

Using real time PCR to detect shigellosis: *ipaH* detection in Kaeng-Khoi District, Saraburi Province, Thailand

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Abstract

To get a real number of the disease burden caused by *Shigella* in Kaeng-Khoi District, Saraburi Province, Thailand, real time-PCR was used to detect *Shigella*-associated DNA. Randomly selected rectal swabs from 20 *Shigella* culture-positive and from 300 *Shigella* culture-negative patients detected in population-based surveillance of patients seeking care for diarrhea were processed using real time PCR. The target of the primer pair is the invasion plasmid antigen H gene sequence (*ipaH*) carried by all 4 *Shigella* species and enteroinvasive *Escherichia coli*. *IpaH* was detected in 19 out of 20 (95%) *Shigella* culture-positive specimens compared to 48 out of 300 (16%) culture-negative patients free of dysentery ($p < 0.001$). The data from real time PCR amplification indicate that the culture-proven prevalence of *Shigella* among diarrheal patients may underestimate the true prevalence of shigellosis by an order of magnitude.

Keywords: Shigellosis, *ipaH* detection, real time PCR

1. Introduction

Shigella spp. are exquisitely fastidious Gram-negative organisms which cause an estimated 164.7 million cases of shigellosis annually resulting in 1.1 million deaths [1]. The traditional identification of these organisms relies on culture methods that detect only a small fraction of the actual shigellosis cases [2]. But cultures can only detect live organisms. *Shigella* strains which have been killed by exposure to antimicrobials, inappropriate changes in ambient temperature or pH during transport will not be detected by culture methods [3]. Recently developed molecular diagnostic methods can overcome some of the shortcomings of traditional culture methods [4].

Several polymerase chain reaction (PCR) protocols have been used for the detection of *Shigella* spp. and related organisms in fecal, food and environmental specimens [5-16].

One popular PCR assay, based on the amplification of the invasion plasmid antigen H gene sequence (*ipaH*) is used for the diagnosis of dysentery cases [8]. *IpaH* is carried by all 4 *Shigella* species, as well as by enteroinvasive *Escherichia coli* (EIEC). Because EIEC are extremely rare in South East Asia, it is thought that most organisms detected by *ipaH* PCR in this region are *Shigella* spp. [17]. Studies using this *ipaH* based- PCR have been published from several Asian countries including Thailand, Bangladesh and more recently India and

Vietnam [4,10,14,18]. These studies suggest that PCR is positive in a large portion of patients who are culture-negative for *Shigella*. In an earlier study from Thailand, traditional microbiology detected 50 shigellosis cases among 119 patients (42%) with dysentery while DNA amplification by PCR detected 72 cases (61%) [10]. In another study from Thailand the *ipaH* PCR system increased the detection rate over 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 [12]. In a study from Kolkata, India, 22 out of 46 PCR positive- specimens (48%) were culture-negative [18].

In this study, we used real time TaqMan PCR (real time PCR), a modified version of the *ipaH* PCR, to detect *Shigella*/EIEC in rectal swabs obtained from diarrhea patients presenting for care.

2. Materials and Methods

The study was conducted in a semi-rural site in Kaeng-Khoi District, Saraburi Province, Thailand. Twenty community health care centers (CHC), two district hospitals, and one provincial hospital participated in the surveillance. For the purpose of this study, patients enrolled in the passive surveillance from May 2002 through May 2003 were included. According to the data collected by the Thai Public Health survey, 80,141 individuals were residing in the study area and 5686 were under 5 years of age [19]. Informed consent was obtained from patients or their parents or guardians. The study was approved by the National Ethics Review Committee of the Ministry of Public Health of Thailand (No. 0314/ 2545, on July 31, 2001).

Individuals of all ages who had three or more loose bowel movements in the preceding 24 hours were enrolled. Two rectal swabs were obtained from each patient using a cotton tipped- applicator. One rectal swab was placed into a buffered peptone water (BPW) solution medium, the other into a buffered glycerol saline (BGS) solution and refrigerated until collection by a courier. The swabs were then transported in a cool box to a central laboratory (Saraburi Provincial hospital), once a day, for bacteriological examination. The time span from collection to plating was 1 to 24 hours,

depending on the collection time of each sample.

2.1. Laboratory procedures

The rectal swab stored in BGS was streaked onto MacConkey and *Salmonella-Shigella* (SS) agar. The rectal swab transported in PBS was stored at -70°C until the time of the real time PCR assay. After overnight incubation at 37°C , the SS and MacConkey plates were checked for non-lactose fermenting colonies. Suspected *Shigella* colonies were inoculated into TSI, LIM, SIM, citrate and urea biochemical test media. After incubation for 18 to 24 hours at 37°C the test media were read for characteristic *Shigella* reactions. Slide agglutination was performed on suspicious colonies with commercially available *Shigella* antisera (Denka Seiken, Japan).

Fluorogenic probe, primers and PCR conditions have been recently described [4]. For real time PCR detection, 0.5 ml of rectal swab PBS suspensions were pipetted into 1.5 mL microcentrifuge tubes. The tubes were incubated in boiling water for 30 minutes to lyse bacterial cells. The lysate was subjected to centrifugation at 10,000 rpm for 1 minute. 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, 1X TaqMan buffer A (Applied Biosystems, Foster City, CA, USA), 2 mM MgCl_2 , 100 nM each of dNTPs, 200 nM of primers (*ipaH*-U1 and *ipaH*-L1), 40 nM of fluorogenic probe, *ipaH*-P1 (TET-labeled) and 1.25 units of AmpliTaq Gold (Applied Biosystems) in 25 μL of total reaction volume. The TaqMan assays were conducted using an ABI 7700 Sequence Detection System (Applied Biosystems). The amplification profile consisted of heat activation at 95°C for 10 minutes; of 40 cycles of denaturalization at 95°C for 30 seconds; and of annealing, extension and fluorogenic probe hybridization at 60°C for 1 minute. The assay was considered positive when the number of cycles to detection was 38 or less. PCR-negative samples found to contain inhibitors were further purified using Qiagen Stool Kit (Qiagen Inc., Valencia, CA, USA). This PCR assay was conducted at the Enteric bacterial section, Armed Forces Research Institute of Medical Sciences, Bangkok, using their protocol [4, 8, 10].

2.2. Sample size

All 20 randomly selected stored culture positive *Shigella* specimens were tested. In addition, 300 randomly specimens from *Shigella* culture-negative diarrhea patients were tested to estimate the percentage of patients who did not grow *Shigella* by cultures but were positive by real time PCR assays. For this purpose, 50 specimens were randomly selected from 2 categories of patients: 1) children under 5 years of age with bloody diarrhea, 2) children with non-bloody diarrhea and 100 specimens were randomly selected from 1) children and adults 5 years and older with bloody diarrhea and 2) children and adults 5 years and older with non-bloody diarrhea. A computer generated list of random numbers was used to select the specimens.

2.3. Analysis

For analytic purposes, patients were divided into 8 groups according to status of their *Shigella* cultures, a history of blood in the bowel movements, and their age group. Comparisons of categorical data were made using Fisher's exact or the χ^2 test as appropriate. A logistic regression model was used to test the association between clinical features which could be associated with the real time PCR detection of *ipaH* in *Shigella* negative specimens. The model was adjusted for variables independently associated with the detection of *ipaH*. Diarrhea was defined as 3 or more loose bowel movements within any 24 hour period. Any loose bowel movement containing blood was defined as dysentery. A patient with a history of fever reported by the patient or a care provider, on presentation, and confirmed by their body temperature by thermometer measurement, was considered a febrile patient for analytic purposes. Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC, USA). P-values less than 0.05 (2-tailed) were considered statistically significant.

3. Results

During the 12 months surveillance period, 2719 individuals presented with diarrhea to a health care provider participating in the surveillance, and rectal swabs were obtained. *Shigella* spp. could be isolated from the rectal swabs obtained from 37 patients (1.5%), of

which 28 were identified as *S. sonnei* and 9 as *S. flexneri*.

ipaH was detected in 19 of 20 (95%) *Shigella* culture-positive specimens (95% confidence interval [CI], 77% to 100%) and in 48 of 320 (16%) *Shigella* culture-negative specimens (95% CI, 12% to 21%; Table 1).

The real time PCR detection rate was 100% in diarrhea patients with bloody stool (or dysenteric patients) from whom *Shigella* had been cultured (95% CI, 59% to 100%). The detection rate was lower in non-dysenteric patients, with culture-confirmed shigellosis (92%; 95% CI, 64% to 100%). In *Shigella* culture-negative patients real time PCR detection rates were significantly higher in rectal swabs from dysenteric patients (24%; 95% CI, 16% to 34%), compared to non-dysenteric patients (12%; 95% CI, 8% to 17%; $p = 0.02$).

The number of real time PCR cycles required to detect a real time PCR product, which is inversely related to the load of DNA in the fecal specimen, was highest for culture-negative, non-bloody diarrheas (mean number of cycles to detection 38.8) and was lowest for children with culture-positive, bloody diarrheas (mean number of cycles 22.5; Table 1). A test for trend for increasing number of real time PCR cycles across disease categories was highly significant ($p < 0.001$).

4. Discussion

The *ipaH* real time PCR assay was found to be a highly sensitive method for *Shigella* spp. detection when compared to conventional culture methods. The sensitivity of the assay reached to 100% in patients with dysentery. The most likely explanation for the observed differences in detection rates by real time PCR are variations in bacterial load. *Shigella* is more difficult to detect in patients who shed only a few organisms compared to individuals who shed a large bacterial load and thus a large amount of *Shigella* DNA. Bloody diarrhea is associated with the rupture of the intestinal epithelial barrier, followed by the invasion and destruction of intestinal mucosa, resulting in a faster proliferation of the pathogens compared to the milder forms of the disease [20]. Patients who suffer from the most severe form of shigellosis, therefore, also shed the most organisms. A direct relationship between bacterial load, detection by culture and disease

Table 1: Description of 8 groups of patients who presented with diarrhea

Group	<i>Shigella</i> culture	Clinical symptoms	Age	N	real time PCR positive ^a	%	real time PCR cycles ^b
1	positive	diarrhea with bloody stool	5 years or older	4	4	100%	22.5
2	positive	diarrhea with bloody stool	<5 years old	3	3	100%	24.4
3	positive	diarrhea without bloody stool	5 years or older	9	8	89%	25.9
4	positive	diarrhea without bloody stool	<5 years old	4	4	100%	27.1
5	negative	diarrhea with bloody stool	5 years or older	50	12	24%	36.1
6	negative	diarrhea with bloody stool	<5 years old	50	12	24%	36.8
7	negative	diarrhea without bloody stool	5 years or older	100	14	14%	38.2
8	negative	diarrhea without bloody stool	<5 years old	100	10	10%	38.8
total				320	67	21%	37.3

^aPositive for *ipaH* ^bMean number of real time PCR cycles required for detection; test for trend for increasing number of real time PCR cycles across groups $p < 0.00$.

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 non-bloody diarrhea probably indicating a scanty presence of organisms.

The real time PCR assay detected *ipaH* in a high number of *Shigella* culture-negative patients. 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 detects exclusively organisms which contain *ipaH* [18]. Apart from *Shigella* spp. the only other organisms that are known to have *ipaH* are EIEC, which are thought to have an exceedingly low prevalence in the region where the study was conducted [17]. Frequent therapy with antibiotics prior to presentation may have contributed to the findings. However *Shigella* culture positive patients with *ipaH* in fecal specimens did not report significantly more frequent use of antibiotics in this study.

Of interest is the comparison of data collected in Thailand with the data from Nha Trang, Central Vietnam using the same real time PCR methodology [4]. The sensitivity of the real time PCR is higher than conventional culture methods in both of them. It can be concluded that the real number of Shigellosis in Kaeng-Koi district, Saraburi province, Thailand should be

higher than the number of Shigellosis from the Public's routine report.

In summary this study shows that real time conclusions PCR has 95% sensitivity in detecting shigellosis cases in Saraburi Province, Thailand. While traditional culture methods detected shigellosis strains in 1.5% of diarrhea stool specimens and the real time PCR detected evidence of *Shigella* infections in 16% of the *Shigella* culture negative diarrhea specimens. These findings may indicate that the true proportion of diarrhea episodes related to *Shigella* infections is one order of magnitude higher than previously thought, based on culture proven shigellosis cases.

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6. References

- [1] Kotloff, K.L., Winickoff, J.P., Ivanoff, B. *et al.* Global burden of Shigella Infections: Implications for Vaccine Development and Implementation of Control Strategies, Bull. World Health Organ; Vol. 77, pp.651-666, 1999.
- [2] Echeverria, P., Sethabutr, O., Pitarangsi, C., Microbiology and Diagnosis of Infections with Shigella and Enteroinvasive Escherichia Coli, Rev. Infect. Dis; Vol.13 Suppl 4, pp. S220-225, 1991.
- [3] Taylor ,W.I. and Schelhart, D. Effect of Temperature on Transport and Plating Media for Enteric Pathogens. J. Clin. Microbiol; Vol.2, No.4, pp. 281-286, 1975.
- [4] Thiem V, Sethabutr O, Von Seidlein L, *et al.* Detection of *Shigella* by a PCR Assay Targeting the *ipaH* Gene Suggests Increased Prevalence of Shigellosis in Nha Trang, Vietnam, J. Clin. Microbiol; Vol. 42, No. 5, pp. 2031-2035, 2004.
- [5] Frankel, G., Riley, L., Giron, J.A., *et al.* Detection of *Shigella* in Feces Using DNA Amplification, J. Infect. Dis., 1990; 161: 1252-6.
- [6] Lampel, K.A., Jagow, J.A., Trucksess, M., Hill, W.E., Polymerase Chain Reaction for Detection of Invasive *Shigella flexneri* in Food. Appl. Environ. Microbiol; Vol.56, pp. 1536-1540, 1990.
- [7] Islam, D. and Lindberg, A.A., Detection of *Shigella Dysenteriae* type 1 and *Shigella Flexneri* in Feces by Immunomagnetic Isolation and Polymerase Chain Reaction. J. Clin. Microbiol., Vol.30, pp.2801-2806,1992.
- [8] Sethabutr, O., Venkatesan, M., Murphy, G.S., Eampokalap B, Hoge CW, Echeverria P., Detection of Shigellae and Enteroinvasive *Escherichia coli* by Amplification of the Invasion Plasmid Antigen H DNA Sequence in Patients with Dysentery. J. Infect. Dis; Vol.167, pp. 458-61, 1993.
- [9] Ye, L.Y., Lan, F.H., Zhu, Z.Y., Chen, X.M., Ye, X.L., Detection of *Shigella* and Enteroinvasive *Escherichia Coli* using Polymerase Chain reaction. J. Diarrheal Dis. Res; Vol.11, pp. 38-40, 1993.
- [10] Sethabutr, O., Echeverria, P., Hoge, C.W., Bodhidatta, L., Pitarangsi, C., Detection of *Shigella* and Enteroinvasive *Escherichia Coli* by PCR in the Stools of Patients with Dysentery in Thailand, J. Diarrheal Dis. Res; Vol.12, pp. 265-269, 1994.
- [11] Oyofe, B.A., Mohran, Z.S., el-Etr, S.H., Wasfy, M.O., Peruski, L.F.Jr., Detection of Enterotoxigenic *Escherichia Coli*, *Shigella* and *Campylobacter* spp. by Multiplex PCR Assay, J. Diarrheal Dis. Res; Vol.14, pp. 207-10, 1996.
- [12] Gaudio, P.A., Sethabutr, O., Echeverria, P., Hoge, C.W., Utility of a Polymerase Chain Reaction Diagnostic System in a Study of the Epidemiology of Shigellosis Among Dysentery Patients, Family Contacts, and well Controls Living in a Shigellosis-endemic area, J. Infect. Dis; Vol. 176, pp. 1013-1018, 1997.
- [13] Houg, H.S., Sethabut, O., Echeverria, P., A Simple Polymerase Chain Reaction Technique to Detect and Differentiate *Shigella* and Enteroinvasive *Escherichia coli* in Human Feces, Diagn. Microbiol. Infect. Dis, Vol. 28, pp.19-25, 1997.
- [14] Islam, M.S., Hossain, M.S., Hasan, M.K., *et al.* Detection of *Shigellae* from Stools of Dysentery Patients by Culture and Polymerase Chain Reaction Techniques. J. Diarrheal Dis. Res., Vol.16, pp. 248-251, 1998.
- [15] Villalobo, E. and Torres, A., PCR for Detection of *Shigella* spp. in Mayonnaise, Appl. Environ Microbiol, Vol. 64, pp. 1242-1245, 1998.
- [16] Lindqvist R. Detection of *Shigella* spp. in Food with a Nested PCR Method-Sensitivity and Performance Compared with a Conventional Culture Method, J. Appl. Microbiol., Vol. 86, pp.971-978, 1999.
- [17] Phantouamath, B., Sithivong, N., Insiengmay, S., *et al.* The Incidence of *Escherichia Coli* Having Pathogenic Genes for Diarrhea: a Study in the People's Democratic Republic of Lao, Jpn. J Infect, Dis; Vol.56, pp.103-106, 2003.
- [18] Dutta, S., Chatterjee, A., Dutta, P., *et al.* Sensitivity and Performance Characteristics of a Direct PCR with Stool Samples in Comparison to Conventional Techniques for Diagnosis of *Shigella* and Enteroinvasive *Escherichia Coli* Infection in Children with Acute Diarrhea in Calcutta,

India, J. Med. Microbiol; Vol.50, pp.667-74, 2001.

[19] Samosornsuk, S., Jitsanguansuk, S., Sirima, N., *et al.* A Community-based Cluster Survey on Treatment Preferences for Diarrhea and Dysentery in Kang-Koi District, Saraburi Province, Thailand, J.

Health Popul. Nutr., Vol.22, No. 2, pp.113-118, 2004.

[20] Sansonetti, P.J., Tran Van Nhieu, G., Egile, C., Rupture of the Intestinal Epithelial Barrier and Mucosal Invasion by *Shigella flexneri*, Clin. Infect. Dis; Vol.28, pp.466-475, 1999.