Detection of *Shigella* by a PCR Assay Targeting the *ipaH* Gene Suggests Increased Prevalence of Shigellosis in Nha Trang, Vietnam

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Shigella **spp. are exquisitely fastidious gram-negative organisms which frequently escape detection by traditional culture methods. To get a more complete understanding of the disease burden caused by** *Shigella* **in Nha Trang, Vietnam, real-time PCR was used to detect** *Shigella* **DNA. Randomly selected rectal swab specimens from 60** *Shigella* **culture-positive patients and 500** *Shigella* **culture-negative patients detected by population-based surveillance of patients seeking care for diarrhea were processed by real-time PCR. The target of the primer pair is the invasion plasmid antigen H gene sequence (*ipaH*), carried by all four** *Shigella* **species and enteroinvasive** *Escherichia coli.* *Shigella* **spp. could be isolated from the rectal swabs of 547 of 19,206 (3%) patients with diarrhea.** *IpaH* **was detected in 55 of 60 (93%)** *Shigella* **culture-positive specimens, whereas it was detected in 87 of 245 (36%) culture-negative patients free of dysentery ($P < 0.001$). The number of PCR cycles required to detect a PCR product was highest for culture-negative, nonbloody diarrheal specimens (mean number of cycles to detection, 36.6) and was lowest for children with culture-positive, bloody diarrheal specimens (mean number of cycles, 25.3) ($P < 0.001$). The data from real-time PCR amplification indicate that the culture-proven prevalence of** *Shigella* **among patients with diarrhea may underestimate the prevalence of** *Shigella* **infections. The clinical presentation of shigellosis may be directly related to the bacterial load.**

Shigella spp. are exquisitely fastidious gram-negative organisms which annually cause an estimated 164.7 million cases of shigellosis worldwide, resulting in 1.1 million deaths (9). The traditional identification of these organisms relies on culture methods, which detect only a small fraction of the actual shigellosis cases (2). Common reasons for the low sensitivities of traditional culture methods include the low number of causative organisms, competition from other commensal organisms, as well as inappropriate changes in ambient temperature and pH during specimen transport (17). The growth, and thus the detection, of the organism is frequently further impaired by the use of antibiotics prior to specimen collection. More recently developed molecular diagnostic methods can overcome some of the shortcomings of culture methods. Several PCR protocols have been used for the detection of *Shigella* spp. and related organisms in fecal, food, and environmental specimens (3, 4, 6–8, 10–12, 15, 16, 18, 19).

One popular PCR assay, based on the amplification of the invasion plasmid antigen H (*ipaH*) gene sequence, is used for the diagnosis of dysentery (16). *IpaH* is carried by all four *Shigella* species as well as by enteroinvasive *Escherichia coli* (EIEC). Because EIEC is rare in fecal specimens from patients

with diarrhea in Southeast Asia, it is thought that most organisms detected by *ipaH*-specific PCR in this region are *Shigella* spp. (13). As part of a large shigellosis surveillance study in Nha Trang, Vietnam, we used the real-time TaqMan PCR (real-time PCR), a modified version of the *ipaH*-specific PCR, to detect *Shigella* and EIEC in rectal swab specimens from patients with diarrhea presenting for care. We compared the epidemiological features of *Shigella* culture-positive patients with the features of real-time PCR-positive but *Shigella* culture-negative patients.

MATERIALS AND METHODS

The study was conducted in a semirural site in Khanh Hoa Province, Nha Trang, on the coast of central Vietnam. The climate in the area is tropical, with a rainy season from September to December and a dry season for the rest of the year. The ongoing surveillance for the detection of shigellosis cases was started in April 2000. For the purposes of this study, patients enrolled through November 2002 were included. Prior to the start of the study, a census of all residents within the catchment area was completed. The census was then updated every year. As of August 2002, 201,978 individuals resided in the catchment area, of whom 15,073 were under 5 years old. The study used passive surveillance. All residents in the catchment area were encouraged through information campaigns to attend any of the 21 participating health care centers (commune health centers, polyclinics, or hospitals) in the catchment area if they had an episode of diarrhea. Individuals of all ages presenting with three or more loose bowel movements in the preceding 24 h were enrolled. The clinical history and physical findings for the patients were captured through a case report form. Two rectal swab specimens were obtained from each patient by using a cotton-tipped applicator (Solon Manufacturing Co., Solon, Maine). One rectal swab specimen was inserted into Cary-Blair medium, and the other was inserted into buffered

TABLE 1. Description of eight groups of patients who presented with diarrhea in Nha Trang, Vietnam,
between April 2000 and November 2002

Group	*Shigella* culture result	Clinical finding	Age (yr)	No. (%) of patients		No. of real-time PCR cycles[b]
				Total	Real-time PCR positive[a]	
1	Positive	Dysentery	<5	15	15 (100)	25.3
2	Positive	Dysentery	≥5	15	14 (93)	27.0
3	Positive	No dysentery	<5	15	13 (87)	28.5
4	Positive	No dysentery	≥5	15	13 (87)	28.4
5	Negative	Dysentery	<5	139	85 (61)	32.7
6	Negative	Dysentery	≥5	116	59 (51)	34.7
7	Negative	No dysentery	<5	128	43 (33)	36.1
8	Negative	No dysentery	≥5	117	44 (38)	36.6
Total				560	286 (51)	

[a] Positive for *ipaH*.
[b] Mean number of real-time PCR cycles required for detection; $P < 0.001$ by test for trend for increasing number of real-time PCR cycles across groups.

glycerol saline. The swab specimens were refrigerated until collection by a courier. The swabs were then transported in a cool box to a central laboratory (Institute Pasteur, Nha Trang, Vietnam) for bacteriological examination. The average time span from collection to plating was 8.0 h (interquartile range, 5.8 to 9.5 h).

Laboratory procedures. The rectal swab stored in buffered glycerol saline was inoculated in MacConkey and salmonella-shigella agars. The rectal swab transported in the Cary-Blair medium was dipped into a 0.8-ml cryovial containing phosphate-buffered saline (PBS) and was stored at −70°C until the real-time PCR assay was performed. After overnight incubation at 37°C, the salmonella-shigella and MacConkey agar plates were checked for non-lactose-fermenting colonies. Suspected *Shigella* colonies were inoculated into Kligler iron agar, mannitol, citrate, urea, and lysine biochemical test media. After incubation for 18 to 24 h at 37°C, the test media were read for characteristic *Shigella* reactions. Slide agglutination with commercially available *Shigella* antisera (Denka Seiken, Tokyo, Japan) was performed for suspicious colonies.

The fluorogenic probe, primers, and PCR conditions used in this study have recently been described (O. Sethabutr, H.-S. H. Houng, S. S. Silapong, and C. C. Mason, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. C-149, 2003). Briefly, the fluorogenic probe (6-carboxyfluorescein-CGC CTT TCC GAT ACC GTC TCT GCA-6-carboxytetramethylrhodamine) and its flanking primer pair (forward primer ipaH-U1 [5′-CCT TTT CCG CGT TCC TTG A-3′] and reverse primer ipaH-L1 [5′-CGG AAT CCG GAG GTA TTG C-3′]) were designed on the basis of *ipaH* gene sequences (GenBank accession no. M32063) previously described by Hartman et al. (5). For real-time PCR detection, 0.5 ml of a rectal swab suspension in PBS was pipetted into a 1.5-ml microcentrifuge tube. The tube was incubated in boiling water for 30 min to lyse the bacterial cells. The lysate was subjected to centrifugation at 10,000 rpm for 1 min. The lysate was either used directly for real-time PCR or stored at −70°C. The working cocktail for the detection contained 1 μl of DNA template, 1× TaqMan buffer A (Applied Biosystems, Foster City, Calif.), 2 mM $MgCl_2$, 100 nM each deoxynucleoside triphosphate, 200 nM primers (primers ipaH-U1 and ipaH-L1), 40 nM fluorogenic probe, ipaH-P1 (tetrachloro-6-carboxy-fluorescein labeled), and 1.25 U of AmpliTaq Gold (Applied Biosystems) in a total reaction volume of 25 μl. The TaqMan assays were conducted with an ABI 7700 sequence detection system (Applied Biosystems). The amplification profile consisted of heat activation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 30 s and annealing, extension, and fluorogenic probe hybridization at 60°C for 1 min. The assay result was considered positive when the number of cycles to detection was 38 or less. Two real-time PCR-negative samples were found to contain inhibitors and were further purified by use of a stool kit (Qiagen Inc., Valencia, Calif.).

Sample size. Overall, 560 specimens from eight categories of patients were tested, as illustrated in Table 1. A sample size of 60 was sufficient to detect a 95% prevalence of *ipaH* within a 95% confidence interval from 86 to 99%, and a sample size of 125 was sufficient to detect a 35% prevalence of *Shigella* DNA within a 95% confidence interval from 26 to 43%.

Analysis. For analytical purposes, the patients were divided into eight groups according to the *Shigella* culture result, a history of blood in their bowel movements, and age. Comparisons of categorical data were made by Fisher's exact test or the χ^2 test, as appropriate. Spearman's test was used to test the statistical significance of the observed correlations. A logistic regression model was used to test the association between characteristics which could influence organism detection by real-time PCR, such as *Shigella* culture positivity and disease severity. The model was adjusted for *Shigella* culture results, fever, and dysentery, variables that were independently associated with the detection of *ipaH*. Diarrhea was defined as three or more loose bowel movements within any 24-h period. Any loose bowel movement containing blood was defined as dysentery. A patient with a history of fever on presentation, as reported by the patient or a care provider, was considered febrile for analytic purposes. Statistical analyses were performed by use of STATA (version 7) software (STATA Corporation, College Station, Tex.). P values (two tailed) less than 0.05 were considered statistically significant.

Informed consent was obtained from patients or their parents or guardians. The study was approved by the National Ethics Review Board of the Government of Vietnam and the World Health Organization (Geneva, Switzerland).

RESULTS

During the 19-month surveillance period, 19,206 individuals with diarrhea presented to a health care provider participating in the surveillance, and a rectal swab specimen was obtained. *Shigella* spp. could be isolated from the rectal swab specimens from 547 patients (3%), of which 435 (80%) were identified as *Shigella flexneri*, 108 (19%) were identified as *Shigella sonnei*, and 4 (1%) were identified as *Shigella boydii*. Females comprised 53% of the patients with diarrhea and 54% of the patients with shigellosis.

IpaH was detected in 56 of 60 (93%) randomly selected *Shigella* culture-positive specimens (95% confidence interval [CI], 84 to 98%) and in 231 of 500 (46%) *Shigella* culture-negative specimens (95% CI, 42 to 51%) (Table 1). The real-time PCR detection rate was highest for dysenteric patients from whom *Shigella* had been cultured (29 of 30 [100%]; 95% CI, 83 to 100%). The detection rates were lower for nondysenteric patients with culture-confirmed shigellosis (26 of 30 [87%]; 95% CI, 70 to 96%). Among the *Shigella* culture-negative patients, real-time PCR detection rates were significantly higher with rectal swab specimens from dysenteric patients (144 of 255 [57%]; 95% CI, 50 to 63%) than with rectal swab specimens from diarrheal patients without dysentery (87 of 245 [36%]; 95% CI, 30 to 42%) ($P < 0.001$). The number of real-time PCR cycles required to detect a real-time PCR product, which is inversely related to the DNA load in the fecal specimen, was highest for patients with culture-negative, nonbloody diarrhea (mean number of cycles to detection, 36.6)

TABLE 2. Variables which predicted or failed to predict the detection of *ipaH* by real-time PCR amplification in 560 specimens tested

Variable	Real-time PCR negative	Real-time PCR positive	Crude odds ratio (95% CI)	Adjusted odds ratio[a] (95% CI)
No. (%) of patients				
Total	274 (100)	286 (100)		
Shigella culture positive	5 (2)	55 (19)	12.8 (5.0–32.5)	12.5 (4.9–32.1)
S. flexneri culture positive	5 (2)	40 (14)	8.7 (3.4–22.5)	9.2 (3.5–24.1)
S. sonnei culture positive	0 (0)	15 (5)		
With a history of fever	21 (8)	63 (22)	3.4 (2.0–5.8)	2.4 (1.4–4.2)
With dysentery	113 (41)	173 (61)	2.2 (1.6–3.1)	2.1 (1.5–3.1)
Of female gender	140 (51)	152 (53)	1.1 (0.8–1.5)	1.1 (0.8–1.6)
Maximum no. of bowel movements[b]	5.0	6.4	1.1 (1.1–1.2)	1.1 (1.0–1.2)
No. of days of diarrhea prior to presentation	1.6	1.6	1.0 (0.9–1.1)	1.0 (0.9–1.0)
No. (%) of patients with self-reported antibiotic use[c]	14 (5)	12 (4)	0.9 (0.4–2.0)	0.9 (0.4–2.2)

[a] Adjusted for culture-proven shigellosis, a history of fever, and dysentery.
[b] The maximum number of bowel movements during any 24-h period prior to presentation.
[c] History of self-medication with antibiotics prior to presentation.

and was lowest for children with culture-positive, bloody diarrhea (mean number of cycles to detection, 25.3) (Table 1). A test for trend for increasing number of real-time PCR cycles across disease categories was highly significant ($P < 0.001$). For all groups except the nondysenteric, culture-negative patients (groups 7 and 8), real-time PCR detection rates tended to be higher for children than for adults. No statistically significant difference could be observed between the rates of *S. flexneri* detection (40 of 45 [89%]; 95% CI, 76 to 96%) and the rates of *S. sonnei* detection (15 of 15 [100%]; 95% CI, 78 to 100%) ($P = 0.2$).

Three variables independently predicted *ipaH* detection by real-time PCR amplification: a positive culture for *Shigella* (*S. flexneri* or *S. sonnei*), a history of fever, and dysentery (Table 2). No statistically significant interaction between these variables could be detected. Among the 563 patients with diarrhea, 84 (15%) presented with a history of fever, and *ipaH* was detected by real-time PCR in 63 of the 84 (75%) febrile patients with diarrhea. Similarly, real-time PCR detected *ipaH* in the stool specimens from 173 of 285 (61%) patients who presented with dysentery. The gender of the patient, the duration of diarrhea prior to presentation, and self-reported use of antibiotics did not predict *ipaH* detection by real-time PCR.

We performed additional analyses to examine the plausibility of the assertion that real-time PCR-positive but culture-negative patients indeed had shigellosis. We found a bimodal age distribution among the total of 547 patients with shigellosis confirmed by culture during the surveillance period (Fig. 1). The percentage of *Shigella* culture-confirmed cases was highest among children 2 to 10 years of age. A second peak was observed in patients over 60 years of age. The distribution of 226 real-time PCR-positive, culture-negative cases by age group was similar to that for the culture-confirmed cases, and there was a significant correlation between the age-specific occurrence of culture-positive cases and real-time PCR-positive, culture-negative cases (Spearman rho value = 0.8; $P = 0.004$). Culture-confirmed shigellosis cases peaked between September and November, and a period with low numbers of cases was found between March and May. The seasonality of the 226 real-time PCR-positive, culture-negative cases correlated closely with that of the culture-confirmed cases (Spearman rho value = 0.6; $P = 0.04$).

DISCUSSION

The *ipaH*-specific real-time PCR assay was found to be highly sensitive in relation to culture for the confirmation of shigellosis. The sensitivity of the assay was increased for children and patients with dysentery and reached 100% for children with dysentery. The most likely explanation for the observed differences in detection rates by real-time PCR is the variation in bacterial loads. *Shigella* is more difficult to detect in patients who shed only a few organisms than in individuals who shed large bacterial loads and, thus, a large amount of *Shigella* DNA. Young children tend to have less effective immune responses to *Shigella* infections than adults and are thus less likely to control bacterial infection and proliferation. Dysenteric patients have a more severe form of shigellosis in a clinical spectrum ranging from watery diarrhea to diarrhea with mucus and frank bloody diarrhea. Bloody diarrhea is associated with the rupture of the intestinal epithelial barrier, followed by the invasion and destruction of the intestinal mucosa, resulting in the proliferation of the pathogens faster than that which occurs in patients with the milder forms of the disease (14). Patients who have the most severe form of shigellosis also shed the most organisms. A direct relationship between bacterial load, detection by culture, and disease severity was shown by the number of PCR cycles required to detect a PCR product. The number of cycles was lowest for culture-positive individuals with bloody diarrhea, indicating a high bacterial load in the specimen. In contrast, the number of cycles required for detection was highest for culture-negative individuals with nonbloody diarrhea, which probably indicates the presence of scant numbers of organisms.

The real-time PCR assay detected *ipaH* in a surprisingly high number of *Shigella* culture-negative patients. Studies that have used this *ipaH*-based PCR have been published from several Asian countries, including Thailand, Bangladesh, and, more recently, India (1, 8, 15). Those studies suggest that PCR tests are positive for a large portion of patients who are culture
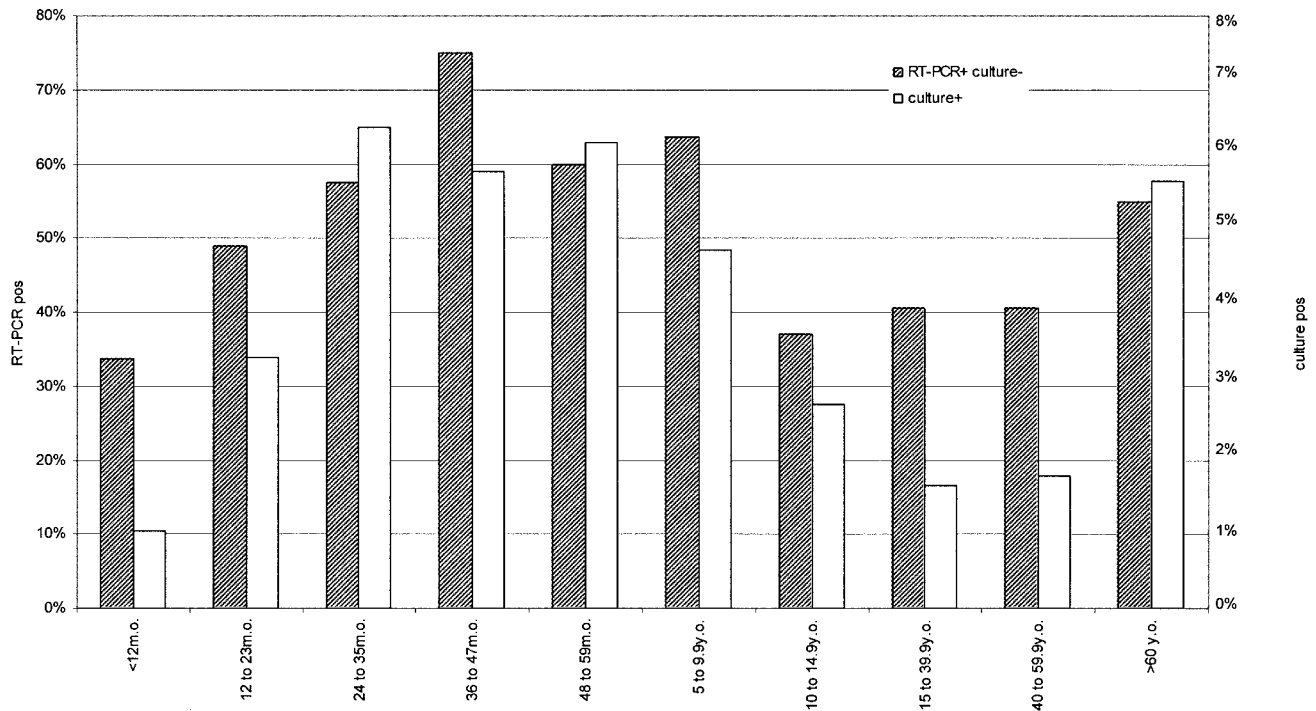
FIG. 1. Ages of 547 patients with culture-confirmed *Shigella* infection (open bars) and 231 selected patients with *Shigella* culture-negative, real-time PCR (RT-PCR)-positive results (striped bars) at the time of presentation. A significant correlation was observed between the age-specific percentage of real-time PCR-positive, culture-negative specimens and the age-specific percentage of culture-positive specimens (Spearman rho value = 0.8; P = 0.004). Symbols and abbreviations: + and pos, positive; −, negative; m.o., months old; y.o., years old.

negative for *Shigella*. In Thailand, traditional microbiology methods detected 50 shigellosis cases among 119 patients (42%) with dysentery, while PCR amplification detected 72 cases (61%) (15). In another study from Thailand, the *ipaH*-specific PCR system increased the detection rate over that by culture alone from 58 to 79% among patients with dysentery and from 6 to 22% among 527 family contacts; 75% of infections in family members were asymptomatic (4). In a study from Kolkata, India, 22 of 46 PCR-positive specimens (48%) were culture negative (1).

One possible explanation for this finding is the detection of DNA from an organism other than *Shigella* by the assay. However, the assay is highly specific and exclusively detects organisms which contain *ipaH* (1). Apart from *Shigella* spp., the only other organism that is known to have *ipaH* is EIEC, which is thought to have an exceedingly low prevalence in the region where the study was conducted (13). Frequent therapy with antibiotics prior to presentation may have contributed to the findings. Specimens from premedicated patients are less likely to grow *Shigella*, as the antibiotic has killed the organisms. In contrast, real-time PCR can detect the genetic material of dead organisms. In this study *Shigella* culture-positive and *Shigella* culture-negative, real-time PCR-positive individuals reported similar rates of antibiotic use prior to presentation. However, the overall percentage of diarrhea patients who reported premedication (6%) is low by Vietnamese standards. Self-reporting of antibiotic use before a visit to the treatment center is subject to reporting bias, and patients may forget or

do not want to admit that they tried alternative treatment options prior to presentation.

The frequent detection of real-time PCR-positive, culture-negative specimens could have resulted from false-positive results. Such false-positive results could have been caused by accidental contamination, a laboratory hazard which can occur when PCR is performed without the utmost diligence. If these real-time *ipaH*-positive, *Shigella* culture-negative cases were due to contamination, they should occur randomly, independent of the patient characteristics or seasonality. To investigate this hypothesis, we compared culture-positive and *ipaH*-positive, culture-negative cases for their occurrence by age and season. The distributions of culture-proven cases of *Shigella* infection and *ipaH*-positive, culture-negative cases correlated closely with both of these features. This finding makes it unlikely that these *ipaH*-positive, culture-negative cases were due to contamination.

Assuming that the large majority of the patients with real-time PCR-positive, culture-negative diarrhea are infected with *Shigella* spp., infections with these organisms may be responsible for 35% or more of the diarrhea episodes in Nha Trang, much more than was previously thought on the basis of the 3% detection rate by culture.

Further research is needed to assess the incidence of culture-negative shigellosis in other areas where *Shigella* is endemic. The International Vaccine Institute is conducting several studies in Asia to estimate the proportion of *Shigella* culture-negative, *ipaH*-positive diarrhea episodes.

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