PREVALENCE OF ENTEROTOXIGENIC *ESCHERICHIA COLI* IN PATIENTS WITH DIARRHOEA IN BANGKOK

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INTRODUCTION

It has been well documented that enterotoxigenic *Escherichia coli* (ETEC) are responsible for acute diarrhoea in children, adults and travellers who have recently arrived in tropical countries (Gorbach and Khurana, 1972; Merson *et al.*, 1979a). ETEC produces a heat labile (LT) and a heat stable (ST) toxin or both. LT toxin has two antigenic moieties with the molecular weights of 44,000 and 33,000 (Kunkel and Robertson, 1979). Its mode of action is similar to that of *Vibrio cholerae* in activating the enzyme adenyl cyclase thus increasing intracellular cyclic AMP and causing active secretion of chloride ion and inhibition of sodium adsorption (Field, 1979). ST toxin is a low molecular weight, non-antigenic protein of 4,000 - 5,000 daltons (Alderete and Robertson, 1978; Takeda *et al.*, 1979) which acts by stimulation of guanylate cyclase to generate cyclic GMP and inhibits the transport of NaCl across the brush border of mucosal epithelium without stimulating the secretary activity (Field, 1979). Evidence has been accumulated to suggest the ETEC producing LT or LT and ST are responsible for a large proportion of cases of traveller's diarrhoea (Shore *et al.*, 1974; Gorbach *et al.*, 1974) as well as endemic diarrhoea (Ryder *et al.*, 1976a). ST + ETEC has also been implicated as a cause of traveller's diarrhoea in adults in tropical countries (Sack *et al.*, 1975b), and in an outbreak of diarrhoea in infants in the United States (Ryder *et al.*, 1976b).

Although ETEC is a relatively common cause of diarrhoea in many parts of the world, the prevalence of this enteric pathogen in Thailand is unknown. The purpose of this study was to determine the prevalence of ETEC in association with the clinical spectrum of the disease, and the age group most frequently affected. The relationship between enteropathogenic serotypes, ability to produce enterotoxins and susceptibility to antibiotics was also examined.

MATERIALS AND METHODS

Stool or rectal swabs were collected from 82 patients, whose ages ranged between 2 months to 82 years, admitted to Bamrajnaradura Hospital during February to April 1978. All patients had sudden onset of watery diarrhoea within 24 hours prior to admission with mild to severe degree of dehydration observed clinically. Routine bacteriological isolation was negative for *Shigella, Salmonella, Vibrio cholerae* and *Vibrio parahaemolyticus*.

Stool or rectal swab was plated on MacConkey agar (Difco). After over night incubation, lactose positive colonies from each subject were randomly picked and identified by standard biochemical tests according to the method of Edwards and Ewing (1972). Ten *E.coli* colonies were collected from each patient. The organisms were maintained in nutrient agar stab cultures at room temperature until testing for enterotoxin production. No more than 2 transfers were made before the isolates were tested for enterotoxin production.

Y-1 adrenal cell assay for heat labile enterotoxin

The technique of Sack and Sack (1975) was followed throughout. A loopful of an overnight culture of *E.coli* in 1% peptone water was inoculated into 6 ml of 2% peptone water in a 125-ml Erlenmeyer flask, followed by incubation at 37°C for 18 to 24 hours. An aliquot of 0.05 ml from each *E.coli* culture was added in duplicate into each well of a 96-well Dynatech Microtiter plate (M29AR) containing a confluent monolayer of Y-1 adrenal cell culture grown in Ham F 10 medium (Flow Laboratories, Rockville, Md, U.S.A.) supplemented with 15% heat inactivated horse serum, 2.5% fetal calf serum plus gentamycin at a concentration of 40 μg per ml. After 5 minutes, the tissue culture medium containing bacteria was removed with a Pasteur pipette. The tissue culture wells were washed twice with phosphate buffered saline pH 7.5 and fresh medium replaced. The Y-1 adrenal cells were observed in 18 to 24 hours for typical rounding. *E.coli* 47-1, a heat labile and a heat stable enterotoxin positive *E.coli* kindly provided by Dr.John H. Cross, NAMRU-2, then at Taipei, was used as a positive control, and a non-enterotoxin producing strain, isolated from a healthy Thai, was used as a negative control.

Suckling mouse assay for heat stable enterotoxin

The technique of Dean *et al.*,(1972) was used. The isolates of *E.coli* were inoculated into 2% peptone water and incubated at 37°C in a tissue culture roller drum at eight revolutions per minute for 18 hours. The culture broth was centrifuged at 2000 g for 10 minutes at room temperature and 0.1 ml of the supernatant together with 0.1 ml of Evan's blue was injected into the stomach with a No.26 gauge needle through the anterior abdominal wall of 4 day old suckling mice. After 4 hours, the mice were killed by chloroform and the intestine removed. The intestine and the rest of the mouse body were weighed separately, and the ratio of the weight of the intestine and the weight of the remaining body was determined. Animals with no dye in the intestine were discarded. A gut/body weight ratio of greater than 0.085 was considered positive, a ratio of less than 0.080 was considered negative, and a ratio of 0.080 to 0.085 as borderline and the isolate was retested. *E.coli* 47A-1 and *E.coli* K-12 (kindly provided by Dr. P. Echeverria, AFRIMS, Thailand) were used as positive and negative controls respectively.

Serotyping for enteropathogenic E. coli

E.coli were serotyped by standard methods (Edwards and Ewing, 1972). Three polyvalent (I, II and III) and seventeen individual type sera were used. The type sera included 026: K60 (B6), 044 : K74(L), 055 : K59(B5), 078 : K80(B-), 086 : K61(B7), 0111 : K58(B4), 0114 : K-(B-), 0119 : K69 (B14), 0124 : K72 (B17), 0125 : K70 (B15), 0126 : K71 (B16), 0127 : K63 (B8), 0128 : K67 (B12), 018a018c : K77, 020a020b : K84, 028 : K73, 0112a0112c : K66. All antisera were prepared locally and have been used in the routine serotyping service of enteropathogenic *E. coli* isolated in Thailand. Thirty four isolates of ETEC were sent for typing by Drs. ϕrskov, International Escherichia and Klebsiella centre, Copenhagen.

Antibiotics susceptibilities

The disc method of Bauer *et al.*, (1966) was used. The antibiotics sensitivity discs used were ampicillin 10 μg (Bristol laboratory), chloramphenicol 30 μg (Lepetit), streptomycin 10 μg (Difco), kanamycin 30 μg (Difco), cotrimoxazole 25 μg (Burroughs Wellcome Co.), and gentamicin 10 μg (Schering). Zone sizes were interpreted according to standard recommendations. Isolates showing inter-

mediate resistance were considered susceptible.

RESULTS

Escherichia coli isolates from 17 of 82 patients (20.7%) were positive in the Y-1 adrenal cell and/or suckling mouse assays. The number of positive isolations out of 10 colonies of *E. coli* from each patients is shown in Table 1. Altogether 63 ETEC colonies were isolated consisting of 29 colonies of LT + ST +, 24 colonies of LT + ST – and 10 colonies of LT – ST +. Three patients (3.7%) were infected with ten ETEC while the 14 others were infected with one to five ETEC.

The association between the age group of the patients and isolation of ETEC is shown in Table 2. Chi square analysis shows no significant difference between the age group of the patients and isolation of ETEC ($P > 0.05$).

As shown in Table 3, ETEC produces diarrhoea with severity varying from mild to rice watery diarrhoea with a number of motions ranging from two to more than ten times. Stool was non-bloody and without inflammatory exudate. Fever was present in two infants with the age of 8 and 10 months with LT + ETEC and in another infant with the age of $4\frac{1}{2}$ months with ST + ETEC infections. None of the adults with diarrhoea were febrile. There was no difference in clinical manifestations among the patients with LT + ETEC, ST + ETEC and LT + ST + ETEC.

Serotyping of ETEC is shown in Table 4. Serotyping could be done only in 14 of 17

Table 1

Frequency of positive isolations of LT and ST enterotoxigenic *E.coli* from 82 patients with acute diarrhoea.

No. positive colonies	Number of patients with					Cumulative frequency (%)
	LT + ST +	LT + ST –	LT – ST +	Mixed ETEC infection	Total (%)	
10	2	-	-	1*	3 (3.7)	3.7
9	-	-	-	-	-	3.7
8	-	-	-	-	-	3.7
7	-	-	-	-	-	3.7
6	-	-	-	-	-	3.7
5	-	-	-	1**	1 (1.2)	4.9
4	-	3	-	1***	4 (4.9)	9.8
3	-	-	1	-	1 (1.2)	11.0
2	-	-	1	-	1 (1.2)	12.2
1	-	3	4	-	7 (8.5)	20.7
0	-	-	-	-	65 (79.2)	100
Total	2	6	6	3	82 (100)	

*6 colonies with LT + ST + and 4 colonies with LT + ST –
** 3 colonies with LT + ST + and 2 colonies with LT + ST –
*** 3 colonies with LT + ST – and 1 colony with LT – ST +

Table 2

The prevalence of ETEC in patients of various age groups.

Age (year)	No. studied	No. posit. for LT + ETEC	No. posit. for ST + ETEC	No. posit. for LT+ST+ETEC	No. with mixed ETEC infections	Total
≤ 1	30	2 (6.7)*	1 (3.3)	1 (3.3)	1**(3.3)	5 (16.7)
2 - 13	17	1 (5.9)	3 (17.6)	1 (5.9)	-	5 (29.4)
≥ 13	35	3 (8.6)	2 (5.7)	-	2***(5.7)	7 (20)
Total	82	6 (7.3)	6 (7.3)	2 (2.4)	3 (3.7)	17 (20.7)

* percentage positive
** infection with LT + ST − and LT − ST + ETEC
*** infection with LT + ST + and LT + ST − ETEC

Table 3

Clinical symptoms in 17 patients with ETEC infections.

Symptoms	Types of ETEC LT+ST+ 4[a]	LT + 7	ST + 6
Frequencies of diarrhoea within 24 hours prior to admission :-			
3 times	0	1	1
4-9 times	1	2	1
10 times	3	4	4
Dehydration[b]			
mild	0	0	1
moderate	2	5	4
severe	2	2	1
Fever	-	2[c]	1[d]
Vomiting	-	-	1
Headache	-	1	-

a. Number of patients. For analysis, mixed infection with LT + ST + and LT + ST − is categorised as LT + ST +, whilst LT + ST − and LT − ST + is treated as LT + ST −.
b. Determined by clinical observation. Mild dehydration = a fluid loss of less than 5% of the total body surface, and the patient appeared normal. Moderate = a fluid loss of 5−7% of the total body surface, the patient could sit in the wheelchair, but could not walk. In severe dehydration, the fluid loss was more than 8% of the total surface area. The patient could not sit, the extremities cool, the eyes sunken
c. All are infants (8 months and 10 months).
d. An infant with the age of $4\frac{1}{2}$ months.

Table 4

Serotyping of ETEC.

Toxin produced	No. of ETEC colonies isolated	No. sent for serotyping or typable	No. of patients from whom serotyping were done	colony code number	Serotypes
LT + ST +	29	13	3	24-1*	O_{78} · H spont. ag.
				24-3	,, ,,
				24-5	,, ,,
				24-8	,, ,,
				24-9	,, ,,
				44-3	O_{78} : H spont. ag.
				44-8	O_{78} : H spont. ag.
				49-1	O_{20}, O_{44} : H spont. ag.
				49-2	O_{114} : H_{49}
				49-3	O_{114} : H_{49}
				49-4	O_{20}, O_{44} : H spont. ag.
				49-5	O_{114} : H_{49}
				49-9	O_{20}, O_{44} : H spont. ag.
LT +	24	13	7	M-1	O_{20}, O_{44} : H spont. ag.
				M-7	O_{20}, O_{44} : H spont. ag.
				12-4	O_{55} : K_{59}
				17-1	O_{80} : H_{26}
				24-6*	O_{78} . H spont.ag.
				24-7	,, ,,
				24-10	,, ,,
				31-7	O_{150} : H_6
				31-10	,, ,,
				32-5	O spont. ag. : K_{14} : H_4
				65-2	O_1 : H_{34}
				65-8	,, ,,
				65-10	,, ,,
ST +	10	7	5	11-7	O_{21} : K_{54}, K_{96} : H-
				34-3	O_{85} : H_{11}
				37-5	O spont. ag. : K_{14} : H_4
				78-5	O_{15} : H_{11}
				82-3	O_{78} : H_{12}
				82-5	,, ,,
				82-6	,, ,,
Total	63	33	15		

* one is mixed infection.

Table 5

Resistance to antibiotics of 62 ETEC isolates from 82 patients.

Toxins produced	No. of isolates tested	Number of isolates resistant to (% resistant)						
		Ap	Cm	Gm	Kn	Sm	Tc	Co
LT + ST +	29	25 (86.2)	19 (65.5)	0	0	18 (62.1)	22 (75.9)	0
LT + ST -	23	14 (60.7)	10 (43.5)	0	5 (21.7)	14 (60.9)	15 (65.2)	0
LT - ST +	10	10 (100)	8 (80)	0	1 (10)	8 (80)	8 (80)	0
Total	62	49 (79)	37 (59.7)	0	6 (9.7)	40 (64.5)	45 (72.6)	0

Ap = ampicillin, Cm = chloramphenicol, Gm = gentamicin,
Kn = kanamycin, Sm = streptomycin, Tc = tetracycline,
Co = cotrimoxazole.

Table 6

Resistance of ETEC to multiple and single antibiotics.

Toxin produced	No. of isolates tested	Number resistant to (% resistant)						No. sensitive to Ap, Cm, Sm and Tc (% sensitive)
		Ap, Cm, Sm, Tc	Ap, Sm, Tc	Ap, Sm	Ap. Sm	Ap only	Tc only	
LT + ST +	29	16 (55.2)	5 (17.2)	0	1 (3.4)	3 (10.3)	0	4 (13.8)
LT + ST -	23	10 (43.5)	3 (13.0)	1 (4.3)	0	0	2(8.7)	7 (30.4)
LT - ST +	10	6 (60)	2 (20)	2(20)	0	0	0	0
Total	62	32 (51.6)	10 (16.1)	3 (4.8)	1 (1.6)	3 (4.8)	2 (3.2)	11 (17.7)

Ap = ampicillin, Cm = chloramphenicol, Gm = gentamicin,
Kn = kanamycin, Sm = streptomycin, Tc = tetracycline,
Co = cotrimoxazole.

patients with ETEC infection, one of whom had mixed ETEC infection. It was noted that 078 ETEC was responsible in 2 patients with LT + ST + ETEC, whereas serotyping in patients with LT + and ST + ETEC were widely scattered in many different 0 groups. In one patient with LT + ST + ETEC infection, 2 serotypes (020, 044 : H spont. ag. and 0114 : H49) were found. Of the remaining 65 patients negative for ETEC, only 4 colonies could be typed. Their serotypes were 0124 : K72 (B17) (two patients), 020a020b : K84 (one patient) and 044 : K74 (L) (one patient).

The antimicrobial susceptibilities of 62 of 68 ETEC isolates are shown in Table 5, and the numbers resistant to multiple as well as single drugs are shown in Table 6. As many as 51.6% of the isolates showed multiple resistance to ampicillin, chloramphenicol, streptomycin and tetracycline. None of them was resistant to gentamicin and cotrimoxazole. LT + ST + ETEC with 078 serotypes from 2 of 4 patients were resistant to these four antibiotics.

DISCUSSION

This study demonstrated that ETEC was isolated from 20.7% of 82 patients with diarrhoea in whom other bacterial agents had been excluded. Such prevalence may be an underestimate, since some ETEC can not be detected by either the Y-1 adrenal cell culture or the infant mouse assay (Klipstein *et al.*, 1978). Regional prevalence rates of ETEC infection in patients with diarrhoea vary. As high as 80% of hospitalized children in Chicago were infected with enterotoxigenic strain of *E.coli* as assayed in the infant rabbit (Gorbach and Khurana, 1972), but a rate of only 18% was found in hospitalized Apache children with diarrhoea as assayed with the Y-1 adrenal cell and in suckling mouse, (Sack *et al.*, 1975a) The prevalence rate of ETEC in children with diarrhoea in Taiwan and in the Philippines were only 16% and 11% respectively (Echeveria *et al.*, 1977; 1978a).

Four or more ETEC colonies were found 8 of 17 patients, whereas in the remaining 9 patients, $\leq$ 3 colonies were detected. This finding raised doubt as to whether the ETEC isolated were causing diarrhoea in these 9 patients, since positive isolation of $\leq$ 3 of 10 colonies were found in as many as 13% of healthy children in Taiwan (Echeveria *et al.*, 1977) and 6% of asymptomatic travellers (Echeverria and Cross, 1977).

Analysis between the age group of the patients and positive isolations of ETEC indicates that ETEC can inflict illness in all age group with equal frequency. Diarrhoea due to ETEC varied in severity as assessed by the degree of dehydration and the number of motions prior to admission. Fever was not common, and it was found only in three infants $\leq$ 10 months of age. Vomiting was rare.

Of 63 ETEC isolates, 33 could be typed (table 4), of which only 1 colony belonged to the classical enteropathogenic serotype of 055 : K9. This result confirms the lack of correlation between common enteropathogenic serotypes and pathogenicity (Sack, 1975; Goldschmidt *et al.*, 1976; ϕrskov *et al.*, 1976). ϕrskov *et al.*, (1976) found that ETEC are restricted to a relatively small number of serotypes (06: H16 ; 08 H9 ; 025: H42; 078 : Hi11 and 078 : H12). A recent report from Bangladesh showed that 86% of LT + ST + ETEC belonged to four "O" serotypes (06;08; 078 and 0115) whereas LT + ETEC and ST + ETEC were widely distributed among 15 and six serotypes respectively (Merson *et al.*, 1979b). In our study, two of four patients with LT + ST + ETEC (50%) had 078 : H spontaneous agglutination confirming results of ϕrskov *et al.*, (1976) and Merson *et al.*, (1979b). In one patient in whom 10 lactose positive colonies were all ETEC seven colonies were typed and found to be 078 eventhough four of which were LT + ST + and the remaining three were LT + ST–. This finding suggested that all these 7 colonies were derived from the same clone but some of them might have lost plasmid that controls production of ST toxin. In another patient withLT + ST + ETEC infection, 2 serotypes of 020, 044: H spontaneous agglutination and 0114: H49 were found. In LT + and ST + ETEC their serotypes appear to vary which again confirm the results of Merson *et al.*. (1979b).

Antibiotics sensitivety testing showed that 51.2% of 62 ETEC isolates had multiple resistance to ampicillin, chlorampenicol, streptomycin and tetracycline. Two of four LT + ST + ETEC having 078 serotypes were resistant to these four antibiotics confirming the report by Smith *et al.*, (1979) showing that four of five isolates of LT + ST + ETEC from Thailand belonging to 078 had multiple drug resistance to chloramphenicol, streptomycin, sulfathiazole and tetracycline. Furthermore ETEC belonging to 078 but were isolated from different regions of the world had different susceptibilites to antibiotics (Smith *et al.*, 1979). ETEC to isolated from the Far

East have been found to be resistant to multiple antibiotics (Echeverria *et al.*, 1978b). Whether regional differences in resistance is due to the extent of local antibiotic selective pressure or to the compatibilities of the local R plasmid and plasmids coding for LT and/or ST is unclear.

SUMMARY

Enterotoxigenic *Escherichia coli* (ETEC) were recovered from 17 of 82 patients (20.7%) with diarrhoea admitted to Bamrajnaradura Hospital. Six patients (7.3%) were infected with LT + ETEC, 6 patients (7.3%) with ST + ETEC, 2 patients (2.4%) withLT + ST + ETEC and 3 patients with mixed ETEC infection, two of them had LT+ST + and LT+and one had LT + and ST + ETEC infections. There was no significant difference between the age group and the positive isolation of ETEC. Only 33 of 63 isolates could be typed. Two of 4 patients with LT + ST + ETEC had 078 serotype. There was no correlation between enteroxigencity and enteropathogenicity as determined by the antisera used. Antibiotic sensitivity testing showed that 51.6% of ETEC isolates were resistant to ampicillin, choramphenicol, streptomycin and tetracycline, and all ETEC were sensitive to gentamicin and cotrimoxazole.

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