopful of the *E.coli* culture grown overnight on ox blood agar was suspended in a small tube in 100 ul of extraction solution provided by the manufacturer. Then it was incubated at 37°C for 30 minutes in a water bath. The suspension was centrifuged at 2000 g for five minutes and a drop of the supernatant was used for the agglutination test on the card provided. The test was done according to the manufacturers' instructions and any coagglutination reaction that occurred within one minute was taken as positive.

Infant Mouse Assay: Infant mice of one to four days old were used. 0.1 ml of the supernatant prepared as for the VET-RPLA test was used to inject through the body wall directly into the milk-filled stomach with a 27G needle and tuberculin syringe. Prior to the intragastric injection, a drop of 2% Evan Blue dye was added to each inoculum. Three to four mice were used for each sample. Only successful inoculations were considered. After inoculation, the mice were kept for four hours at room temperature and then killed with chloroform. The abdomen was dissected and the small intestine was examined for distention and removed with the forcep to be weighed immediately. The intestines from four mice were weighed together and the rate of the gut weight to remaining body was calculated. Ratios of less than 0.070 were considered negative. Those in the range of 0.070–0.080 were questionably positive. Those above 0.080 were considered positive as the known positive control strain used in the tests gave a range of ratios between 0.081 to 0.1040. Ratios ranging from 0.075 to 0.080 were repeated.

Antibiotic Susceptibility Tests: The disc diffusion method of Kirby Bauer was used to determine the antibiogram of the six LT producing strains, five ST producing strains and one LT and ST producing strain. 22 different antibiotics were used. They were amikacin, ampicillin, aztreonam, ceftazidime, cephalothin, ceftriazone, cefoperazone, cephalexin, cefotaxime, chloramphenicol, carbenicillin, gentamicin, kanamycin, nalidixic acid, netilmicin, nitrofurantoin, norfloxacin, cotrimoxazole, streptomycin, tetracycline, trimethoprim and rifampicin.

Results

During the study period, a total of 433 stool samples from diarrhoeal cases were examined. About half of these cases were from the age group below ten years (Table 1). *E.coli* was isolated from 204 cases, of which seven (i.e. 1.6% of diarrhoeal cases) were EPECs and 16 (3.7%) were ETECs. Of the 16 ETECs nine were producing ST alone, six LT alone and only one was producing both LT and ST.

The number and percentage of the bacterial enteropathogen isolated in this study are shown in Table 2.

Both the VET-RPLA and Phadebact ETEC-LT tests used in detecting the LT were comparable in sensitivity. Out of the 204 *E.coli* strains tested, seven were positive and the rest negative by both methods. Details of these seven cases are in Table 3. The Phadebact ETEC-LT method was found to be simpler and faster to perform whereas the VET-RPLA required the culture to be grown on a special media overnight before extracting the toxin and the reading could only be taken after overnight incubation. The result was also easier to read with the Phadebact ETEC-LT method, the coagglutination usually occurred within one minute of rocking the card. It was also more practical to use when only a few strains were tested at a time.

Table 1
Age distribution of diarrhoeal cases and ETEC isolates

Age (years)	Number (percentage)	
	Diarrhoeal cases	ETEC isolates
1 – 10	229 (52.9)	12 (75)
11 – 20	34 (7.8)	0 (0)
21 – 30	48 (11.1)	0 (0)
31 – 40	37 (8.5)	2 (12.5)
41 – 50	27 (6.2)	1 (6.2)
51 – 60	20 (4.6)	1 (6.2)
⩾ 60	32 (7.4)	0 (0)
Unknown	6 (1.4)	0 (0)
Total	433	16 (3.7)

Table 2
Bacterial Enteropathogens isolated from 433 diarrhoeal cases

Organisms	Number	Percentage
Shigella spp	19	4.4
Salmonella spp	17	3.9
ETEC	16	3.7
EPEC	7	1.6
Aeromonas spp	5	1.2
Campylobacter jejuni	2	0.5
Vibrio cholerae	1	0.2
Total	67	15.5

In the detection of the ST, initial results showed five strongly positive cases which gave a bioassay ratio of ⩾ 0.080. Those that gave a bioassay ratio between 0.075 to 0.080 or the weight of the intestine was less than 0.2 gm were repeated. There were 50 such strains. Five more strains were eventually found to be positive on the second repeat.

The antibiogram of the isolates are listed in Table 4. Two out of the 12 strains tested were sensitive to all the 22 antibiotics tested. Three showed resistance to only one antibiotic. The rest were multiply resistant to the antibiotics.

Table 3
Results of the seven ETEC-LT strains by the VET-REPLA and Phadebact ETEC-LT tests

Strain no.	Results	
	VET-RPLA (titre value)	Phadebact ETEC-LT
75	1 : 128	+ ve
76	1 : 4	+ ve
92	1 : 16	+ ve
171	1 : 4	+ ve
243	1 : 8	+ ve
258	1 : 8	+ ve
266	1 : 4	+ ve

Table 4
Antibiogram of 12 ETEC strains

	Strains	Resistance Phenotype
	75	Am, C
	76	Am, C
	*92	–
LT	171	Am, Car, C, Te, Sm, Cf
	243	Am
	258	Am
	125	Te, C
	*153	–
ST	176	Am, Car, Sm
	179	Am, C, Te, Sm, Car, Km, Sxt
	278	Am, Te, Car, CF, Sm
LT/ST	266	Sm

Am – Ampicillin
Cf – Cephalothin
Car – Carbenicillin
C – Chloramphenicol
Te – Tetracycline
Sm – Streptomycin
Sxt – Trimethoprin

* Strains 92 and 153 were sensitive to all the 22 antibiotics tested.

Discussion

In the present study, the prevalence of ETEC amongst diarrhoeal cases in Malaysia, using the infant mouse assay and two immunological methods was found to be only 3.7%. It may seem low when compared to other Asian studies where ETECs were found in 11% of children with diarrhoea in a study conducted in the Philippines,[14] 18% in Thailand,[15] 25% in Bangladesh[1] and 7% in Taiwan.[16] However when it was compared to the other bacterial pathogens in this study it was almost as common as the salmonellas and shigellas which were the most common isolates from our diarrhoeal cases and was even more common than the EPECs (Table 2). Therefore relative to other pathogens, it does contribute to a significant cause of diarrhoea.

The study in Bangladesh done in children aged between 2–60 months showed that the highest incidence of ETEC diarrhoeas was amongst the 9–10 months old children.[17] In our study, although the majority of the strains were detected in children below ten years of age, it was not possible to draw any reasonable conclusions as this age group also contributed to the highest number of cases studied plus the total number of positive isolates was too small.

Antimicrobial drugs have been recommended for treatment of moderate to severe traveller's diarrhoea.[18] The choice of antibiotic would depend on the sensitivity pattern of the local isolates. Echeverria had shown in his study that 72% of the isolates of ETECs from the far east, was resistant to at least one or more antibiotics, and 44% were resistant to four or more antibiotics.[19] In our series, ten out of 12 (i.e. 83%) strains tested were resistant to one or more antibiotics and three out of 12 (i.e. 25%) were resistant to four or more antibiotics. Resistance to ampicillin was high, eight out of 12 strains (i.e. 67%).

A suitable method for detection of ETECs in a routine laboratory should be easily available, simple, rapid and cost effective as well as sensitive and specific. We did not study the sensitivity and specificity of the two test kits used here as previous studies by other works have found them to have good sensitivity and specificity when compared with the standard tissue culture techniques.[20] Of the two kits used in this study, the Phadebact ETEC-LT was found to be more suitable for a routine diagnostic laboratory as it was simpler and faster to perform. The only disadvantage is that it cannot detect ST producing strains, and in this study, strains producing ST alone constituted more than half the number of the total ETEC strains.

The study in Bangladesh also has more strains producing ST alone than LT or both.[1] Therefore in this country, if routine testing for ETEC strains in diarrhoeal cases is to be introduced, a simple and convenient test kit that can detect both LT and ST should be used, and until such a kit is available we feel that testing for ETEC for routine diagnostic purpose should not be encouraged.

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