Journal of Infection (2010) 61, 471–475

BIA British Infection Association

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Surveillance of shigellosis by real-time PCR suggests underestimation of shigellosis prevalence by culture-based methods in a population of rural China

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Accepted 8 October 2010
Available online 15 October 2010

KEYWORDS
Disease burden;
Shigella;
Real-time PCR;
ipaH;
Culture

Summary *Introduction:* Shigellosis is a leading public health issue in China, especially in Children under 5 years of age. The disease burden of shigellosis is usually underestimated by conventional culture. In this study, real-time PCR was applied to detect Shigella infection in parallel with routine culture, to investigate the true burden of disease caused by *Shigella* spp.
Methods: Rectal swab specimens of 39 *Shigella* culture positive and 298 *Shigella* culture negative patients from a population-based surveillance study were selected randomly. Real-time PCR targeting the invasion plasmid antigen H gene sequence (*ipaH*) was used to detect DNA sequences characteristic for *Shigella* spp.
Results: ipaH were detected in 174 of 298 (58%) randomly selected *Shigella* culture negative specimens and in 38 of 39 (97%) *Shigella* culture positive specimens ($p < 0.001$). Among 10 variables, culture results was the strongest predictive factor (OR = 15.5; 95% CI: 2.0–119.0), followed by a clinical presentation of diarrhea with fever (OR = 2.8; 95% CI: 1.2–6.2), epidemic season (OR = 2.4; 95% CI: 1.4–4.3), and female gender (OR = 1.8; 95% CI: 1.1–3.0).

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doi:10.1016/j.jinf.2010.10.004

Conclusion: The high detection rate of *ipaH* in culture negative specimens through use of real-time PCR suggests that earlier estimates of shigellosis burden measured by conventional culture may have underestimated the true disease burden.
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Introduction

Diarrhea is a leading cause of morbidity and mortality in developing countries. Dysentery caused by the *Shigella* spp. remains a major source of diarrhea, especially in developing countries.[1,2] China is undergoing rapid social and economic changes. Water supply and sanitation, factors intimately linked to the prevalence of enteric diseases, have improved in urban as well as in many rural areas. With the widespread use of hepatitis A vaccine, shigellosis has replaced hepatitis as a leading public health issue in China, especially in children under 5 years of age.[3]

The lack of efficiency of conventional culture methods may be one of the reasons for underestimating the burden of shigellosis. The traditional stool culture detects only a small fraction of the actual shigellosis cases.[4] Low bacterial load of stool specimens, competition from other commensal organisms, inappropriate transportation of specimens and prior antibiotic use are the most common factors impairing the detection of *Shigella* spp. by traditional culture methods.[5] Several PCR protocols have been developed for the detection of *Shigella* spp. in food, environmental specimens and feces. These molecular techniques have overcome some of the problems posed by conventional culture methods.[6–8] The assay is based on the detection of an invasion plasmid antigen, *ipaH*, a gene found in all *Shigella* species as well as in enteric invasive *Escherichia coli* (EIEC). Because EIEC is uncommon in Asia including China,[9–13] it is thought that the majority of *ipaH* detected in stool are derived from *Shigella* spp.

From 1st January to 31st December in 2002, a population- and treatment center-based surveillance study was conducted in a rural area of China to estimate the disease burden due to shigellosis. The surveillance findings have been reported previously.[2,3] Here we compare real-time TaqMan PCR with conventional stool culture and analyze predictive factors for PCR detection of *ipaH* in fecal specimens from Zhengding, China.

Methods

Study design

The study design has been described previously.[3] Briefly diarrhea cases were detected through a population- and treatment center-based surveillance system, which consisted of 29 villages in four rural townships in Zhengding County, with a population of 75,630. The study followed a standardized protocol, based on a generic protocol.[14] Consenting patients of all ages with diarrhea or dysentery presenting to the participating health care providers were included in the study. The clinical history and physical findings of each participating patient were captured through case report forms completed by a clinician at presentation. Two rectal/stool swabs or a stool specimen were obtained. One swab was placed in buffered glycerol saline (BGS) for conventional culture and the other in phosphate buffered saline (PBS) for PCR assay. The specimens were stored refrigerated until they were transported in a cool box to the central laboratory by motorcycle, usually within 4 h of collection. Diarrhea was defined as three or more loose bowel movements during a 24-h period. Dysentery was defined as one or more loose bowel movements with visible blood.

Microbiology

Upon reception in study laboratory the specimens in BGS were plated immediately on MacConkey agar and *Salmonella–Shigella* agar. The PBS specimens were stored at minus 40 centigrade for PCR assays after study completion. Biochemical reactions of microbial colonies were evaluated in Kligler's iron agar and motility indole urease medium as described previously.[3] All *Shigella* isolates collected during the surveillance were confirmed at a reference laboratory in Shanghai (Fudan University, Shanghai).

PCR assay

Fluorogenic probe, primers and PCR conditions and summary results have been previously described.[2,15] 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.[16] For real-time PCR detection, 0.5 mL of rectal swab PBS suspensions was pipetted into 1.5-mL microcentrifuge tubes. The tubes were incubated in boiling water for 30 min to lyse bacterial cells. The lysate was subjected to centrifugation at 10,000*g* 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, 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 was as follows: heat activation at 95 °C for 10 min, 40 cycles of denaturalization at 95 °C for 30 s, and annealing, extension and fluorogenic probe hybridization at 60 °C for 1 min.

The assay was considered positive when the number of cycles to detection was 38 or fewer. An exogenous internal control was spiked into each reaction to detect the presence of any inhibitors. PCR-negative samples found to

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