Case-Control Study of Diarrheal Disease Etiology in a Remote Rural Area in Western Thailand

Ladaporn Bodhidatta,* Philip McDaniel, Siriporn Sornsakrin, Apichai Srijan, Oralak Serichantalergs, and Carl J. Mason
Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand; Kwai River Christian Hospital (KRCH), Sangkhlaburi, Kanchanaburi, Thailand

Abstract. The objective was to assess the association of enteric pathogens in diarrheal disease in a remote rural area in Thailand. Stool specimens were collected from 236 children aged 3 months to 5 years with acute diarrhea (cases) and from 236 asymptomatic controls. Standard microbiologic methods, and enzyme immunoassay for viral pathogens, *Giardia*, and *Cryptosporidium*, were used to identify enteric pathogens with susceptibility testing by disk diffusion. *Campylobacter*, *Plesiomonas*, *Salmonella*, and enterotoxigenic *Escherichia coli* were commonly isolated from cases and controls (22% versus 25%, 10% versus 11%, 6% versus 9%, and 10% versus 6%, respectively). Only *Shigella*, rotavirus, and adenovirus were identified significantly more frequently in cases than controls (9% versus 0%, 18% versus 3%, and 16% versus 2%, respectively), whereas *Giardia lamblia* was detected less often in cases than controls. Most pre-school children were infested with enteric pathogens; laboratory-based studies are important to understand the epidemiology of enteric pathogens in remote areas among marginal populations.

INTRODUCTION

With over 1.8 million children under 5 years of age dying of diarrheal disease, accounting for 19% of all childhood deaths,¹ acute diarrheal disease is a major cause of morbidity and mortality. The mortality of diarrheal disease has declined substantially from a median of annual death of 4.6 million reported in 1982² to 3.0 million and 2.5 million reported in 1992³ and 2003,⁴ respectively. Because 78% of childhood mortality in developing countries occurred in World Health Organization (WHO) African and southeast Asia regions,¹ it is vital to gain knowledge of the etiologic and epidemiologic features of diarrheal diseases in that area to make it possible to devise diarrheal disease-specific therapeutic measures and control strategies, most prominent of which is the development of a vaccine that could save millions of lives. The capability to detect an etiologic agent is unfortunately lacking in remote rural areas in many parts of the world where diarrheal disease is most prevalent and where most of the mortality occurs.

In this year-long study of diarrheal disease conducted between October 16, 2001 and October 22, 2002, the researchers established a field microbiology laboratory at the Kwai River Christian Hospital (KRCH) in a rural community in western Thailand on the Thai–Myanmar border to obtain stool specimens to describe and compare the distribution of enteric pathogens as well as the antimicrobial susceptibility pattern among pre-school children with and without diarrhea.

METHODS

Study site. This study was conducted from October 2001 to October 2002 in Kanchanaburi province, Sangkhlaburi district, Nongloo subdistrict, located 365 km northwest of Bangkok, Thailand on the Thai–Myanmar border. In this district, six villages are located within a 9-km radius of KRCH and share a main market. Stool samples were collected from pre-schoolers seen at KRCH or one of three nearby health centers.

Enrollment and specimen and data collection. This study was approved by the Ethical Review Committee of Research

in Human Subjects, Ministry of Public Health, Nonthaburi, Thailand and the Human Subjects Research Review Board, US Army Medical Research and Material Command, Fort Detrick, MD.

After obtaining informed consent from a parent or guardian, a stool sample was collected from children aged 3 months to 5 years who came to the hospital or one of the three nearby health centers with complaints of acute diarrhea. Specimens were collected from age-matched children (± 3 months) who came to the hospital or health centers for reasons other than diarrhea within 1 week after the case was enrolled. Controls had no history of diarrhea for the 2 weeks before enrollment. Information on demographic and historical clinical data were obtained from parents or caregivers of both cases and controls and recorded in questionnaires.

Specimen processing and examination. Fresh stool was inoculated onsite onto MacConkey (MC), Hektoen Enteric (HE), thiosulfate citrate bile salts sucrose (TCBS), and modified semi-solid Rappaport Vassiliadis (MSRV) agars and into four enrichment broths (0.5% alkaline peptone water [APW], selenite broth [SB], buffered peptone water [BPW], and modified Doyle). Then, they were incubated at 37°C overnight, except for MSRV, which was incubated at 42°C. Millipore-filtered (0.65 μ m) stool suspension was inoculated on *Brucella* agar with 5% sheep blood (BAP), and stool suspension was inoculated on modified charcoal-cefoperazone deoxycholate agar (mCCDA) for detection of *Campylobacter*; both were incubated under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) at 37°C for at least 48 hours. After overnight incubation of enrichment broths, aliquots of APW were inoculated to TCBS; aliquots of BPW and SB were inoculated to MC, HE, and MSRV; and aliquots of Doyle were inoculated to BAP (after millipore filtration) and mCCDA. Subculture plates were incubated as previously described. *Campylobacter*, *Salmonella*, *Shigella*, *Plesiomonas*, *Aeromonas*, *Vibrio*, and *Arcobacter butzleri* were subsequently identified by standard microbiologic methods at Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand. *Campylobacter* isolates were identified at species level by using conventional phenotypic tests for *Campylobacter*.⁵

For diarrheagenic *Escherichia coli* identification, five lactose-fermenting and up to five non-lactose-fermenting *E. coli* colonies isolated from each child were spotted on nylon membranes N+ (GE Healthcare, Buckinghamshire, UK). The

*Address correspondence to Ladaporn Bodhidatta, Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences (AFRIMS), 315/6 Rajvithi Road, Phayathai, Bangkok 10400, Thailand. E-mail: ladapornb@afirms.org

membranes were processed for hybridization assay with specific Digoxigenin-labeled DNA polynucleotide probes. A heat-labile toxin (LT) and two heat-stable toxin (STIa and STIb) probes were used to identify Enterotoxigenic *E. coli* (ETEC).^{6,7} Invasion-associated locus (ial) probe was used to identify Enteroinvasive *E. coli* (EIEC).⁷ Shiga-like toxin I and II (SLTI and SLT II) probes were used to identify Shiga-like toxin-producing *E. coli* (STEC).⁷ Effacing attachment (EAE), Enteroadherent factor (EAF), and bundle-forming pilus A (bfpA) probes were used to identify Enteropathogenic *E. coli* (EPEC).^{8,9} Three reference *E. coli* strains derived from plasmid harboring enterotoxin genes, pEWD299 (LT), pDAS100 (STIa), and pDAS101 (STIb), were included as positive controls for ETEC. Six reference *E. coli* strains harboring virulence plasmid or virulence DNA fragments, 933J (SLTI), 933W (SLTII), AS-04-1 (EAE), pMSD 207 (*BfpA*), pMAR-22 (EAF), and *E. coli* No. 2 (EIEC), were included as positive controls for the STEC, EPEC, and EIEC, respectively. *E. coli* K-12 Xac was used as a negative control. Digoxigenin-labeling and hybridization assay were carried out according to manufacturer instruction (Roche Diagnostics, Indianapolis, IN).

Rotavirus, astrovirus, and adenovirus were identified by enzyme immunoassay (EIA) test kits (RIDASCREEN; R-Biopharm, Darmstadt, Germany). *Giardia lamblia* and *Cryptosporidium* were also detected by EIA kits (ProSpecT; Remel, Lenexa, KS).

Susceptibility testing. Antibiotic susceptibility testing was performed by a standard disk diffusion technique on all identified bacterial isolates. In the absence of Clinical and Laboratory Standards Institute (CLSI, Wayne, PA) definitive standards for interpreting *Campylobacter* susceptibility results, breakpoints for the family Enterobacteriaceae were used. For *Campylobacter* and other bacterial enteropathogens, azithromycin interpretive standards as published by the disk manufacturer for *Staphylococcus* spp. were used (BBL package insert; Becton-Dickinson, Sparks, MD).

Statistical analysis. Statistical analysis was performed by using a McNemar test on the case-control data. A *P* value of < 0.05 was considered significant.

RESULTS

From October 16, 2001 to October 22, 2002, stool specimens were collected from a total of 472 pre-school children (233 males and 239 females); 236 presented with diarrhea (cases), and 236 were asymptomatic (controls). The median age for cases was 15 months and 14 months for controls; 78% of cases and 79% of controls were less than or equal to 2 years of age, and 53% of cases and 46% of controls were male. Eighty-seven percent (205/236) of controls were children attending a well-child clinic for check-up or routine immunization. The remaining controls were children without diarrhea but with other medical problems. Two percent of controls had received antibiotics by report before the enrollment. Among cases, the mean duration of diarrhea was 48.2 hours, and 69% and 35% were reported to have had a history of fever and vomiting, respectively.

The most common organisms identified in diarrhea cases among these pre-school children were *Campylobacter* spp. (22%), rotavirus (18%), and adenovirus (16%) (Table 1). Although *Shigella*, rotavirus, and adenovirus were detected significantly more often from diarrhea cases as opposed to

TABLE 1
Number and percentage of isolation of enteropathogens in children with diarrhea and non-diarrhea controls in Sangkhlaburi, Thailand

	Cases (N = 236)	Controls (N = 236)	P value*
<i>Campylobacter</i>	51 (22%)	59 (25%)	NS
<i>Salmonella</i>	15 (6%)	21 (9%)	NS
<i>Shigella</i> *	21 (9%)	1 (0.4%)	< 0.0001
Enterotoxigenic <i>E. coli</i>	20/208 (10%)	13/203 (6%)	NS
Enteroinvasive <i>E. coli</i>	4/219 (2%)	0/219 (0%)	—
Enteropathogenic <i>E. coli</i>	33/219 (15%)	31/219 (14%)	NS
<i>Plesiomonas</i>	23 (10%)	27 (11%)	NS
<i>Aeromonas</i>	6 (3%)	8 (3%)	NS
<i>Arcobacter butzleri</i>	2 (1%)	2 (1%)	NS
Rotavirus	41/232 (18%)	7 (3%)	< 0.0001
Astrovirus	7/207 (3%)	3/227 (1%)	NS
Adenovirus	33/207 (16%)	4/227 (2%)	< 0.0001
<i>Giardia lamblia</i>	31/207 (15%)	53/227 (23%)	0.04
<i>Cryptosporidium</i>	4/207 (2%)	12/227 (5%)	NS
No pathogens identified	72 (31%)	76 (32%)	NS

* Analysis for matched-pair case-control data using McNemar test with significant *P* value at < 0.05; NS = not significant.

asymptomatic controls (9% versus 0%, 18% versus 3%, and 16% versus 2%, respectively), *Campylobacter*, *Salmonella*, *Plesiomonas*, *G. lamblia*, and *Cryptosporidium* were identified less often in case patients than in controls (22% versus 25%, 6% versus 9%, 10% versus 11%, 15% versus 23%, and 2% versus 5%, respectively). Of these, *G. lamblia* was the only enteropathogen detected significantly more often in controls than cases (*P* < 0.05). ETEC and EPEC were detected nearly as often in controls as in cases (6% versus 10% and 14% versus 15%, respectively). More than one organism was identified in 82 of 236 (35%) diarrhea cases and 60 of 236 (25%) of controls. No *Vibrio* species or STEC was detected in this study. No enteric pathogens were identified in 31% and 32% of cases and controls, respectively (Table 1).

Among 57 *Campylobacter* isolates from 51 cases and 67 isolates from 59 controls, 70% versus 54%, 12% versus 24%, and 16% versus 22% were *C. jejuni*, *C. coli*, and *C. upsalienses*, respectively. Eighty-six percent (18/21) of *Shigella* isolates from cases were *S. flexneri*, and the rest were *S. sonnei*. The most common serotype was *S. flexneri* 3a, identified in 13 of 21 (62%) cases. The one *Shigella* isolate from one control was *S. dysenteriae* type 4. Of the 23 ETEC isolates from 20 cases, 48% had ST, 17% had LT, and 35% had LT and ST. Of the 13 ETEC isolates from 13 controls, 31% had ST, 54% had LT, and 15% had LT and ST.

The age-specific prevalence of identified organisms is given in Table 2. There was no significant difference in prevalence in cases compared with controls for all age groups for *Campylobacter*, *Salmonella*, *Plesiomonas*, and EPEC. Rotavirus and adenovirus were most commonly found in children less than 12 months old with diarrhea (20%), and controls clearly had low recovery (0–6%). *Shigella* was most often (23%) found in the case children over 2 years of age with diarrhea, whereas none was found in controls. *G. lamblia* was more commonly detected among controls than in cases, and interestingly, prevalence increased with age.

The pattern of antibiotic resistance for bacterial pathogens isolated from cases and controls is shown in Table 3; because the pattern differed little in cases and controls, data were not analyzed separately. Ciprofloxacin was very effective against all bacterial pathogens except *Campylobacter*, for which resistance was detected in 38% of isolates. No resistance to azithromycin was observed among *Campylobacter* isolates. Nalidixic

TABLE 2
Number and percentage of pathogens isolated from children by age in Sangkhlaburi, Thailand

Age	3–12 months		13–24 months		25–59 months	
	Cases (N = 98)	Controls (N = 109)	Cases (N = 86)	Controls (N = 78)	Cases (N = 52)	Controls (N = 49)
<i>Campylobacter</i>	21 (21%)	27 (25%)	21 (24%)	24 (31%)	9 (17%)	8 (16%)
<i>Salmonella</i>	6 (6%)	6 (6%)	4 (5%)	9 (12%)	5 (10%)	6 (12%)
<i>Shigella</i>	3 (3%)	1 (1%)	6 (7%)	0 (0%)	12 (23%)	0 (0%)
Enterotoxigenic <i>E. coli</i>	6/84 (7%)	0/97 (0%)	6/78 (8%)	6/65 (9%)	8/47 (17%)	7/41 (17%)
Enteropathogenic <i>E. coli</i>	12/91 (13%)	19/105 (18%)	13/80 (16%)	8/71 (11%)	8/48 (17%)	4/43 (9%)
<i>Plesiomonas</i>	5 (5%)	9 (8%)	5 (6%)	9 (12%)	13 (25%)	9 (18%)
Rotavirus	19/97 (20%)	7 (6%)	14/85 (17%)	0 (0%)	8/50 (16%)	0 (0%)
Adenovirus	18/85 (21%)	3/103 (3%)	8/79 (10%)	0/77 (0%)	7/43 (16%)	1/47 (2%)
<i>Giardia lamblia</i>	5/85 (6%)	10/103 (10%)	12/79 (15%)	28/77 (36%)	14/43 (33%)	15/47 (32%)
No pathogens identified	35 (36%)	39 (36%)	27 (31%)	25 (32%)	10 (19%)	12 (25%)

acid resistance was observed in 18% of *Salmonella*, 46% of *Campylobacter*, 12% of *Plesiomonas*, and 0% of *Shigella* or ETEC isolates (Table 3).

DISCUSSION

Determination of the true etiological role of enteric pathogens detected in children with diarrhea in a developing country is difficult, because enteric pathogens are also frequently identified in apparently healthy children. Moreover, it is not uncommon to find more than one pathogen in one child, and therefore, it is rarely possible to define which is causing disease. Asymptomatic infections are widely reported in developing countries in both community and clinic-based studies.^{10–16} Acquired immunity from frequent previous exposures, long-term convalescent shedding of the organism, or exposure to insufficient amounts of an organism to cause disease are the main explanations for asymptomatic carriers.^{15,17–19} Several studies have reported a high prevalence of *Salmonella*, *Giardia*, and *Campylobacter* in children without diarrhea in developing countries.^{16,20,21} Recent data from Vietnam reported 23% of diarrheagenic *E. coli* in both diarrhea cases and controls.¹² The data obtained in this study support those findings, and *G. lamblia* was detected in a significantly higher proportion of controls than in cases. This was also observed in a case-control study in Bangladesh with no clear explanation.¹⁶

The proportions of cases and controls with pathogens identified were similar at approximately 70%. A previous clinic-based study of diarrhea etiology conducted in Bangkok in 1985 reported lower pathogen carriage in controls of 30%. In the same study, *Shigella*, *Campylobacter*, *Salmonella*, ETEC, EIEC, *Cryptosporidium*, rotavirus, and adenovirus were all found to be causative agents of diarrhea illness.¹⁴ Organisms

that have been consistently and substantially reported more frequently in children with diarrhea than in controls were ETEC, *Shigella*, and rotavirus.^{11,16,21,22} In this study, only four organisms, *Shigella*, rotavirus, adenovirus, and ETEC (in children less than 12 months), were significantly associated with acute diarrheal disease in this pre-school population, but these 4 pathogens accounted for approximately 50% of all cases. Among these, antibiotic administration is required only for children with shigellosis as per WHO treatment guideline; for acute diarrhea, the recommendation is the use of antibiotic only in suspected cases of shigellosis and cholera.²³

This draws attention to the potentially unnecessary administration of antibiotics for the treatment of diarrheal disease. This can be problematic, both in terms of economic burden related to cost of medications and healthcare and as a contributor to antibiotic resistance. For example, *Shigella* isolates had a high prevalence of resistance to trimethoprim-sulfamethoxazole (TMP-SXT), the antibiotic previously used for treatment of diarrheal disease in Thailand. This study also observed resistance to nalidixic acid among all bacterial species, although ciprofloxacin resistance was detected only in *Campylobacter* isolates (38%). A high prevalence of nalidixic acid and fluoroquinolone-resistant *Campylobacter* has been well-documented in Thailand.^{24–26} Resistance of *Campylobacter* isolates to nalidixic acid increased significantly during 1991–2000, with isolates resistant to both nalidixic acid and ciprofloxacin observed significantly more frequently in urban than in rural settings.²⁴ Ongoing antimicrobial surveillance may permit more effective therapy in remote areas.

In conclusion, this study, undertaken in a remote area of Thailand, found the major causes of acute diarrhea in children less than 5 years of age to be rotavirus, adenovirus, and *Shigella* spp. Although a high proportion of mixed infections and asymptomatic carriers were reported, similar to other case-control studies in developing countries, 70% of pre-school children in our study were infested with at least one potential enteric pathogen, a greater number than expected. Laboratory-based studies that include non-diarrhea controls are important to gain a more complete understanding of the epidemiology and distribution of enteric pathogens to enable development of effective programs aimed at disease prevention and health promotion targeting remote areas and marginal populations.

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TABLE 3
Number and percentage of antibiotic resistance of bacterial pathogens isolated from children in Sangkhlaburi, Thailand

Pathogen	<i>Salmonella</i> (N = 38)	<i>Shigella</i> (N = 22)	<i>Campylobacter</i> (N = 126)	<i>Plesiomonas</i> (N = 50)	ETEC* (N = 44)
Ampicillin	11 (29%)	9 (41%)	14 (11%)	13 (26%)	24 (55%)
TMP-SXT†	8 (21%)	21 (95%)	56 (44%)	4 (8%)	18 (41%)
Tetracycline	19 (50%)	21 (95%)	27 (22%)	12 (24%)	30 (68%)
Nalidixic acid	7 (18%)	0 (0%)	58 (46%)	6 (12%)	0 (0%)
Ciprofloxacin	0 (0%)	0 (0%)	48 (38%)	0 (0%)	0 (0%)
Azithromycin	2 (5%)	1 (5%)	0 (0%)	0 (0%)	2 (5%)

* Enterotoxigenic *E. coli*.

† Trimethoprim-sulfamethoxazole.

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Authors' addresses: Ladaporn Bodhidatta, Siriporn Sornsakrin, Apichai Srijan, Oralak Serichantalergs, and Carl J. Mason, Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences, Phyathai, Bangkok, Thailand, E-mails: ladapornb@afirms.org, siriporns@afirms.org, apichais@afirms.org, oralaks@afirms.org, and carl.mason@afirms.org. Philip McDaniel, Kwai River Christian Hospital, Sangklaburi, Kanchanaburi, E-mail: krchphil@gmail.com.

Reprint requests: Ladaporn Bodhidatta, Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences (AFRIMS), 315/6 Rajvithi Road, Phyathai, Bangkok 10400, Thailand, E-mail: ladapornb@afirms.org.

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