Iranian J. Publ. Health
1982, Vol. 11, No.3,4(77 - 88)

THE FIRST REPORT ON THE ISOLATION OF ENTEROTOXIGENIC ESCHERICHIA COLI AS A CAUSE OF INFANTILE DIARRHEA IN IRAN*

S.Mohadjer,Ph.D.;A.Bidanjiri,M.S;.R.Hafezi,B.S. and A.H. Hamidi D.V.M**

key words: Enterotoxigenic E.coli , Infantile diarrhea, Enteropathogenic E.coli , Iran.

ABSTRACT

The role of enterotoxigenic E.coli as a causative agent in diarrheal disease was studied among 100 cases of infant and children 0-2 years of age. Routine bacteriological methods were used for identification enteropathogenic E.coli, Salmonella, Shigella and Vibrio cholera. The ability of E.coli strains to produce toxin was assayed in animal models (rabbit-ileal loop and suckling mice)and in tissue- culture (Y_1-adrenal cell).

A total of seven enterotoxin producing strains of E. coli were isolated. Three of these strains were producing both (heat-labile-LT) and heat-stable(ST)enterotoxin).The other four strains were producing only heat-stable enterotoxin which was lost during storage.The rate of isolation for other enteropathogenic bacteria such as Salmonella , Shigella and enteropathogenic E.coli was 7% , 4% and 13% respectively.No Vibrio cholera or yersinia enterocolitica was isolated from the age group under the study.

---

* *This study has been partly supported by the research funds of the Ministry of Science and Higher Education, contract No. 400- 6 -36/1 and in part by the School of Public Health and Institute of Public Health Reasearch, University of Teheran.*

** *Microbiology,School of Public Health,University, of Tehran P.O. Box 1310. Tehran,Iran.*

INTRODUCTION

A previous report(18) and other unpublished data from our laboratory in accordance with the most published surveys of sporadic enteritis in children (26-4), shows that is known classical enteric pathogens can not be isolated from more than 25-30% of children with diarrhea . Recent studies of the etiology of acute diarrhea in children have focused attention on enterotoxin-producing strains of E. coli(14,6,24). Studies reported from developed countries indicate that sporadic among infants and children due to enterotoxigenic E. coli appears to be a rare event(15,10) whereas it seems to be an important enteric pathogen among children and adults in developing countries (19,21,9).

This study was carried out to determine the importance of enterotoxigenic E.coli in sporadic diarrhea in children 0-2 years of age in Iran.

MATERIALS AND METHODS

Hundred children of 0-2years of age who were referred to a children hospital in Tehran were studied.They suffered from diarrheal symptoms with different degrees of severity. Most of them had previously been treated with antibiotice.

Stools were collected from the diper on admission by means of a cotton swab which was put in screw-capped tubes containing 10 ml.of Cary-Blair Transport Medium. Up on arrival at the laboratory, specimens were immediately cultured for Shigella, Salmonella, enteropathogenic E. coli and Vibrio cholera as well as for Yersinia enteropathogenic E. according to standard methods (11-1). Desoxycolate citrate agar (Difco), Salmonella-Shigella agar(SS-Difco), enrichment broth Selenit-F(Difco),alkaline pepton(PH=8.5) and T.C.B.S. medium (B.B.L.), were used for primary isolation of Salmonella, Shigella and Vibrio cholera. Biochemical and serological identification tests were performed with Difco, Pasteur Institute(Paris)and Behring antisera.

For the isolation of E. coli blood-agar and Endo-agar (Difco) media were used. These were characterized by their biochemical reaction and tested by slide agglutination with antisera against enteropathogenic E. coli strains

(O_{26}: B_6, O_{55}: B_5, O_{111}: B_4, O_{119}: B_{14}, O_{127}: B_{17}, O_{126}: B_{16}, O_{128}: B_{12}, O_{123}: B_{15}, O_{86}: B_7).The results of slide agglutinations were confirmed by tube-agglutination tests Identification of enterotoxigenic E. coli was performed according to the method described initially by Donta and modified by Sack (8-23). One to three colonies from each patient showing the typical appearance of E . coli were subcultured after 24hr incubation at (37°C, and the tubes were kept at room temperature for determination of enterotoxin production.

ENTEROTOXIN PRODUCTION: E. coli strains were inoculated into 5ml. of Trypticase soy broth (TSB)and incubated at 37°C for 24 hours. Two mililitres aliquot of these suspensions were further inoculated into 10 ml (of TSB in 250ml flasks) These were incubated at 37°C for 18-24hours in a rotary shaker (180 r.p.m.). Afterwards the cultures were centrifuged at 3500 r.p.m. at 4°C to remove the Bacteria.The resulting supernatant was then filtered through a 0.22 m/μ membrane filter (millipore) for use in ileal loop of adult rabbits suckling mice or tissue culture tests.

ENTEROTOXIN ASSAY

a. Assay of heat-stable enterotoxin (ST)

The suckling mice model described by Dean et al. (6) was used for the detection of heat-stable enterotoxin.Two to 4 days old suckling mice were separated from their mothers shortly before testing.Five to six mice used for each assay.Each of the animals were injected intragastric with, 0.1 ml. of the filtered superatant containing Evans blue (1.25%)as an indicator.They were kept separated from their mothers and sacrificed within three to four hours of inoculation.

After opening the abdomen, the intestine was examined for the presence of blue colouration and distention.Those showing no colouration of the intestine were not considered.The coloured intestine of each group of mice were carefully removed and weighed . The carcasses were also weighed together and the ratio of the weight of the inte-

stine to remaining body weight was calculated . If these were fewer than four successful introgastric inculation the test was repeated. A ratio of less than 0.07 was considered to be negative, those between 0. 07 - 0.09 as suspicious and those with a ratio of more than 0.09 as positive.

b. Assay of Heat- labile Enterotoxin (LT)

The Y_1 mouse adrenal tissue culture cells was kindly provided by Dr. Donta (Vererans Administration Hospital, Iowa City, Iowa) and was used according to the methods described by him and Sack (8,23). Aliquots of 0.5 ml. of the sterile culture filtrate,prepared as described before was added to the monolayer cultures of Y_1-cells grown for 3-4 days in 30mm petri-dishes (Falcon). After 5-10minutes of incubation at 37°C, the remainder of the filtrate was removed by suction, the plates washed with P.B.S.and replaced with fresh tissue-culture media (MEM - Egle's Base Containing, 10% inactivated fetal calf serum , 40/μg/ ml gentamycin and glutamine). The plates were examined after 4 and 24 hours of incubation at 37°C for the presence of rounding cells indicating presence of enterotoxin.Positive and negative controls were included in each test. In case of using mini-culture (monolayer prepared in flat- bottom microtiter plates) 0.05 ml. of test material was used.
E.coli strains demonstrating enterotoxin activity were further tested in rabbit ileal loops according to the method described by kasai and Burrows (16). Albino rabbits (8-9 weeks old) were used. Six intestinal loops , each 10 cm long were made in each rabbit. These loops included , two negatives and one positive control. Negative control loops were injected with 2 ml. of sterile TSB or 2 ml . of a culture filtrate of an E. coli devoid of enterotoxin plasmid.The positive control loop was injected with 2 ml. of a culture filtrate of E. coli(H-10407) possesing enterotoxin plasmid.Both of the strains,negative and positive for enterotoxin were kindly provided by Dr. Orskov,Serum Staten Institute in kopenhagen.

All the isolated strains were also tested, according to the method described by Fredrique(12)for colicinogenic activity.

Sensitivity tests were carried out using the single-disk technique of Bauer and kirbey (2).

RESULTS

A total of 100 cases of gastroenteritis among chidlren under 2 years of age was studied.
Table 1. shows the number and percentage of enteropathogens isolated.

Enteropathogenic E. coli(EPEC) was found in 13 cases: Theses patients has $O_{126}:B_{16}$, two had $O_{86}:B_7$, two others had $O_{26}:B_6$ and each one of the rest had one of the following $O_{111}:B_4$, O_{55}: B_5, O_{125}: B_{15}, $O_{119}:B_{14}$ and O_{127}: B_{17}. None of these classical strains of enteropathogenic E. coli was found capable of producting either LT or ST.

Table 1. Number and percent of enteropathogens isolated from 100 cases of children with gasteroenteritis.

Pathogen	No.	Percentage
Enteropathogenic E. coli	13	13%
Salmonella	7	7%
Shigella	4	4%
Enterotoxigenic E. coli	7	7%
Total number of pathogens	31	31%

Table I-Seven cases were positive for Salmonelleae of which 5 strains were S.typhimurium, 1 strain was S.havana and 1 strain was S.monotevideo. Among the 4 isolated Shigelease there were 2 strains of Sh. sonnei , 1 strain of Sh. flexneri and one strain of Sh.boydii . All cases of gastroenteritis examined were neither positive for V.cholera and its biotype EL- Tor nor for Yersinia enterocolitica.

Table 2. O: H serotypes and the type of toxin elaborated among 7 enterotoxigenic strains isolated from100 cases of children with gasteroenteritis.

Pathogen No.	O: H Serotype*	Enterotoxine type	
		Heat-labile (LT)	Heat-stable (ST)
1	$O_5:H^-$	+	+
2	$O_?:H_?$	+	+
3	$O_7:H^-$	+	+
4	$O_{27}:H^-$	-	+
5	$O_{27}:H_{20}$	-	+
6	$O_?:H^-$	-	+
7	$O_?:H_{10}$	-	+

* - *The O: H serotyping was kindly performed by Dr. Qrskove in Kopenhagen.*

Table 2. shows the isolated enterotoxigenic E. coli(ETEC) strains with their O:H serotypes and the type of elaborated toxin. The three straine producing heat-labile and or heat-stable toxins were of O:H serotypes of $O_5:H^-$, O_7: H^- and $O_7:H?$. The other four strains belonging to O_{27} : H^- , O_{27}: H_{20}, $O?:H^-$ and $O_?:H_{10}$were producing only heat-stable toxin. The ability to produce heat-stable toxin was lost during 6-8 months of storage.

Table 3. Clinical patterns of cases of gasteroenteritis due to E. coli that elaborate both, heat-labile and heat-stable enterotoxins.

Patients No.	Age/ Month	Sex	period of illness before admission/ days	No. of stool/day	Fever	Appearance of stool	Vomitting
5	24	M	5	6	+	watery	-
11	11	M	11	5-6	+	watery	-
150	12	M	4	4-5	-	watery	+

Table 4. Clinical patterns of cases of gasteroenteritis due to E.coli that elaborate only heat-stable enterotoxin.

Patients No.	Age/ Months	Sex	Period of illness beford admission/ days	No. of stool/day	Fever	Appearance of stool	Vomitting
22	6	M	1	7-9	+	watery	-
23	12	M	2	10-12	+	watery	-
36	24	M	2	more than 12	-	watery	-
123	24	F	7	6-7	+	watery	+

Tables 3 and 4 show the clinical patterns of the 7 cases of gastroenteritis due to ETEC elaborating either the two kinds of toxins (ST and LT) or only one kind (ST).Judging by the number of stool specimen per day and the duration of illness before admission to the hospital it seems that enteritis due to ETEC producing only ST is more severe

than due to strains producing both kinds of toxins. All 7 ETEC strains isolated were harboring R-plasmids and only two were colicinogenic (Table 5).

Table 5. R-type and colinogenic of 7 ETEC strains isolated from 100 cases of gasteroenteritis among infants and children

No.	R-type	Enterotoxin		Colicin - type
		ST	LT	
1	ASSuT*	+	+	-
2	ACKSSuT	+	+	I_b
3	T	+	+	-
4	CSSuT	+	-	-
5	kSSuT	+	-	-
6	CKSSuT	+	-	-
7	T	+	-	+(untypable)

* = *A=Ampicillin, C=Chloromycetin, K=Kanamycin S=Streptomycin, Su=Sulfonamide, T=Tetracycline*

DISCUSSION

During the last few years enterotoxigenic E.coli(ETEC) has been shown to cause diarrhea in adults (22,13 and to be a significant cause of severe diarrhea among infants and children particularly in developing countries (14,26, 9).

This report demonstratis potent enterotoxin producing straine of E. coli isolated from cases of childhood diar-

rhea in Iran.In our study a potential pathogens was found in (31%) patients with diarrhea. Among these, enterotoxigenic straine of E. coli (ETEC) were encountered in 7% of the cases.

Strains of salmonella were also isolated from 7% of the cases, whereas Shigelleae was isolated less frequent (4%).

On the other hand the rate of isolation of enteropathogenic E. coli(EPEC) was higher than any other pathogens isolated (13%). However, none of these strains were found capable of producting either LT or ST. Among the isolated ETEC, 4 strains were found to produce heat-stable enterotixin. This capability, however, was lost during 6 to 8 months of storage.

The spontanous loss of ST plasmid during storage has been reported by others (17) and needs further studies. A possible explanation for this phenomena could be, that the gene confering ST production to the strain is located on the transposan and therefore easy to lose (3,5).

The O:H serotypes of the enterotoxigenic strains of E. coli isolated in our study, were different from those isolated from other parts of the world (20). Thus, it seems that there is no association between O : H serotypes of strains of E.coli and the ability to accept the plasmid necessary to provoke the production of an enterotoxin.

The Japanese have examined and reported satisfactory result with the Bikent Test . At the Present, however,the tissue culture Tecnic remains the method of choice for the detection of Enderotoxigenic Ecoli. (25)

REFERENCES

1 - *Barua, D. and W. Burrows, (1974). "Cholera" W.B. Sannders Comp. Philadelphia , London, Toronto.*

2 - *Baur, A. W. Kirby, W.M.M., Sherris, J.C.et al.(1966). Antibiotic susceptibility testing by a standardized single disk method. Am.J.Clin. Pathol. 45:493-496.*

3 - Cohen, S.N. (1976).
Transponsable genetic elements and plasmid evolution. Nature, 263:731.

4 - Crammblett. H.G.,Azimi, P., Hayness.R.E.(1971).
The etiology of infectious diarrhea in infancy, with special reference to entero-pathogenis E.coli. Ann.N.Y. Acad. Sci. 176:80-92.

5 - Datta,N. (1977).
Classification of plasmids as an did to understanding their epidemiology and evolution.J.Antimicrob.Chemoth 3: Suppl. C. 19.

6 - Dean AG,Ching Y-C, Williams G, et al. (1972).
Test for E.Coli enterotoxin using infant mice: Application in a studied diarrhea in children in Honolulu. J.Infect. Dis, 125: 407-411.

7 - Dean,A.G.Ching, Y.C. and G.Williams, et al. (1972).
Test for Eschrichia coli enterotoxin using infant mice: Application in study of diarrhea in children in Honululu. J.Infect. Dis, 125: 407-411.

8 - Donta, S.T. and H.W.Monn. (1974).
Detection of Heat-Labile Eschericia coli Enterotoxin with the use of Adrenal Cells in Tissue Culture.Science, 183:334-336.

9 - Donta, S.T.Wallace, R.B Whipp, Sh. C. and J . Olarte (1977).
Enterotoxigenic E. Coli and Diarrheal Disease in Mexican Children. J. Infect. Dis. 135: 482-485.

10- Echverria, P., Blacklow N.R., Smith. (1975).
Role of heat-labile Toxigenic E.Coli and Reovirus - Like agent in diarrhea-in Boston children. Lancet, i.1113-1116.

11- Edwards.R.R. and Ewing, W.H. (1972).
(4th edition) Identification of Enterobacteriaceae. Burgen Publ. Co. Minneapolis 15. Minnesota.

12- *Fredericq. P. (1948).*
Actions antibiotiques chz les Enterobacteriaceae. Revue lelge de pathologie et de Medicine experimentale. 19 , Supptement IV, 1.

13- *Gorbach, S.L. Kean.B.Evans. D.g. Evans.Jr.D,J. and D. Bessudo (1970).*
Travelers Diarrhea and Toxigenic E.Coli. The New.Engl. J. Med. 292: 933.

14- *Gorbach SL,Khurana CM.(1972).*
Toxigenic E.Coli: A cause of infantile diarrhea in Chicago. New Engl. J. Med. 287: 791-795.

15- *Kapikian A.Z.Kim.H.W., Wyatt.R.G., Cline.W.L. , et al (1976).*
Human Reovirus-Like Agent as the major pathogen associated with "Winter" gastroenteritis in hospitalized infants and young children. New Engl.J. Med. 294:965.

16- *Kassi.G.J. and W.Burrows (1966).*
The titration of cholera toxin and antitoxin in the rabbit ileal-loop.j. Infect. Dis 116: 6-6-614.

17- *Levine, M.M.,Caplan, E.S.Waterman D., Cash, R.A.,Hornick R.B. and M.J. Snyder (1977).*
Diarrhea caused by E.Coli that produce only Heat - stable enterotoxin. Infection and Immunity 17 : 78.

18- *Mohadjer, S.and Badalian K. (1969).*
Studies of Diarrheal Diseases in Iran . I. Occurence of baterial infection in pre-school children on the central plateau of Iran. J.Trop. Med.and Hyg.72: 265-270.

19- *Nalin D.R.Mclangblin J.C., Rahaman.M., Yunus,M.Curlin G.(1975).*
Enterotoxigenic E.Coli and idiopathic diarrhea in Bangladesh. Lancet, i. 116-1119.

20- *Qrskow, F. and I. Qroskow (1976)..*
Special E.Coli Serotypes among enterotoxigenic strains from Diarrhea in adults and children.

Med, Microbiol. Immunol. 162: 73.

21- *Ryder, R.W. Sack. D.A,Kaprikian, A.Z., et al.(1976). Enterotoxigenic E.Coli and Reoviruse like Agent in Rural Bangladesh. Lancet. i. 659-663.*

22- *Sack, R.B.(1975). Human diarrheal disease caused by enterotoxigenic E. Coli Annual Review of Microbiology, 29: 333-353.*

23- *Sack. D.A., Sack, R.B.(1975) A test for enterotoxigenic E. coli using Y_1 adrenal cells in miniculture. infect. Immun. 11: 334-336.*

24- *Sebodo, T.Soenato. Y.Rohde, J.E. and et al. (1977). Etiology of Diarrhea in Children below the age of two in Cental Java. The Lancet, 26: 490-491.*

25- *Takeshi Honda, Michiko Arita, Yoshifumi Takeda, and Toshio Miwatani Journal of Clinical Microbiology July 1982 P.6.62. Further Evaluation of Biken test for Detection of Enterotexigenic Esherichia coli producing Heat- labil Enterotoxin and Application of the test to Sampling of Heat-Stable Entrotoxin.*

26- *Yow, M.D. Melnick, J.L., Blattner, R.J., Stephenson , W.B., Robinson N.M. and M.A. Burkhardt (1970). The Association of Viruses and Bacteria with Infantile Diarrhea. Am. J. Epid. 92: 33-39.*

Received. Jun. 1980