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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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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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 diarrheal 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 TTT TCG GAT ACC GTC TCT GCA-6-carboxytetramethylrhodamine) and its flanking primer pair (forward primer *ipaH*-U1 [5'-CTT TTT CGG CGT TCC TTG A-3'] and reverse primer *ipaH*-L1 [5'-CCG 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 10,000g 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 MgCl₂, 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 denaturation 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
contain inhibitors were further purified with the Qiagen Stool Kit (Qiagen Inc., Valencia, CA, USA).

Sample size

In total, 337 specimens from eight categories of patients were tested (Table 1). A sample size of 60 was sufficient to detect a 95% prevalence of ipaH with 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%. A computer-generated list of random numbers was used to select the specimens.

Data management and analysis

All case report forms were double entered into a custom-made data entry programs (FoxPro, Microsoft, Seattle, USA). And the SAS program (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. For analytic purposes, patients were divided into 8 groups according to Shigella culture status, a history of visible blood in bowel movements and age group (See Table 1). The t-test was used to compare the mean number of cycles in each group. For categorical data, Fisher’s exact or the χ² test was used. A stepwise logistic regression model was used to test the association between clinical features that could be associated with the real-time PCR detection of ipaH. And a significant level of p ≤ 0.15 was specified for co-variables to be included in the model. The explanatory variables included culture results, onset month, age, sex, dysentery, fever, premedication with antibiotics, maximum number of bowel movements, diarrhea prior to presentation and duration of diarrhea. Age, maximum no. of bowel movements, diarrhea prior to presentation and duration of diarrhea were included as continuous variable. The remaining variables were included as binary categorical variable. A p-value <0.05 (two-tailed) was considered statistically significant.

Ethics

The study received approval from the local government, Hebei province, China and the Secretariat Committee for Research Involving Human Subjects, WHO, Geneva, Switzerland.

Results

ipaH could be detected in 97% (38/39; 95% CI: 92–100%) culture positive specimens compared to 58% (174/298; 95% CI: 52–64%) culture negative specimens using real-time PCR (p < 0.0001) (Table 1). The highest detection rate was found in culture positive patients with dysentery, which was 100% (20/20); conversely, the lowest detection rate, 54% (112/206; 95% CI: 47–61%) was seen in culture negative patients without dysentery (p < 0.0001).

The cycle threshold (Ct) of real-time PCR required for the detection of ipaH was highest in culture negative, non-dysenteric patients over 5 years of age (Mean = 36.5), and was lowest in children less than 5 years of age with bloody stool (Mean = 20.7) (p < 0.0001). Overall, the mean Ct tended to be lower in patients less than 5 years of age than patients over 5 years (p < 0.0001). There was a significant trend of increasing Cts across 8 categories (p < 0.001) (Table 1).

Among those potential variables, which might affect the detection of ipaH, culture results had the strongest predictive factor (OR = 15.5; 95% CI: 2.0–119.0), followed by diarrhoea 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) (Table 2).

Between 1 January and 31 December 2002, 10,105 patients from catchment area consented and provided a rectal swab. Based on the stool culture results, the incidence rate of treated shigellosis was highest in children less than 5 years of age (19.4/1000/year) and lowest in people aged 30–39 years (2.6/1000/year). The incidence increased significantly after age 30 years (p < 0.001). Individuals over age 50 years had a shigellosis rate of 6.3/1000/year (Table 3). The increase in detection rates between culture and PCR was 3.7-fold increase in the 40–49 yrs age group up to a 9-fold increase in the 10–19 yrs age group and on average 5.4-fold. After adjusting shigellosis incidence based on PCR detection rates, the highest incidence of treated shigellosis was in children less than 5 years of age (420.4/1000/year), followed by people over 50 years of age (89.1/1000/year). The PCR adjusted shigellosis incidence in people aged 30–39 year was 40.8/1000/year. PCR detected Shigella in 63% of diarrhea episodes in the catchment area during study period.

Table 1  Clinical and demographic variables related to the detection of ipaH in Zhengding 2003.

<table>
<thead>
<tr>
<th>Shigella culture result</th>
<th>Dysentery</th>
<th>Age (yr)</th>
<th>Total</th>
<th>No. of tested</th>
<th>PCR positive (%)</th>
<th>Ct value (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Yes</td>
<td>&lt;5</td>
<td>21</td>
<td>10</td>
<td>10 (100)</td>
<td>20.7 (0.76)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥5</td>
<td>126</td>
<td>10</td>
<td>10 (100)</td>
<td>21.8 (0.77)</td>
</tr>
<tr>
<td>Negative</td>
<td>Yes</td>
<td>&lt;5</td>
<td>37</td>
<td>9</td>
<td>9 (100)</td>
<td>22.6 (1.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥5</td>
<td>147</td>
<td>10</td>
<td>9 (90)</td>
<td>24.6 (2.33)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>&lt;5</td>
<td>55</td>
<td>45</td>
<td>31 (69)</td>
<td>31.8 (1.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥5</td>
<td>249</td>
<td>47</td>
<td>31 (66)</td>
<td>32.6 (1.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;5</td>
<td>1738</td>
<td>102</td>
<td>63 (62)</td>
<td>36.1 (0.37)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥5</td>
<td>7732</td>
<td>104</td>
<td>49 (47)</td>
<td>36.5 (0.45)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>10,105</td>
<td>337</td>
<td>212 (63)</td>
<td></td>
</tr>
</tbody>
</table>

Mean as well as standard error of Ct value required for detection.
Data not available for 8 patients. Corrected by the PCR positive rate of randomized sampling.

Data not available for 10 patients. Data not available for 2 patients.

were culture negative for PCR assays can detect Shigella specific DNA in patients who culture.15 There is now a range of studies which show that magnitude more than 3% detection rate detected by stool diarrhea episodes in Nha Trang, Vietnam, an order of infection may be responsible for 35% or more of the

Using a similar approach Thiem et al. found shigellosis increase 20-fold from 4.4/1000/year to 84.1/1000/year. shigellosis the incidence rate in catchment area would

confirmed by stool culture and 212 were positive by real-time PCR. If PCR would be used for the detection of culture positive 38 1 15.5 (2.0 11.4) 113 (68.1) 19.4 420.4

With dysentery 38 1 15.5 (2.0 11.4) 113 (68.1) 19.4 420.4

No. of days of diarrhea (Median)c 2 2 1.0 (0.6 2.2) 0.511

Data not available for 8 patients.

Data not available for 10 patients.

Data not available for 2 patients.

Table 2 Variables related the detection of ipaH by real-time PCR.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PCR positive</th>
<th>PCR-negative</th>
<th>Odds ratio (95%CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shigella culture positive</td>
<td>38</td>
<td>1</td>
<td>15.5 (2.0–119.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>With fever</td>
<td>53</td>
<td>9</td>
<td>2.8 (1.2–6.2)</td>
<td>0.015</td>
</tr>
<tr>
<td>Specimens occurred in epidemic season (June to October)</td>
<td>166</td>
<td>71</td>
<td>2.4 (1.4–4.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>Female gender</td>
<td>100</td>
<td>46</td>
<td>1.8 (1.1–3.0)</td>
<td>0.029</td>
</tr>
<tr>
<td>With dysentery</td>
<td>82</td>
<td>30</td>
<td>1.7 (0.9–3.0)</td>
<td>0.083</td>
</tr>
<tr>
<td>With self-reported antibiotic use before presentationa</td>
<td>46</td>
<td>24</td>
<td>1.2 (0.6–2.2)</td>
<td>0.613</td>
</tr>
<tr>
<td>Age (Mean)</td>
<td>18.9</td>
<td>20.5</td>
<td>1.0 (0.9–1.0)</td>
<td>0.106</td>
</tr>
<tr>
<td>Maximum no. of bowel movements (Median)b</td>
<td>4</td>
<td>4</td>
<td>1.1 (0.6–2.3)</td>
<td>0.723</td>
</tr>
<tr>
<td>No. of days of diarrhea prior to presentation (Median)c</td>
<td>1</td>
<td>1</td>
<td>1.4 (0.6–3.0)</td>
<td>0.431</td>
</tr>
<tr>
<td>No. of days of diarrhea (Median)</td>
<td>2</td>
<td>2</td>
<td>1.0 (0.6–2.2)</td>
<td>0.511</td>
</tr>
</tbody>
</table>

a Data not available for 8 patients.
b Data not available for 10 patients.
c Data not available for 2 patients.

Discussion

The real-time PCR detected a much higher number of shigellosis episodes in comparison with conventional stool culture. Out of 337 stool specimens examined, 39 were confirmed by stool culture and 212 were positive by real-time PCR. If PCR would be used for the detection of shigellosis the incidence rate in catchment area would increase 20-fold from 4.4/1000/year to 84.1/1000/year. Using a similar approach Thiem et al. found shigellosis infection may be responsible for 35% or more of the diarrhea episodes in Nha Trang, Vietnam, an order of magnitude more than 3% detection rate detected by stool culture.15 There is now a range of studies which show that PCR assays can detect Shigella specific DNA in patients who were culture negative for Shigella.6,17–19

ipaH detection was found to be related to culture results, clinical manifestation, gender and season of specimen collection. ipaH was more frequently detected in female patients compared to male patients. In rural Zhengding women are responsible for the cooking, housekeeping and caring for children. Women are therefore more frequently exposed to children. In our study, the highest incidence rate (32/1000/year) by stool culture was found in children aged 3–4 years.3

The detection rate of ipaH was also significant higher in during the epidemic season compared to the non-epidemic season, and in diarrhea patients with fever compared to afebrile patients. Higher detection rates of ipaH could also be found in young children compared to older children and dysenteric diarrhea compared to non-dysenteric diarrhea, though these differences were not statistically significant. The most likely explanation for the relationship between detection rate by PCR and clinical manifestation is the variation of bacterial loads. The clinical spectrum of shigellosis is broad, extending from subclinical infections and mild watery diarrhea to fulminating dysentery characterized by high fever.20 Severe shigellosis is associated with the rupture of the intestinal epithelial mucosa, resulting in a high bacterial load in the stool.21 The number of PCR cycles directly related to the original amount of target present in the PCR reaction.22 The number of cycles required for the detection of ipaH was lowest for in culture-positive dysenteric patients and highest for culture negative and non-dysenteric individuals.

Our study has several limitations. Apart from Shigella spp., the ipaH detected by PCR could originate from EIEC. However, the prevalence of EIEC reported was exceedingly low in comparison with that of Shigella spp. in China as well as Asia.9–13 It seems highly unlikely that the ipaH detected by this study was

Table 3 Incidence rate and corrected incidence rate of Shigella.

<table>
<thead>
<tr>
<th>Age</th>
<th>Population</th>
<th>No. of diarrhea cases</th>
<th>No. of culture positive</th>
<th>Randomized sampling from diarrhea cases Total</th>
<th>No. of culture positive (%)</th>
<th>No. of PCR positive (%)</th>
<th>Incidence rate of Shigella (/1000/year)</th>
<th>Corrected incidence rate of Shigella a (/1000/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–</td>
<td>2997</td>
<td>1851</td>
<td>58</td>
<td>166</td>
<td>19 (11.4)</td>
<td>113 (68.1)</td>
<td>19.4</td>
<td>420.4</td>
</tr>
<tr>
<td>5–</td>
<td>4151</td>
<td>536</td>
<td>15</td>
<td>13</td>
<td>1 (7.7)</td>
<td>6 (46.2)</td>
<td>3.6</td>
<td>59.6</td>
</tr>
<tr>
<td>10–</td>
<td>18,787</td>
<td>1580</td>
<td>52</td>
<td>29</td>
<td>2 (6.9)</td>
<td>18 (62.1)</td>
<td>2.8</td>
<td>52.2</td>
</tr>
<tr>
<td>20–</td>
<td>8988</td>
<td>1125</td>
<td>30</td>
<td>30</td>
<td>4 (13.3)</td>
<td>18 (60.0)</td>
<td>3.3</td>
<td>75.1</td>
</tr>
<tr>
<td>30–</td>
<td>13,675</td>
<td>1535</td>
<td>36</td>
<td>22</td>
<td>2 (9.1)</td>
<td>8 (36.4)</td>
<td>2.6</td>
<td>40.8</td>
</tr>
<tr>
<td>40–</td>
<td>11,504</td>
<td>1440</td>
<td>42</td>
<td>21</td>
<td>3 (14.3)</td>
<td>11 (52.4)</td>
<td>3.7</td>
<td>65.6</td>
</tr>
<tr>
<td>50–</td>
<td>7301</td>
<td>1066</td>
<td>39</td>
<td>25</td>
<td>4 (16.0)</td>
<td>17 (68.0)</td>
<td>5.3</td>
<td>99.3</td>
</tr>
<tr>
<td>60–</td>
<td>4482</td>
<td>594</td>
<td>31</td>
<td>19</td>
<td>2 (10.5)</td>
<td>13 (68.4)</td>
<td>6.9</td>
<td>90.7</td>
</tr>
<tr>
<td>70–</td>
<td>3745</td>
<td>378</td>
<td>28</td>
<td>12</td>
<td>2 (16.7)</td>
<td>8 (66.7)</td>
<td>7.5</td>
<td>67.3</td>
</tr>
<tr>
<td>Total</td>
<td>75,630</td>
<td>10,105</td>
<td>331</td>
<td>337</td>
<td>39 (11.6)</td>
<td>212 (62.9)</td>
<td>4.4</td>
<td>84.1</td>
</tr>
</tbody>
</table>

a Corrected by the PCR positive rate of randomized sampling.
related to EIEC. The frequent occurrence of PCR positive, culture negative specimens could be false-positives due to contamination. If this were the case, the culture negative PCR-positive cases should occur randomly, independent of patient’s clinical manifestations and seasonality. But, this possible explanation was not supported by findings of our analysis. Asymptomatic carriers of Shigella spp. who had a diarrhea episode caused by other organisms could result in an over-estimate of the disease burden. However the culture positive episode negative specimens could be false-positives due to contamination. If this were the case, the culture negative specimens could be false-positives due to contamination.

Acknowledgements

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References


