RECENT ADVANCES IN BACTERIAL DIARRHOEA

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INTRODUCTION

Recent advances in molecular genetic technology have been applied to studies of the pathogenesis and diagnosis of bacterial diarrhoeal disease in Thailand. Our efforts have concentrated primarily on *E. coli* which compromise the largest proportion of the fecal flora. At present four pathogenic mechanisms have been identified by which *E. coli* cause diarrhoea. These include enterotoxin production, enteroinvasion, enteroadherence, and cytotoxin production. This report will review our current knowledge of gastrointestinal infections caused by *E. coli* that possess these enteropathogenic mechanisms in Thailand.

Enterotoxigenic *Escherichia coli*

Enterotoxigenic *E. coli* (ETEC) is a common cause of diarrhoea in Thailand (Leksomboon *et al.*, 1981; Echeverria *et al.*, 1983, 1984a). As shown in Table 1 ETEC infections occur primarily in young children and non-immune adults. ETEC cause diarrhoea by producing a heat-labile toxin (LT), a heat-stable toxin (ST), or both (Sack, 1975). *E. coli* LT is similar but not identical in structure and mode of action to cholera toxin (CT).

Two different forms of LT have been identified. One from ETEC from humans (LT-H) and the other from ETEC from pigs (LT-P). The subunits of CT, LT-H, and LT-P have partially cross reactive determinants.

Several years ago we isolated an *E. coli* from a water buffalo in Korat which we designated *E. coli* SA-53 (Moseley *et al.*, 1982). Culture filtrates of SA-53 caused rounding of Y-1 adrenal cells, typical of the effect of LT or CT. This effect was not, however, inhibited by anti-CT, anti-LT-H, or anti-LT-P. The isolate contained a 60 Mdal plasmid that did not hybridized with the LT probe at high degrees of stringency, but both plasmid and chromosomal DNA did hybridize with the LT probe at lower degrees of stringency. This new toxin is heat-labile, and causes activation of adenylate cyclase in Y-1 adrenal cells. In addition partially purified preparation of this toxin produce a secretory response in rabbit ileal loops.

Table 1

Incidence of enterotoxigenic *Escherichia coli* in different populations in Thailand.

Location	Incidence %
1. Children's Hospital, Bangkok (children< 5 years)	8-33
2. Soongnern Hospital, Korat (all ages)	10
3. Ban Pong village, Korat (all ages)	11
4. Bamrasnaradura Hospital, Bangkok (adults)	5
5. Ban Vinai, Loei (all ages)	10
6. Pramongkutklao Hospital, Bangkok (infant < 5 days)	28
7. Peace Corps volunteers (adults)	39-57

This toxin, now formally designated as LT-II, is composed of 1A and 5B subunits which are similar in size to the subunits of LT-I and CT (about 28 Kd and 12 Kd respectively). The genes that encode LT-II have been cloned are located at contigenous sites within about 1.2 Kb of DNA. A 0.8 Kd Hind III-PstI fragment containing LT-II toxin sequences can be used as a specific probe for LT-II. In Thailand we have found LT-II ETEC in four other water buffalo and in a cow in Korat, but have not found LT-II ETEC in children with diarrhoea. Dr. Trabulsi in Brazil, however, has isolated LT-II ETEC from children with diarrhoea more often that well controls. Human volunteer studies are planned.

The heat-stable toxin of *E. coli* is resistant to inactivation by heat, acid, and proteolytic enzymes. Two different forms of ST have been identified, ST-A and ST-B. ST-A is further subdivided into two closely related toxins ST-A1 and ST-A2 that have similar physiochemical and biological properties. Both ST-A1 (ST-P) and ST-A2 (ST-H) are produced by ETEC isolated from children and adults with diarrhoea. All ST ETEC isolated in animals only contain genes encoding for ST-A1 (Udomporn *et al.*, 1983).

ST-B has been identified in *E. coli* isolated from young pigs with diarrhoea in Thailand and the United States, but did not appear to play a role in diarrhoeal disease in pig handlers in Sri Racha or in a longitudinal study of ETEC in two villages in northeastern Thailand (Echeverria *et al.*, 1984b, 1985).

The genes encoding for LT-I, LT-II, ST-A1, ST-A2, and ST-B have been cloned. Endonuclease digestion fragments of the cloned plasmids have been used as probes to identify these enteric pathogens in Thailand, Bangladesh, and Africa. The use of radiolabeled enterotoxin gene probes enable an investigator to examine many more specimens than could previously be examined with bioassays used to detect enterotoxins. Fig. 1 demonstrates isolates examined with a radiolabeled enterotoxin gene probe. Another approach is to use single stranded synthetic oligonucleotide probes of 27 base pairs that are made from the known DNA sequences of the enterotoxin gene probes. These oligo probes are presently being compared to the DNA plasmid fragment probes in the identification of ETEC. Preliminary experiments suggest that these oligo probes are as sensitive, but may be more specific.

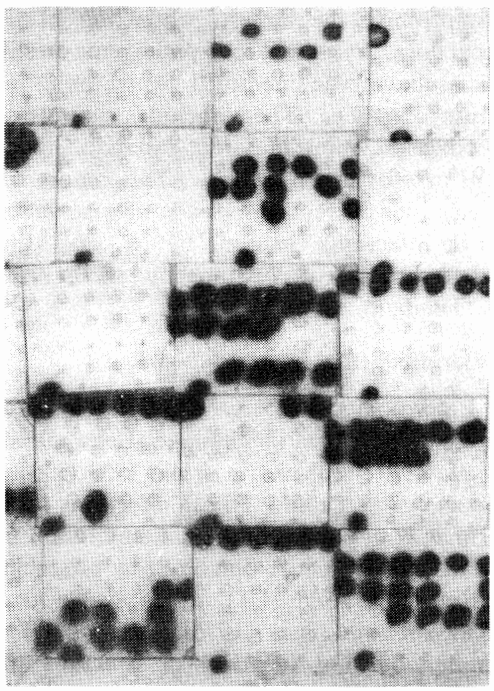

Fig. 1—A large number of *Escherichia coli* tested for hybridization with the radioactively hybridization with the radioactively labeled LT enterotoxin gene probe.

In an attempt to develop non-radioactive DNA probes, we have experimented with biotinylated and photobiotinylated oligonucleotide probes. Although pure DNA can be detected with these probes, they are not

sensitive enough to be used in colony blots. By incorporating alkaline phosphatase directly into the oligonucleotide probe this system seen to be considerable more sensitive, but has not as yet been tested in colony blots or stools.

In addition to producing enterotoxin *E. coli* must attach to the intestinal mucosa to cause disease. This was first shown very convincingly by Smith and Linggood who found that ETEC that had lost adhesive fimbriae (K88) caused less severe diarrhoea or no diarrhoea at all. Evans *et al.*, identified an adhesion factor on human ETEC which they called CFA-I. Strains processing CFA-I are able to colonize the intestine of humans and rabbits. In volunteer studies CFA/I + *E. coli* H10407, but not CFA-I – *E. coli* H10407P caused diarrhoea and was persistently excreted. In Thailand 86% of LTST, 24% of ST only, and no LT only ETEC process CFA-I or CFA-II antigens (Changchawalit *et al.*, 1984). Plasmids encoding for CFA-I antigens carry genes encoding for ST-A2 and plasmids coding for CFA-II antigens carry genes encoding for LT and ST-A2 (Echeverria *et al.*, 1986). Since genes encoding for ST-A2 are closely associated with genes encoding for CFAs, it has been difficult to construct a strain producing CFAs that does not also produce ST. Such a strain would be very useful as a vaccine candidate.

Enteroinvasive *Escherichia coli*

Enteroinvasive *E. coli* (EIEC) and *Shigella* cause dysentery by invading epithelial cells of the colon. EIEC are often non-motile, late or non-lactose fermenting, fail to produce gas from glucose, or to decarboxylate lysine. EIEC belong to a relatively few number of 0 serogroups. These are 0 serogroups 28, 29, 112, 124, 136, 143, 144, 147, 164, and 167. EIEC and *Shigella* produce keratoconjunctivitis when inoculated into the conjunctiva of a guinea pig eye. This assay, the Sereny test, is the definitive laboratory test for EIEC. Alternatively, EIEC can be identified by their ability to invade HeLa cells grown in tissue culture. Neither the Sereny test nor the HeLa cells assay are suitable for testing large numbers of colonies in studies to define the role of this enteric pathogen in diarrhoeal disease.

The genetics of the virulence of *Shigella* and EIEC are similar. Recently, it was shown in both *Shigella* and EIEC that plasmids in the range of 120-140 Mdal are necessary for virulence. The loss of the 120 Mdal plasmid found in *S. sonnei* is associated with the loss of the form I surface antigen and enteroinvasion.

Shigella and EIEC have recently been detected by Dr. Pal in Hungary by an indirect enzyme-linked immunosorbent assay using antisera raised to a virulent EIEC 0143. This antisera, subsequently absorbed with an avirulent derivative of the immunizing strain, contains ELISA reactive antibodies specific for unique antigen site(s) on the virulent strain termed virulence marker antigen (VMA). This ELISA distinguishes virulent *Shigella* and EIEC from their avirulent derivatives. Children infected with different serogroups of *Shigella* develop antibodies to the polypeptides which comprise this VMA antigen suggesting it may be possible to use VMA as a vaccine that would be effective against both *Shigella* and EIEC.

EIEC might also be identified by examining *E. coli* for 120-140 Mdal plasmids. In a study of dysentery in Thailand, 364 (13%) of 2,758 *E. coli* isolated from chldren contained large molecular weight plasmids of approximately 140 Mdal, but only 64 (18%) of these 364 *E. coli* were positive in the Sereny test (Sethabutr *et al.*, 1985 a). Thus screening *E. coli* for plasmids was too time consuming and too non-specific to be practical. The homology between the 120-140 Mdal plasmids

of EIEC and *Shigella* suggested that these plasmids shared DNA sequences that could be used as a specific probe to identify EIEC. A 17 kb EcoRI digestion fragment of pWR100, the 140 Mdal plasmid of *S. flexneri* 5 (M90T), was shown to be specific in differentiating EIEC from non-EIEC. The VMA ELISA, and this DNA probe identified the same 64 EIEC in 200 Thai children with dysentery. A biotinylated rather than a radiolabeled EIEC DNA probe has been developed to examine stools directly (Sethabutr *et al.*, 1985b). Fig 2.

In a study of the etiology of childhood dysentery, EIEC was isolated from 5%. EIEC was a less common cause of dysentery than *Shigella* (5% vs 44%), but was as likely as *S. flexneri* to result in hospitalizations (20% vs 18%). EIEC was identified in 4% of 410 Thai children with diarrhoea, and in 1.5% of children without diarrhoea. At present little is known about sources or modes of transmission of EIEC.

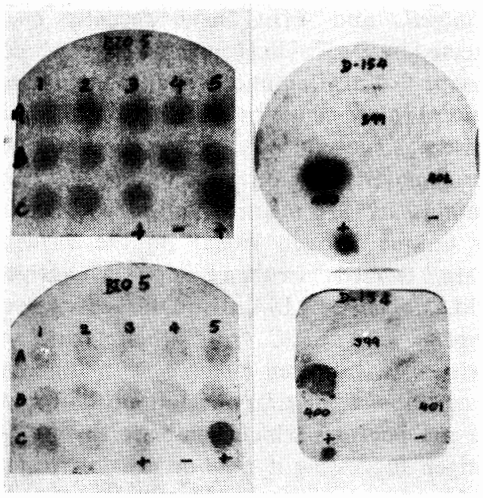

Fig. 2—Top left: Detection of 20 EIEC with the radiolabeled DNA probe. Positive controls were EIEC 115A (left) and-3 *S.flex* 5(M90T) (right). Negative control (middle was *E. coli* K12 Xac).

Bottom left: Same isolates examined with a biotinylated copy of the same probe.

Top right: 3 stool sampes (349,400, and 401) hybridized with the radiolabeled probe. *S. boydii* 14 was isolated from stool 400; neither *Shigella* nor EIEC was isolated from 399 and 401.

Bottom right: The same stools examined with the biotinylated probe.

A year long study is presently being conducted at Children's Hospital Bangkok to determine the relative importance of EIEC relative to other enteric pathogens as a cause of gastroenteritis in children less than 5 years of age.

Enteropathogenic *Escherichia coli*

Certain serotypes of *E. coli* were identified as causes of epidemics of diarrhoea in nurseries and sporadic infantile diarrhoea in England and the United States in the 1940s and 1950s. These *E. coli* serotypes have been incriminated in many studies throughout the world where in outbreaks they have been isolated significantly more often from infants with diarrhoea than from infants without diarrhoea of the same age. Although several EPEC serovars had been shown to cause diarrhoea in adult volunteers, no pathogenic mechanisms or virulence properties were initially demonstrated. With the discovery of LT, ST, and epithelial cell invasion, a number of investigators tested collections of EPEC for these virulence factors and usually found them to be negative (Echeverria *et al.*, 1976).

More recently two possible pathogenic mechanisms for EPEC have been identifiied (Levine and Edelman, 1984). Histopathologic studies in infants and animals with EPEC-associated diarrhoea showed that EPEC strains were adherent to the small bowel mucosa. It was suggested that close adherence was important for the induction of diarrhoea (Rothbaum *et al.*, 1982). Cravioto *et al*,. reported that most EPEC isolated during epidemics of infantile gastroenteritis in the

United Kingdom adhered to HEp-2 cells in tissue culture while most non-EPEC did not. Baldini *et al.*, (1983) subsequently demonstrated that HEp-2 cell adherence was encoded on a 60 Mdal plasmid(pMAR-2) in EPEC E2348 (serotype 0127 : H6). The presence of this plasmid correlated with the ability of *E. coli* E2348 to cause diarrhoea in adult volunteers. The genes for HEp-2 (or HeLa cell) adherence have been cloned and used to identify *E. coli* that adhered to HeLa cells in a localized pattern (Scaletsky *et al.*, 1984). EPEC appear to adhere to HeLa cells in either a localized or diffuse pattern (Scaletsky *et al.*, 1984). EPEC that adhere to HeLa cells in a diffuse pattern do not hybridize with genes coding for localized adherence. EPEC strains adhering in localized and diffuse patterns are shown in Figs. 3 and 4.

In addition to intestinal mucosal adherence EPEC have been reported to produce exotoxins distinct from *E. coli* LT and ST. O'Brien *et al.*, (1982) identified a Shiga-like toxin and Konowalchuk *et al.*, (1977) identified a cytotoxin, which they called verotoxin (VT), produced by EPEC strains. Scotland *et al.*, (1980) reported that 25 of 253 EPEC isolated from infants with diarrhoea in the United Kingdom produced VT. In a survey of 98 EPEC of ten different serotypes isolated from infants under one year of age in Thailand, 69% adhered to HeLa cells in either a localized or diffuse pattern. Eighty-nine percent of 57 *E. coli* 0119 : K69 adhered in a localized adherence pattern. 40% of 101 EPEC identified by the Department of Medical Sciences produced low concentration of cytotoxins. Efforts are underway to determine if these low levels of cytotoxin are Shiga toxin-like. No verocell toxin was produced (unpublished observation). It remains to be proven that EPEC cause diarrhoea by adherence, cytotoxin production, or both . These virulence properties may be related to the serotype of the EPEC.

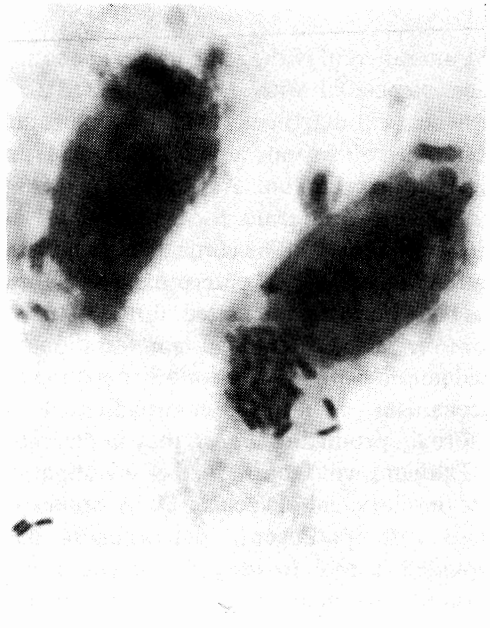

Fig. 3—Adherence of enteropathogenic *Escherichia coli* to HeLa cells in a localized pattern.

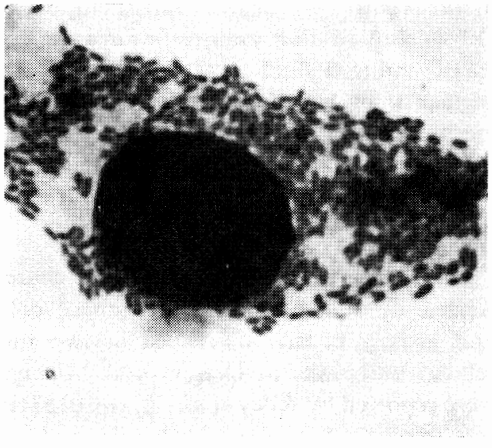

Fig. 4—Adherence of enteropathogenic *Escherichia coli* to HeLa cells in a diffuse pattern.

Enteroadherent *Escherichia coli*

E. coli of non-EPEC serotypes that adhere to HEp-2 cells have been referred to as enteroadherent *E. coli* (EAEC). Cravioto *et al.*, reported that 29% of non-EPEC strains isolated in five different diarrhoeal outbreaks

were adherent to HEp-2 cells. Three of these five outbreaks occurred in adults. More recently Mathewson *et al.*, (1985) identified EAEC in 15% of 188 travellers with diarrhoea in Mexico and 8% of 92 well travellers; EAEC was identified in 30% of 56 travellers with diarrhoea in whom no recognized enteric pathogens could be identified (Mathewson *et al.*, 1985). It was not determined whether this EAEC adherence was similar to the plasmid mediated adherence found in some strains of EPEC. These studies have not yet been repeated elsewhere in either children or adults with diarrhoea.

Cytotoxic *Escherichia coli*

A number of different serotypes of *E. coli* have been reported to produce exoproteins; some of these *E. coli* were shown to cause gastroenteritis either in experimental animals or man. It is not certain whether these exoproteins are distinct or related. With regard to EPEC, Klipstein *et al.*, (1978) showed that culture filtrates of some EPEC induced fluid secretion into perfused segments of rat intestine. As previously mentioned, some EPEC strains produce VT. VT may be similar or even identical to the Shiga-like toxin also reported to be produced by strains of EPEC.

Outbreaks of hemorrhagic colitis characterized by sudden, severe abdominal colic, and grossly bloody diarrhoea in two food related outbreaks in Oregon and Michigan were reported by Riley *et al.* *E. coli* 0157:H7 was implicated in these outbreaks and in a similar outbreak in Canada. Strains of *E. coli* 0157 : H7 isolated in these outbreaks were shown to produce large amount of VT. Karmali isolated *E. coli* 0157:H7 and other VT+ *E. coli* serotypes from 73% of sporadic cases of hemolytic uremic syndrome in Canada. Hemolytic uremic syndrome is the commonest cause of acute renal failure in children in North America. It is an acute febrile illness followed by renal failure and intravascular hemolysis often occurring after an episode of gastroenteritis. In addition to VT and Shiga toxin other cytotoxins have been implicated in diarrhoeal disease in animals. It is not clear how these expoproteins are related.

We have identified as mall number of VT+ 0157 *E. coli* from children with diarrhoea in Thailand. Orasa Suthienkul of the Faculty of Public Health has isolated a phage from the *E. coli* 0157 strain isolated in Michigan and purified and digested this DNA with EcoRI. She is attempting to clone these fragments to determine if any code for VT production. The plan is to develop a specific DNA probe from genes encoding for VT. This would be a valuable tool to access the role of VT- *E. coli* in diarrhoeal disease. She is also comparing the endonuclease digestion fragment pattern of VT-0157 *E. coli* from North America and Thailand.

CONCLUSION

Four different pathogenic mechanisms have been associated with *E. coli* isolated from patients with diarrhoea : enterotoxin production, enteroinvasion, enteroadherence, and cytotoxin production. Assays used to identify the "enteropathogenic *Escherichia coli*" are shown in Table 2. The clinical characteristics and epidemiology of enterotoxigenic *E. coli* has been partially elucidated. Little is known, however, about the clinical manifestations, or epidemiology of *E. coli* with other pathogenic mechanism. What role enteroadherent and cytotoxin producing *E. coli* play in diarrhoea in Thailand will require further investigation. The development of specific DNA probes for these enteropathogenic determinants have provided a tool to identify infections with these *E. coli* that are phenotypically similar to the non-pathogenic *E. coli* that comprise the largest proportion of the bacteria flora of the

gut. Recent development of non-radioactive markers for these probes will allow these methods to be used by investigators without ready access to radioisotopes.

Table 2

Assays used to identify enteropathogenic determinants of *Escherichia coli.*

ETEC

1. Y-1 or CHO cell assay for LT
2. Suckling mouse assay for ST
3. Biken test for LT
4. ELISA for either LT or ST
5. DNA hybridization with enterotoxin gene probes for LT and ST

ETEC

1. Sereny test
2. Invasion of HeLa cells
3. ELISA for VMA
4. DNA hybridization with a 17 kb EcoRI digestion fragment of pWR100

Intestinal adherence*

1. Attachment to HeLa or HEp-2 cells
2. DNA hybridization with probe for localized adherence

Cytotoxin production*

1. Destruction of HeLa cells
2. Destruction of Vero cells
3. ELISA for Shiga toxin (unpublished)
4. DNA hybridization with DNA probes for genes coding for Shiga and Vero toxins (unpublished)

* Both mechanism have been described with *E. coli* of enteropathogenic and non-enteropathogenic serotypes.

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