Characterization of Shigella spp. by antimicrobial resistance and PCR detection of ipa genes in an infantile population from Porto Velho (Western Amazon region), Brazil

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The incidence of Shigella spp. was assessed in 877 infants from the public hospital in Rondônia (Western Amazon region, Brazil) where Shigella represents the fourth cause of diarrhea. Twenty-five isolates were identified: 18 were Shigella flexneri, three Shigella sonnei, three Shigella boydii and one Shigella dysenteriae. With the exception of S. dysenteriae, all Shigella spp. isolated from children with diarrhea acquired multiple antibiotic resistances. PCR detection of ipa virulence genes and invasion assays of bloody diarrhea and fever (colitis) were compared among 25 patients testing positive for Shigella. The ipaH and ipaBCD genes were detected in almost all isolates and, unsurprisingly, all Shigella isolates associated with colitis were able to invade HeLa cells. This work alerts for multiple antibiotic resistant Shigella in the region and characterizes presence of ipa virulence genes and invasion phenotypes in dysenteric shigellosis.

Key words: Shigella spp. - Brazil - multiple antibiotic resistance - diarrhea - ipa genes

Shigelllosis continues to be a major health problem worldwide, occurring predominantly in children younger than five years of age in developing countries. Thus far, the only available information about diarrhea in Rondônia is from previous studies of enteropathogens associated with diarrhea in an infantile population from a district of Porto Velho, where rotavirus appeared as the major etiological agent (Orlandi et al. 2001, 2006).

This study was conducted over a period of 24 months at the Cosme Damião Public Infant Hospital in Porto Velho, Rondônia (Western Amazon region, Brazil) to assess the incidence of enteropathogens in infantile diarrhea. The population was composed mainly of poor inhabitants living in unsanitary conditions. A group of children with nonenteric pathologies was used as a control and PCR detection of ipa virulence genes as well as epithelial invasion assays assessed the presence of wild Shigella spp. This study was approved by the local ethics committee (Center of Tropical Medicine Ethical Committee, Porto Velho, nº 463).

Stool specimens were collected between March 2000-March 2002 using natural or glycerin-induced swabs of 470 children between 0-60 months of age presenting diarrhea. In addition, 407 children of the same age group with nonenteric pathologies were examined. Dysentery or hemorrhagic colitis was confirmed by the appearance of fever or bloody diarrhea traces in the stool (Feca Cult, One Step Test, INLAB Diagnostica).

Shigella spp. in stool samples (cultured in Cary-Blair medium, MacConkey, XLD and Salmonella-Shigella agar) was identified using the 20E System Analytical Profile Index (API-Bio-Merieux). Determination of Shigella serotypes was performed by slide agglutination assays on commercial antisera (Bio-Merieux). Among the Shigella flexneri isolates, 16 were serotype 2a and two were serotype 3a. All Shigella boydii were serotype 4 and Shigella dysenteriae was serotype 1. Shigella sonnei serotyping was not determined.

Antimicrobial sensitivity tests were conducted according to Mates et al. (2000), using commercially available Mueller-Hinton agar disks (Difco) containing antibiotics against enterobacteria (ampicillin, penicillin, amoxicillin-clavulanic acid, azitromycin; nalidixic acid, ciprofloxacin, norfloxacin; ceftriaxone; chloramphenicol and trimethoprim-sulfamethoxazole). To control for sensitivity, we used Escherichia coli ATCC25922 strain and azitromycin sensitivity assay the Staphylococcus aureus ATCC25923 strain.

PCR detection of ipaB, ipaC, ipaD and ipaH was previously described by Faruque et al. (2002). Invasion capacity was assessed by Hep-2 infection assays (Francis et al. 1991) and efficiency of infection was determined by visually scoring ethanol-fixed, Giemsa-stained cells after treatment with a balanced salt solution containing gentamicin (Mantis et al. 1996); this treatment exclusively kills extracellular bacteria and eliminates non-invasive bacteria. Chi-squared analyses were performed using Fisher’s exact tests.

Gastroenteritis was found predominantly in children younger than 24-months of age with Shigella spp., representing the fourth major cause of diarrhea, preceded only by rotavirus, diarrheagenic E. coli and Salmonella sp. (Orlandi et al. 2006). Twenty-four Shigella spp. were isolated, of which 72% were S. flexneri,
12% *S. boydii*, 12% *S. sonnei* and 4% *S. dysenteriae*; the frequency of *Shigella* comparing with all enteropathogens was 5.1%. Slide agglutination assays revealed the presence of 16 *S. flexneri* type II and two type III strains, three *S. sonnei*, three *S. boydii* and one *S. dysenteriae*. However, the low frequency of *S. dysenteriae* may have been due to non-specific *S. dysenteriae* present in the culture media.

High levels of resistance to trimethoprim/sulfamethoxazole, ampicillin, amicacin, penicillin and cotrimoxazol were observed. Eighteen *S. flexneri* isolates displayed resistance to multiple antibiotics, as well as a low frequency of resistance to nalidixic acid and quinolones (ciprofloxacin and norfloxacin).

PCR-based detection of *ipaBCD* and *ipaH* genes showed the presence of *ipaH* in 17 of 18 *S. flexneri* isolates, and all isolates were positive for *ipaBCD*. Two of three *S. sonnei* and *S. boydii* isolates tested positive for *ipaH* and *ipaBCD* virulence genes, while *S. dysenteriae* did not. We observed 19 *ipaH*+ and inv+, three *ipaH*- and inv-, two *ipaH*+ and inv- and one *ipaH*- and inv+ strains by invasion phenotyping, and 20 *ipaBCD*+ and inv+, three *ipaBCD*- and inv- and one *ipaBCD*+ and inv- genotypes. The *S. flexneri* collected from stool of non-enteric subjects was unable to invade HeLa cells.

An association of colitis with bloody diarrhea and fever was observed in 20 patients. Nineteen had *ipaH*+ and inv+, three *ipaH*+ and inv-, two *ipaH*+ and inv- and one *ipaH*- and inv+ strains by invasion phenotyping, and 20 *ipaBCD*+ and inv+, three *ipaBCD*- and inv- and one *ipaBCD*+ and inv- genotypes. The *S. flexneri* collected from stool of non-enteric subjects was unable to invade HeLa cells.

The presence of multiple antibiotic resistant *Shigella* isolates, *Shigella* in children with nonenteric pathologies and identification of diarrhea without dysentery detach-es the presence of *Shigella* within our region.

**REFERENCES**


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**TABLE**

Analysis between colitis presented by infected children and *ipa H* or *ipaBCD* or invasion phenotypes

<table>
<thead>
<tr>
<th></th>
<th>Colitis</th>
<th>Without colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IpaH</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>$\chi^2_{2}=5.38$</td>
<td>p = 0.016</td>
<td></td>
</tr>
<tr>
<td><em>Ipa BCD</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>$\chi^2_{2}=8.55$</td>
<td>p = 0.0043</td>
<td></td>
</tr>
<tr>
<td>Invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>$\chi^2_{2}=19.14$</td>
<td>p = 0.00018</td>
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</tr>
</tbody>
</table>

*a*: colitis characterized by diarrhea with fever and blood in the stools.

While *S. flexneri* and *S. dysenteriae* were found to be quite common in areas with inadequate sanitation, *S. sonnei* prevalence was related more to contaminated food and drink in developed countries (Lima et al. 1997, Faruque et al. 2002). According to the present study, incidence of shigellosis along the Porto Velho border is far worse than in other poorer areas of Brazil due to the presence of the uncommon *S. boydii* strain.

Many studies have previously described Shigellosis frequencies around 3-6% (Leal et al. 1988, 1998, Lima et al. 2000, Medeiros et al. 2001). Orlandi et al (2001) also found a 6.1% frequency of *S. flexneri* in 130 children with diarrhea and the frequencies and predominance of *S. flexneri* were similar to those observed in different states of Brazil (Leal et al. 1988, 1998, Lima et al. 2000) as well as in other tropical countries (Navia et al. 1999).

In our study we have analyzed colitis related to different virulence factors, the presence of *ipaH* or *ipaBCD* genes and the ability to invade HeLa cells. Our data has shown that all these phenotypes are associated with colitis (Table), although the ability to invade HeLa cells was not strictly associated with the presence of *ipa* virulence genes.

The presence of multiple antibiotic resistant *Shigella* isolates, *Shigella* in children with nonenteric pathologies and identification of diarrhea without dysentery detach-es the presence of *Shigella* within our region.


