Epidemiol. Infect. (2001), **127**, 229–236. © 2001 Cambridge University Press
DOI: 10.1017/S0950268801005933 Printed in the United Kingdom

Prospective study of the incidence of diarrhoea and prevalence of bacterial pathogens in a cohort of Vietnamese children along the Red River

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(*Accepted 11 May 2001*)

SUMMARY

We prospectively studied diarrhoea incidence among 1655 children < 5 years of age in northern Vietnam for 1 year using primarily passive surveillance. Standard culture methods were used to detect bacterial pathogens. Overall 2160 cases occurred (1·3 cases/child per year). Peak rates of diarrhoea occurred in children < 12 months old. Rates ranged from 3·3 cases/child per year in children < 1 year old, to 0·7 cases/child per year in 4-year-olds. Campylobacter, shigella and enterotoxigenic *Escherichia coli* were most commonly isolated. Rates detected by active surveillance were about twice those detected passively. *S. flexneri* was the most common shigella serogroup (65%). *S. flexneri* serotypes 6, 4, 1 and Y were most common, but 40% were untypable using commercial antisera. The data illustrate important regional differences in pathogen prevalence and shigella serotype distribution. Shigella vaccine development strategies, commonly targeting *S. flexneri* 2a, *S. sonnei* and *S. dysenteriae* 1, will have little impact on diarrhoea rates in Vietnam.

INTRODUCTION

Much progress has been made relative to treatment and prevention of acute watery diarrhoea in recent years. Aggressive oral rehydration therapy, perhaps in combination with renewed emphasis on the importance of breast-feeding and handwashing has been associated with small, but significant decreases in mortality due to diarrhoeal illness worldwide [1], though incidence of disease remains stable. Concurrently, however, widespread growth of antimicrobial resistance among bacterial enteric pathogens [2] is cause for concern about the ability to extend this success to dysenteric disease. Additionally, socioeconomic conditions and poor hygiene in areas most at risk for diarrhoea suggest that incidence reductions will be difficult and expensive to achieve.

Inexpensive, effective vaccines directed against bacterial enteric pathogens may offer a public health intervention that is cost-effective and attainable, but necessary information relating to diarrhoea burden and prevalence of bacterial pathogens is lacking for many parts of the world. Vaccine protection against shigella, for example, is reportedly serotype-specific [3], but shigella serotype prevalence varies considerably from one region of the world to another and is unknown in many areas. Enterotoxigenic *Escherichia coli* (ETEC) and *Campylobacter jejuni*, both pathogens for which vaccine development efforts are underway, offer similar challenges in terms of immune specificity and inadequacy of prevalence data.

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In a recent review of the global burden of shigella infections, Kotloff et al. [4] attempted to gather the necessary data to support rational shigella vaccine development efforts. Examination of the studies used for this estimate point out some unfortunate limitations in that effort. For example, shigella group and serotype distribution estimates for the East Asia and Pacific region were based on data from 5 studies: 3 from Thailand, 1 from Singapore and 1 from Malaysia. The current burden of disease due to shigella in Thailand and Singapore, however, is relatively low (campylobacter, ETEC and salmonella represent the predominant enteric bacterial pathogens in Thailand [5]. A shigella vaccine effort in those countries would have minimal impact on the burden of disease and is unlikely to be implemented. Furthermore, serotype distribution in relatively developed Thailand is largely irrelevant for other developing countries in the region, such as Vietnam, Laos and Cambodia, where the burden of shigella is presumably far greater due to poverty, malnutrition and other factors [6, 7]. Furthermore, unpublished data from our laboratory in Bangkok suggests that shigella group distribution is changing in Thailand from what it was in the 1980s. *Shigella sonnei* now constitutes approximately two-thirds of shigella isolates, reconfirming the group shift from *S. flexneri* predominance thought to accompany improved hygiene levels associated with industrialization [8]. It is also not clear that serotype distribution is stable even within regions. Studies performed 20 years ago may not reflect current serotype prevalence.

Based on data largely from the 1960s and 1970s [9–11], Vietnam is thought to have a relatively high burden of shigella diarrhoea. Sheehy [9] reported that diarrhoeal diseases were responsible for more hospital admissions than any other disease except 'fevers of undetermined origin' among US Army personnel stationed in Vietnam. Sullivan [10] reviewed over 27000 diarrhoea stool cultures from US soldiers and Vietnamese obtained between 1966 and 1969, finding that *Shigella* spp. and *Vibrio cholera* accounted for most isolates (37% and 39% respectively), followed by enteropathogenic *E. coli* (15%) and salmonellae (9%). Of the 2760 shigella isolates in this study, group B (*S. flexneri*) constituted 85%, with serotype 2 representing 66% of these.

Current data on bacterial diarrhoea in Vietnam are sparse, however. Nishio [12] reported that 50% of 158 Vietnamese infants with diarrhoea in Ho Chi Minh City tested positive for rotavirus, but did not provide data on bacterial pathogens. Ngan [13] described 83 children from Hanoi with persistent diarrhoea (> 14 days duration), finding ETEC in 24%, enteropathogenic *E. coli* in 8%, rotavirus in 5% and giardia in 4%. Surprisingly, no shigella, campylobacter or salmonella were found in this study. Thanh [14], in a retrospective case series of acute and persistent diarrhoea in 3833 persons in Ho Chi Minh City, found that 40% of persistent diarrhoea was grossly bloody, but did not include stool culture data. Van Tran [15] conducted a questionnaire-based survey of morbidity, calculating diarrhoea rates of 1·6 episodes/child-year for children under 5 years of age. Again, no pathogen prevalence data was presented in this study. Finally, Lindberg [16], in a seroepidemiologic case-control study of shigella disease in various communities around Hanoi, found that the most common serotype of *S. flexneri* was type 1b (75%), followed by type 2a (14%) and type 4a (7%). The sampling method used in this study was not well described, however, making it difficult to understand what the true group and serotype prevalence actually was in these communities, and no estimate of incidence was attempted.

No prospective studies describing the incidence of diarrhoea morbidity and mortality, or bacterial pathogen prevalence in Vietnamese cohorts are published in the English language medical literature. The current study examines these issues in a cohort of children less than 5 years of age from communities located along the Red River northwest of Hanoi.

METHODS

This study was conducted from August 1998 through July l999 in three communes (Phu Chao, Phu Phuong, Chao Son) of the Ba Vi District in Son Tay Province, located along the Red River approximately 50 kilometers northwest of Hanoi, Vietnam. Approval was obtained prior to beginning the study from the Scientific Review Board of the Armed Forces Research Institute of Medical Sciences (AFRIMS), and from the Human Use Research Review Board of the Walter Reed Army Institute of Research (WRAIR). A review of human use was also undertaken and approved by the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi, Vietnam. Informed consent for the children to participate in the study was obtained from the children's parents by Vietnamese-language speaking investigators prior to enrolling in the study.

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Ba Vi District is a relatively poor agricultural community including several communes. Each of the communes contains 1–3 villages. Within each village are 4–5 hamlets, with 15–20 households per hamlet. The average size of a household is 4–6 persons. The water supply for 2 of the 3 communes studied (Phu Phuong, Chao Son) is provided by deep wells on each household. One commune (Phu Chao) is located outside the dike that prevents flooding from the Red River, using surface water from this river as it's primary water source. About 10% of households have sanitary latrines. Each commune is served by one health center, staffed by five community health workers. In addition, a District Health Center includes the District Hospital, which serves all of the communes and houses the local study laboratory. As these are the only health care facilities in the District, all cases of diarrhoea serious enough to warrant seeking medical attention should have been captured by the study. Approximately 1800 children under 5 years old live in the three communes, and all were eligible for participation in the cohort.

Passive surveillance (health center and hospital based) was conducted for the entire 12 months of the study. Following enrolment, all study subjects seeking care for diarrhoea, and/or the parent/guardian of the child, were asked about symptoms and signs of enteric infection from a standardized questionnaire, administered by the clinician (physician or nurse) attending to the child. Each child also had a stool or rectal swab sample obtained for culture of bacterial enteric pathogens prior to initiating therapy. Responses and clinical observations were recorded on standardized case report forms, that were later entered into a computer data base custom designed for the study. Medical management of illness was left to individual health care providers, but guidelines of the World Health Organization were generally followed, including the provision of oral rehydration therapy for acute watery diarrhoea, reserving antimicrobial therapy for dysenteric disease. Subjects were then followed up daily at home until resolution, defined as failure to meet the criteria for definition of diarrhoea for a period of three sequential days. Participants in the study were encouraged to seek treatment for diarrhoea, and were provided treatment free of charge at the local health clinics.

Active surveillance supplemented passive surveillance for two of the months (August and January) in a cost-conscious attempt to estimate rates of less severe disease that may not necessitate a visit to the health clinic, thus not be included when using passive surveillance methods. These months were chosen because diarrhoea rates generally peak during the rainy season from May to September, and trough during the dry season from November to March. We chose a representative month in each period. During periods of active surveillance, health workers visited each volunteer home twice weekly, inquiring as to occurrence of diarrhoea since the last visit. If diarrhoea was reported, an evaluation similar to that for passively reported cases was undertaken, including stool or rectal swab obtainment for culture, and daily follow-up until symptom resolution was documented for 3 days.

Diarrhoea was defined as (a) history of an obvious change in the normal stool pattern, characterized by 3 or more loose stools in a 24 h period regardless of other gastrointestinal symptoms; (b) 2 or more loose stools associated with at least 1 other symptom of gastrointestinal infection (abdominal pain, cramping, nausea, vomiting, fever); or (c) passage of a single loose stool with grossly evident blood/mucous. To be considered a new episode of diarrhoea, at least 3 intervening days of normal stools without other gastrointestinal symptoms or fever must have passed between diarrhoea occurrences.

A 10% sample of control stool cultures was obtained from asymptomatic cohort members. Cohort members assigned to each health worker were randomly ordered at the beginning of the study period. For each 10th case of diarrhoea that occurred, a control stool was obtained from the next child on the list who was not (a) living in the same house as the case subject; (b) taking antibiotics in the preceding 2 weeks; (c) previously a provider of a control stool sample; or (d) possessing diarrhoea symptoms during the preceding 2 weeks.

Stool culture methods employed were as follows. Identified cases and controls were asked to provide a stool sample, from which a stool swab was obtained. If the subject could not provide a timely stool sample, a rectal swab was obtained. Presence or absence of grossly evident blood/mucous was noted. The stool/rectal swabs were placed in Cary–Blair transport media and taken to the District Hospital for plating onto MacConkey, Hektoen Enteric, TCBS and Brucella (with 5% sheep blood) agars for overnight incubation. Samples were also inoculated into Selenite F broth, alkaline peptone water and Doyle's enrichment broth. Following overnight incubation, specimens were subcultured and refrigerated at 4 °C

until transport to the National Institute of Hygiene and Epidemiology (NIHE) in Hanoi for definitive identification. Separate swabs in Cary–Blair media were also shipped by overnight airfreight once weekly to the AFRIMS laboratory in Bangkok, Thailand for confirmatory culture and *E. coli* diagnosis. Culture was pursued for *Salmonella*, *Shigella*, *Aeromonas*, *Plesiomonas* and *Vibrio* species. Campylobacter was isolated using a membrane filter method on brucella agar before and after enrichment. All control specimens, and a 20% systematic sample of case specimens were tested for *E. coli* pathogens as follows. Five lactose-fermenting and five non-lactose-fermenting *E. coli* colonies per specimen were tested for production of heat-labile (LT) and heat-stable (STIa, STIb) enterotoxin. Enteroinvasive *E. coli* (EIEC) and enterohaemorrhagic *E. coli* (EHEC) were identified using specific DNA probes for the invasion plasmid, SLT I and SLT II [17, 18].

Diarrhoea cumulative incidence was calculated overall and stratified by specific pathogens, age in years, commune and month of occurrence. Due to 20% sampling of stools for *E. coli* diagnosis, rates due to *E. coli* pathogens were estimated by multiplying the actual number of cases by five. Denominators for each age group were based on age at time of study initiation, rather than actual person-time. Statistical analysis of incidence rates was performed using the asymptotic test (Mantel–Haensel) for person-time data, and stratified Poisson rate analysis (both using StatXact version 4.0.1, Cytel Corp.); and for comparison of proportions, the χ^2 test (with Yates correction as appropriate; Epi info version 6.04).

RESULTS

Of the roughly 1800 children under the age of 5 years living in the three communes, 1655 were enrolled into the study and followed for 1 year (Table 1). The median age of cohort members was 35 months (range 0–59 months), 52·6% of whom were males. Forty-eight percent of subjects were from Phu Chao, 29% from Phu Phuong, and 22% from Chao Son. No dropouts and no deaths occurred among cohort members during the period of follow-up.

Clinical data

Two thousand one hundred and sixty cases of acute diarrhoea occurred among 1055 of the 1655 enrolled children (64%) during follow-up. Of those children with diarrhoea, 531 (50·3%) experienced two or more episodes (range 0–12 cases/child). The median duration of diarrhoea was 2 days (range 1–9 days). The mean maximum number of stools per day was 4·3 (S.D. 1·4). Seven percent of case stools had grossly evident blood, while 47% had mucous. Abdominal pain was reported in 64% of cases, and fever in 22%. Control stools were obtained from 203 unmatched asymptomatic subjects.

Table 1. *Baseline biographical data of enrolled subjects* (n = *1655*)

Age	Median	35 months
	Range	0–59 months
	0–11 months	277 (16·7%)
	12–23 months	289 (17·5%)
	24–35 months	308 (18·6%)
	36–47 months	332 (20·1%)
	48–59 months	449 (27·1%)
Sex	Male	52·6%
	Female	47·4%
Commune	Chao Son	370 (22·4%)
	Phu Chao	798 (48·2%)
	Phu Phong	487 (29·4%)

Diarrhoea incidence

The relationship of age and aetiology with the incidence of diarrhoea is shown in Table 2. All-cause diarrhoea incidence peaked among the youngest children (< 12 months of age), with rates of 3·23 episodes per child-year, dropping off steadily to 0·7 episodes per child-year in 4 year-olds. Pathogen-specific rates due to shigella, campylobacter and ETEC showed a similar age-related pattern, with rates peaking among infants less than 12 months old at about 0·2, 0·3 and 0·3 episodes per child per year respectively. Overall diarrhoea incidence for all children < 5 years old was 1·3 episodes per child per year.

Figure 1 demonstrates the monthly incidence of diarrhoea. The 2 months of active surveillance are indicated by light bars, and demonstrate a roughly two-fold increase in rates compared to adjacent passively surveyed months. During passively surveyed months (dark bars), rates were lowest during the dry season months of November and December, and highest during the wet season in June and September, but otherwise marked seasonal variation was not evident.

https://doi.org/10.1017/S0950268801005933 Published online by Cambridge University Press

Table 2. *Cumulative incidence of diarrhoea* (*cases* (*per child per year*))

Age* (months)	All causes	Shigella	Campylobacter	ETEC (est†)
0–11 ($n = 277$)	901 (3·25)	49 (0·18)	77 (0·28)	70 (0·25)
12–23 ($n = 289$)	416 (1·44)	28 (0·10)	30 (0·10)	40 (0·14)
24–35 ($n = 308$)	279 (0·91)	15 (0·05)	15 (0·05)	5 (0·02)
36–47 ($n = 332$)	246 (0·74)	18 (0·05)	18 (0·05)	15 (0·05)
48–59 ($n = 449$)	307 (0·68)	21 (0·05)	7 (0·02)	10 (0·02)
0–59 ($n = 1655$)	2160‡ (1·31)	133‡ (0·08)	148‡ (0·09)	140 (0·08)

* At time of study onset.
† ETEC diagnosis pursued on a 20% systematic random sample of specimens. Estimated rates are based on actual isolates $\times 5$.
‡ Total exceeds sum of age-specific cases due to age-data collection shortfall.

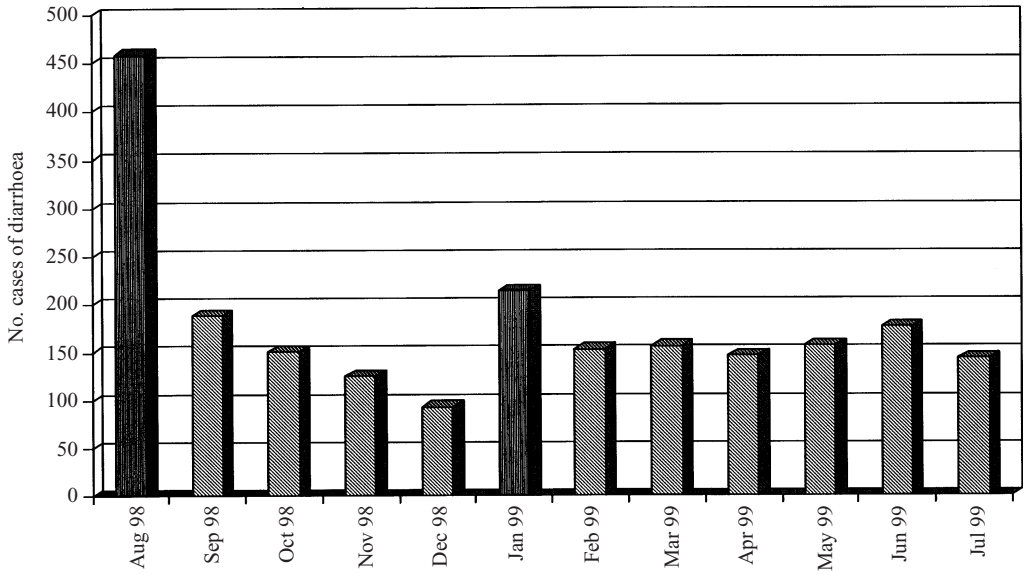

Fig. 1. Monthly cases of diarrhoea in Ba Vi, Vietnam. Dark bars indicate months of active surveillance; light bars indicate months of passive surveillance.

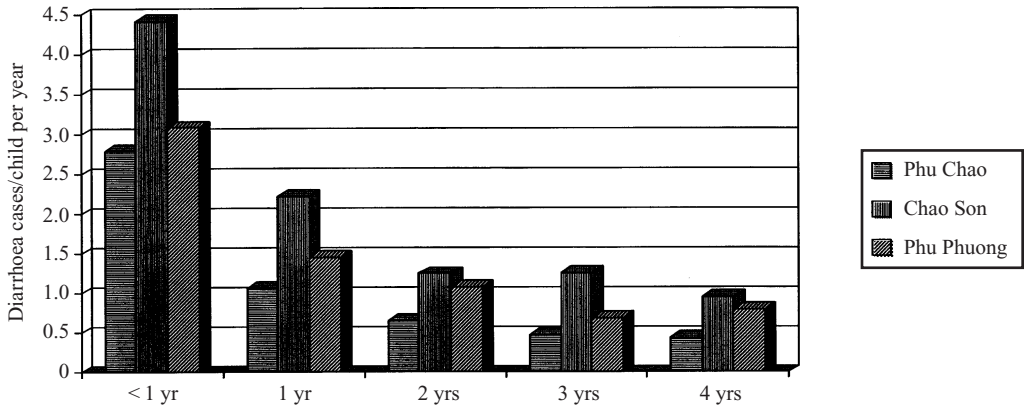

Fig. 2. Diarrhoea cumulative incidence (cases/child per year) stratified by age and commune.

The relationship of commune to diarrhoea rates, stratified by age, is shown in Figure 2. Rates were consistently and significantly lowest in Phu Chao commune, outside the Red River dike (relative risk 1·3; 95% CI 1·1–1·5, versus pooled data from Phu Phuong and Chao Sun). No association was found

https://doi.org/10.1017/S0950268801005933 Published online by Cambridge University Press

Table 3. *Microbiology*

	Cases ($n = 2160$)			Controls ($n = 203$)			
Pathogen	Isolates	% Cases	% Isolates	Isolates	% Controls	% Isolates	PRR
Culture (+) (adjusted*)	347 (484*)	22·4*	—	22	10·3	—	2·0
Shigella	143	6·1†	30	3	1·5	14	4·3
Group A	10	0·5	2	1	0·5	5	1·0
Group B	93	4·3†	19	0	0	0	⩾ 8·2
Group C	24	1·1	5	2	1·0	9	1·1
Group D	16	0·7	3	0	0	0	⩾ 1·4
Campylobacter	150	6·8	31	8	3·9	36	1·8
C. jejuni	126	5·8†	26	5	2·5	23	2·3
C. coli	24	1·1	5	3	1·5	14	0·7
ETEC	25 (140*)	6·5*	29	9	4·4	41	1·5
LT	16 (80*)	3·7	17	6	3·0	27	1·2
ST	6 (30*)	1·4	6	0	0	0	⩾ 2·8
LTST	6 (30*)	1·4	6	3	1·5	14	0·9
EIEC	5 (25*)	1·2	5	0	0	0	⩾ 2·4
Salmonella (NT)	17	0·8	4	2	1·0	9	0·8
Other	9			0			

* Due to 20% sampling of *E. coli* diagnoses in case specimens, adjustment based on multiplying ETEC/EIEC diagnoses × 5.
† $P < 0·05$ compared to controls.

between commune and prevalence of specific pathogen (Mantel–Haensel Summary χ^2, $P = 0·7$).

Microbiology

Of 2160 specimens, 347 (484 after adjustment for *E. coli* sampling) yielded a bacterial pathogen (22·4% adjusted yield) (Table 3). Twenty-two (10·3%) of 203 control stools also grew bacterial pathogens (Table 3). Campylobacter was the most commonly identified pathogen (31% of isolates), followed by shigella (30%) and ETEC (29%). EIEC represented about 5% of isolates, and EHEC was not found in this study. Infections with shigella and *Campylobacter jejuni* were significantly associated with disease.

While shigella, campylobacter and ETEC each represented about a third of isolates overall, there were differences in age-related importance. Shigella represented about 25% of bacterial isolates among 0–11 months old children, but 56% of isolates in 4 year olds. Campylobacter and ETEC represented 39% and 36% respectively of isolates in 0–11 month-olds, but only 18% and 26% respectively in 4 year olds.

S. flexneri constituted 65% of all shigella isolates, followed distantly by *S. boydii*, *S. sonnei* and *S. dysenferiae* (17%, 11% and 7% respectively). Of the *S. flexneri* isolates, serotype 6 was the most common (17%), followed by type 1 (13%), type 4 (10%), variant Y (9%), type 2 (6%) and type 3 (4%). Forty percent of isolates were unable to be typed with commercially available (Denka-Seiken) antisera against types 1-6, X and Y.

DISCUSSION

This study is the first in the literature to evaluate prospectively the incidence of diarrhoea in rural Vietnamese children. Passive surveillance for 1 year, supplemented by active surveillance during August and January, detected 1·3 cases per child per year overall for children under 5 years of age. As active surveillance roughly doubled case-detection rates, the actual overall incidence assuming active surveillance for a full year, is roughly approximated at 2-3 cases per child per year. These rates are intermediate in Southeast Asia between those in relatively developed urban Thailand (0·9 cases/child per year) [19] and undeveloped rural Bangladesh (4·6 cases/child per year) [20]. It should be emphasized that incidence and distribution of pathogens may differ between children in urban versus rural settings. Thus, this study may not be reflective of disease in Hanoi, for example. Rates in our study were heavily age-dependent for all major pathogens, with children less than 12 months of age experiencing nearly five-fold higher incidence

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overall than 4 year olds. This age-related pattern is expected for campylobacter and ETEC, but not for shigella, which generally peaks in incidence during the 2nd and 3rd years of life [20, 21]. It is unknown why this pattern exists in Vietnam and not in other developing countries.

Other studies have also documented the seasonality of diarrhoea incidence in the tropics [21, 22], increasing in the wet rainy season from April to September, and declining in the cooler dry months. This is presumably due to the impact of flooding on the spread of faecal waste into drinking water supplies. While our study demonstrated peak rates of diarrhoea in the wet season, rates were not higher in the commune (Phu Chao) that was most at risk from flooding, due to its unprotected location outside of the Red River dike. Indeed, rates were consistently lowest, across all age strata, in Phu Chao, suggesting that perhaps flooding is not the explanation for increased rates at this time.

Shigella species and serotype prevalence appears to have changed since 1967, when Sullivan [10] found that *S. flexneri* represented 84·7% of shigella isolates overall, *S. sonnei* 9·5%, *S. boydii* 3·0% and *S. dysenteriae* 2·8%. Our study found a significantly higher proportion of *S. boydii* (17%; $P < 0{\cdot}01$), and lower proportion of *S. flexneri* (65%; $P < 0{\cdot}01$). Among *S. flexneri* isolates, Sullivan found that serotype 2 represented 66%, followed by types 3, 1 and 4 (16%, 10% and 5% respectively). Our study found that type 2 was much less common, representing only 6%, and that types 6, 1, 4 and variant Y were most common (17%, 13%, 10%, and 9% respectively). Furthermore, fully 40% of *S. flexneri* were not able to be typed using a standard commercial typing reagent (Denka-Seiken). Closer examination of these untypable isolates is warranted to clarify their relationship to known serotypes.

These serotype data raise concern for vaccine development strategies aimed at preventing bacillary dysentery. Currently, shigella vaccines are being developed which target *S. flexneri* 2a, *S. sonnei* and *S. dysenteriae* 1 [4, 23, 24], none of which caused much disease in this study. The choice of *S. flexneri* 2a as a target is based on data compiled over the past 20–30 years suggesting that type 2a accounts for about half (45%) of Group B shigella disease worldwide (4). Our data from Vietnam raise the possibility that Group B serotype prevalence may be changing in some parts of the world, and that updated prevalence data are needed to aid in vaccine-targeting strategies.

There are several weaknesses in our study design that should be addressed. Firstly, passive surveillance generally identifies disease serious enough to warrant seeking medical attention, and thus underestimates overall morbidity in the community. Though limited by available funds, we attempted to understand true morbidity by conducting active surveillance during 1 month each in the wet and dry seasons. While this allowed us some insight into true incidence rates (roughly double that detected with passive surveillance alone), it does not provide truly accurate rate estimates, thus cannot fully replace a full year of continuous active monitoring. Secondly, sampling of cases (20%) for *E. coli* diagnosis reduces power to detect statistically significant differences in rates for these pathogens. Lastly, our specimen collection and transport requirements, involving use of transport media, inconsistent refrigeration due to power outages, and delayed plating in remote locations, likely were responsible for the relatively low yield of bacterial pathogens. Under optimal conditions, one might expect a roughly 50% yield for bacterial pathogens in acute diarrhoea stool specimens [18], the other half presumably due to unculturable bacteria, viral, protozoal or other causes [12]. Shigella and campylobacter, in particular, are notoriously fragile thus our data likely underestimates their relative importance in this community.

Overall however, these data provide valuable insight into diarrhoeal disease morbidity and aetiology in an understudied area of the world. Further study evaluating antimicrobial resistance patterns, and shigella serotype distribution in Vietnam and elsewhere in South and Southeast Asia could clarify treatment approaches and vaccine development strategies. Closer serologic and molecular analysis of the untypable *S. flexneri* isolates may help understand their relationship to known serotypes. These studies are ongoing.

ACKNOWLEDGEMENTS

We wish to thank Nguyen Mai Lam and Hoang Duc Hanh of the Ba Vi District Hospital, and Nguyen Thi Gam and Nguyen Thi Be of NIHE, for their gracious efforts in support of this study. From AFRIMS, we thank Apichai Srijan, Chittima Pitarangsi and Orapan Chivaratanond for microbiologic specimen processing, Duangchai Lamson for database management, and Anan Wongpinyochit for administrative and logistical support.

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