Nationwide Surveillance Program To Identify Diarrhea-Causing 
*Escherichia coli* in Children in Thailand

RENU SUNTHADVANICH,¹ DUMRONG CHIEWSILP,¹ JITVIMOL SERIWATANA,² RIICHI SAKAZAKI,³ AND PETER ECHEVERRIA²†*

Department of Medical Sciences, Thai Department of Public Health, Nonthaburi,¹ and Armed Forces Research Institute of Medical Sciences, Rajvithi Road, Bangkok 10400,² Thailand, and National Institute of Health, Tokyo, Japan³

Received 31 July 1989/Accepted 14 November 1989

*Escherichia coli* strains isolated from children with diarrhea from 16 hospitals in different districts in Thailand during 1985 and 1986 and submitted to the National Reference Laboratory. Isolates were identified by serogrouping or as enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) adhesin factor (EAF) *E. coli*, or Shiga-like-toxin (SLT)-producing *E. coli* by DNA hybridization. EPEC strains of known serogroups were isolated from 10%, ETEC strains were isolated from 6%, EAF *E. coli* strains were isolated from 4%, EIEC strains were isolated from less than 1%, and SLT-producing *E. coli* strains were isolated from none of 393 children with diarrhea. Among 278 children whose ages were recorded, the highest rate of isolation of EAF *E. coli* was 11% (9 of 85) from children <6 months old. ETEC was isolated from 5% (4 of 85) of children <6 months old, from 10% (12 of 118) of children 6 to 23 months old, and from 1% (1 of 75) of children >23 months old. EPEC strains of known serogroups were isolated from 18% (15 of 85) of children <6 months old, from 11% (13 of 118) of children 6 to 23 months old, and from 9% (7 of 75) of children >23 months old. *E. coli* strains that hybridized with the EIEC probe were isolated from three children who were 20, 36, and 48 months old. Examining *E. coli* for hybridization with DNA probes for virulence determinants is a practical way of conducting nationwide surveillance of diarrhea-causing *E. coli*. Since only 33% (13 of 39) of EPEC serogroups hybridized with the EAF probe and none hybridized with the SLT probes, identification of EPEC by serogroup analysis, followed by serotyping, should continue to be used in the identification of EPEC.

*Escherichia coli* strains that cause diarrhea have been classified into five major categories: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (or Shiga-like-toxin [SLT]-producing *E. coli*), and enteroadherent *E. coli* (18). In South America, most *E. coli* strains that both adhere to tissue culture cells in a localized adherence pattern and hybridize with the EPEC adherence factor (EAF) probe have been reported to be of EPEC serotypes (23, 30). In Thailand, however, only 50% of *E. coli* strains that hybridize with the EAF probe and adhere to HeLa cells in a localized adherence pattern are of EPEC serotypes (7). Most ETEC, EIEC, and enterohemorrhagic *E. coli* strains that have been isolated worldwide are of non-EPEC serotypes (7, 18).

EPEC serogroups are classically identified by agglutination in polyvalent EPEC antisera. Ideally, EPEC serogroups are then serotyped by using monovalent antisera to O and H antigens, but this frequently is not possible because of the cost of antisera and the unavailability of technicians. ETEC, EIEC, and enterohemorrhagic *E. coli* strains and *E. coli* strains that hybridize with the EAF probe (EAF *E. coli* strains) have been identified by in vitro and in vivo assays that require animal or tissue culture facilities (5, 16, 29) or alternatively by enzyme-linked immunosorbent assays that require specific antisera (28, 35). Alternatively, ETEC, EIEC, enterohemorrhagic *E. coli*, and EAF *E. coli* strains can be identified by hybridization with specific DNA probes (1, 3, 7, 8, 22, 23). DNA hybridization assays have been used by research laboratories (6, 27) but not by public health laboratories in tropical developing countries. To evaluate the effectiveness of the use of DNA probes to analyze such isolates, *E. coli* strains isolated from children with diarrhea were forwarded to the Thai National Reference Laboratory for EPEC serogrouping and for hybridization analysis to identify ETEC, EIEC, SLT-producing, and EAF *E. coli*. The hybridization analysis and the in vitro assays were performed by a technician from the National Reference Laboratory under supervision of technicians at the Armed Forces Research Institute of Medical Sciences.

MATERIALS AND METHODS

Specimens. *E. coli* strains were isolated from children with diarrhea at 16 hospitals throughout Thailand between 1 January 1985 and 31 December 1986. Fecal specimens were cultured by standard techniques (17), and five lactose-fermenting *E. coli* strains from each child from whom shigellosa, salmonellae, and vibrios were not isolated were pooled and tested by slide agglutination with polyvalent *E. coli* O:K antisera. Whenever a pool agglutinated, individual colonies were tested for agglutination in *E. coli* O:K polyvalent antisera. Antisera were supplied by the Department of Medical Sciences of the Thai Department of Public Health and were prepared by immunizing rabbits as described by Ewing (13) with Formalin-killed *E. coli* serogroups obtained from the Robert Koch Institute, Berlin, Federal Republic of Germany. These pools contained antisera to 18 *E. coli* O:K serogroups: pool 1, serogroups O25:K11, O26:K60, O44:K74, O55:K59, O78:K80, O111:K38, O114:K--; and O119:K69; pool 2, serogroups O86:K61, O124:K72, O125:K70, O126:K71, O127:K63, and O128:K67; pool 3, serogroups O18a:K77, O20a:K84, O28:K73, and O112a:K66. Strains

* Corresponding author.
† Address for correspondence from the United States: Armed Forces Research Institute of Medical Sciences, APO San Francisco, CA 96346-5000.
that agglutinated in these antisera were mailed to the Department of Medical Sciences for confirmation, usually within 1 week of isolation. These isolates were tested in monovalent O:K antisera before and after being heated at 100°C for 1 h. In this study, *E. coli* O111b, O122, O26, O44, O45, O86, O111, O114, O119, O125, O126, and O127 were considered as EPEC serogroups; *E. coli* O25, O78, and O128 were considered as ETEC serogroups; and *E. coli* O28, O112, and O124 were considered as EIEC serogroups (13).

**Colony hybridization assays.** *E. coli* strains were spotted on MacConkey agar plates (20 isolates per plate), incubated at 37°C overnight, and transferred to Whatman 541 filters by firmly pressing the filters even over the bacterial colonies. The filters were placed in a glass petri dish on Whatman no. 3 paper saturated with 1.0 M ammonium acetate (pH 7) for 4 min and air dried (20). Colonies to be examined with the synthetic heat-stable A (STA) oligonucleotide gene probe were spotted on a nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.) layered on MacConkey agar and incubated at 37°C overnight. Filters were placed on Whatman no. 3 paper saturated with 0.5 N NaOH and 1.5 N NaCl and steamed for 3 min in an autoclave. Filters were then immersed in 1 M Tris–2 M NaCl (pH 7) for 4 min and air dried (20). Colonies to be examined with the synthetic heat-stable A (STA) oligonucleotide gene probe were spotted on a nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.) layered on MacConkey agar and incubated at 37°C overnight. Filters were placed on Whatman no. 3 paper saturated with 0.5 N NaOH for 10 min and then transferred to Whatman no. 3 paper saturated with 1.0 M ammonium acetate–0.2 N NaOH for 1 min. This process was repeated four times, and after the last transfer, the filters were left for 10 min. The filters were then air dried and baked overnight at 65°C (15).

The polynucleotide probes for genes that code for heat-labile toxin (LT) (4). EAF. *E. coli* (23), EIEC (34), and SLTs I and II (25) have previously been described. After purification of plasmid DNA by cesium chloride-ethidium bromide ultracentrifugation, the plasmid DNA was digested with the appropriate endonuclease and separated by polyacrylamide gel electrophoresis. The specific endonuclease digestion fragments used as probes were removed by electroelution and nick translated to incorporate α-32P-labeled nucleotides (21). A 25-mer oligonucleotide probe (5′ AGT CCT GAA AGC 3′) to detect genes that code for STA was constructed by using phosphoramidite chemistry on a DNA synthesizer (Applied Biosystems, Foster City, Calif.) (2) and end labeled with γ-32P-labeled nucleotides (9).

For hybridization with polynucleotide DNA probes, filters were incubated at 37°C for 3 h in 10× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll 400, and 0.02% polyvinylpyrrolidone)–4× SET (1× SET is 0.15 M NaCl, 0.03 M Tris [pH 8], and 1 mM EDTA)–0.1% sodium dodecyl sulfate–10 g of heat-denatured calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) per ml. The filters were transferred to 50% formamide–2× Denhardt solution–4× SET–0.1% sodium dodecyl sulfate–10 µg of heat-denatured calf thymus DNA per ml–6% polyethylene glycol 6000–50 µg of heparin per ml–10× cpm of a DNA probe per ml and incubated at 37°C overnight (10).

For hybridization with the STA oligonucleotide probe, colonies processed on nitrocellulose paper were prehybridized for 2 h at 50°C in 6× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate)–5× Denhardt solution–1 mM EDTA–0.2 mg of heat-denatured calf thymus DNA per ml. DNA hybridization was performed at 50°C overnight in the same buffer with 10× cpm of the radiolabeled oligonucleotide probe per ml. After hybridization, the filters were washed twice in 6× SSC at 25°C for 10 min and then three times in 6× SSC for 30 min at 50°C (9).

Filters were air dried and exposed to X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex Lightning-Plus intensification screen (Du Pont Co., Wilmington, Del.) at −70°C for 24 h. Films were developed as recommended by the manufacturer.

**Standard assays.** To verify the results of nucleotide analysis of isolates that had been stored for over 6 months, *E. coli* strains were tested for enterotoxin production in the Y-1 adrenal cell (29) and suckling mouse (5) assays. In addition, ETEC isolates were tested for mannose-resistant hemagglutination of human group A and bovine erythrocytes and agglutination in colonization factor antigen (CFA) I, CFA II, and E8775 antisera (11, 12, 36); *E. coli* strains that hybridized with the SLT I and II probes were tested for verocytotoxin production (16); *E. coli* strains that hybridized with the EAF probe were tested for mannose-resistant adherence to HeLa cells as described by Scaletsky et al. (31); *E. coli* strains that hybridized with the EIEC probe were tested in the Sereny test (33). ETEC strains and *E. coli* strains that hybridized with EAF and EIEC probes were serotyped at the National Institute of Health in Japan by standard procedures (26).

**RESULTS**

*E. coli* isolates (one per patient) were received from 16 hospitals. EPEC strains of known serogroups were isolated from 18%, ETEC strains were isolated from 6%, EAF. *E. coli* strains were isolated from 4%, EIEC strains were isolated from less than 1%, and SLT-producing *E. coli* strains were isolated from none of 393 children with diarrhea. The isolation rates by age of ETEC and EAF *E. coli* strains as identified with DNA probes and known EPEC serogroups among 278 children whose ages were recorded are shown in Fig. 1. The highest isolation rate of EAF *E. coli* strains was 11% (9 of 85) in children <6 months old. ETEC strains were isolated from 5% (4 of 85) of children <6 months old, 10% (12 of 118) of children 6 to 23 months old, and 1% (1 of 75) of children >23 months old. EPEC serogroups were isolated from 18% (15 of 85) of children <6 months old, 11% (13 of 118) of children 6 to 23 months old, and 9% (7 of 75) of children >23 months old. Three children were infected with *E. coli* isolates that hybridized with the EIEC probe. These *E. coli* strains were isolated from three children who were 20, 36, and 48 months old. One isolate, which could not be serotyped, was positive in the Sereny test, but the other two *E. coli* strains were Sereny test negative. One of these *E. coli* isolates was serotype O28:H12, and the other was serotype O128:H12.
Forty-two percent (164 of 393) of the isolates initially identified at the referring hospitals with pools of *E. coli* O:K polyvalent antisera were confirmed by the Department of Medical Sciences with monovalent *E. coli* O:K antisera after heating of the organisms (Table 1). Of 112 ETEC serogroups identified, 20 were toxigenic; of 41 EPEC serogroups identified, 13 hybridized with the EAF probe; and of 7 EIEC serogroups identified, none hybridized with the EIEC probe. One *E. coli* strain that hybridized with the EIEC probe was identified as *E. coli* O28:H- at the National Institute of Health in Japan. All of the isolates that hybridized with the LT and STA gene probes produced enterotoxin. All of 15 LT- and heat-stable-toxin (ST)-producing ETEC strains, 1 of 3 ST-producing ETEC strains, and none of 6 LT-producing ETEC strains were agglutinated in antisera to CFA I. Eighty-six percent (12 of 14) of *E. coli* strains that hybridized with the EAF probe adhered to HeLa cells in a localized adherence pattern. The *E. coli* serotypes that hybridized with the LT, STA, EAF, and EIEC probes were as follows. Of 24 ETEC isolates, 2 were serotype O78:H- and CFA negative and produced ST, 1 was serotype O126:H12 and CFA I positive and produced ST, 3 were serotype O114:H- and CFA negative and produced LT, 3 were serotype O128:H12 and CFA negative and produced LT, 1 was serotype O128:H- and CFA I positive and produced LT and ST, and 14 were serotype O128:H12 and CFA I positive and produced LT and ST. Of 14 EAF *E. coli* isolates, 1 was serotype O114: H-, 6 were serotype O114:H2, 1 was serotype O115:H45, and 6 were serotype O119:H6. Of three EIEC isolates, one was not typeable and Sereny test positive, one was serotype O28:H- and Sereny test negative, and one was serotype O128:H12 and Sereny test negative.

**DISCUSSION**

The percent isolation by age of ETEC, EAF *E. coli*, and EIEC strains as identified with DNA probes in this national surveillance program was similar to that of prospectively designed studies of diarrheal disease in children in Thailand (6, 7). In this national surveillance program, 17% (4 of 24) of *E. coli* strains that hybridized with probes for enterotoxin genes were EPEC serogroup O114 (n = 3) or O126 (n = 1). These *E. coli* strains were also shown to be enterotoxigenic. In 1976, Gangarosa and Merson (14) argued against continuing to search for EPEC in nonpedemic diarrhea because EPEC strains isolated in the United States and Europe were usually not enterotoxigenic or enteroinvasive. However, conclusions about the virulence determinants of EPEC serogroups isolated in developed countries may not be valid worldwide because some EPEC serogroups isolated in this study in Thailand were enterotoxigenic. Recently, enteropathogenic (23) and, in certain serotypes, SLT production (32) have been identified as virulence determinants of EPEC. EPEC strains have been epidemiologically associated with diarrheal disease most convincingly in young children (19) and were most frequently found in children <6 months old in this nationwide surveillance program.

The two *E. coli* strains that hybridized with the EIEC probe but were Sereny test negative and the two *E. coli* strains that hybridized with the EAF probe but did not adhere to HeLa cells in a localized adherence pattern were presumably mutants that were virulent at the time of initial isolation. Pal et al. (27) reported EIEC isolates that had been stored for extended periods and continued to hybridize with the 17-kilobase probe but became Sereny test negative. The 17-kilobase EIEC probe was, however, 100% specific in identifying EIEC strains tested soon after isolation (7, 8, 33).

Initially, *E. coli* strains were serogrouped by the referring hospitals with pools of polyvalent *E. coli* O:K antisera. However, fewer than 50% of these serogroup results were confirmed at the Department of Medical Sciences; thus, serogrouping by the referring hospitals was discontinued. As with commercial EPEC polyvalent antisera, Thai *E. coli* serogrouping pools contained not only antibodies to EPEC but also antibodies to ETEC and EIEC serogroups. If polyvalent pools are to be used to identify EPEC, they should be EPEC specific.

Hospital laboratories are now asked to submit five lactose-fermenting *E. coli* isolates (if available) from a MacConkey plate culture from each child with diarrhea. All of the *E. coli* strains submitted will be examined with the six DNA probes described above. Two lactose-positive *E. coli* strains and one lactose-negative *E. coli* strain will be examined by the Department of Medical Sciences by agglutination for EPEC serogroups. ETEC, EAF *E. coli*, EIEC, and SLT-producing *E. coli* strains identified with DNA probes and EPEC serogroups will be serotyped by an *E. coli* reference laboratory. In addition, colonization factors and antimicrobial susceptibility will be determined for ETEC, EAF *E. coli*, and EPEC strains. A report of each yearly national surveillance will be compiled and distributed to Thai physicians. This information also can be used to guide the selection of appropriate vaccines for reducing the numbers of infections by diarrhea-causing *E. coli* strains among children in Thailand.

**ACKNOWLEDGMENTS**

We are grateful to the technicians and physicians who collected the specimens examined in this study. We also thank Ornita Sethabutr, Suchitra Changchawali, Orapan Chivoratanond, Thanma Sakulkaipaera, Vitaya Khungvalert, and Songmuang Piyaphong for assistance in this study and Nattakarn Siripraivan for preparing the manuscript.

**LITERATURE CITED**


