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 phenotypesin dysenteric shigellosis.*

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

Shigellosis 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-Merrieux). Among the *Shigella flexneri* isolates, 16 were serotype 2a and two were serotype 3a. All *Shigella boydii* were serotype 4 and *Shigella disenteriae* 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, azitromicin; nalidixic acid, ciprofloxacin, norfloxacin; cefitriaxone; chloranphenicol and trimethoprim-sulfamethoxazole). To control for sensitivity, we used *Escherichia coli* ATCC25922 strain and azitromicin 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*,

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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*+ (χ^2_{2t} 5.38, p = 0.016) (Table) and among the five patients without colitis, two contained the *ipaH* gene. All 20 colitis patients were *Shigella ipaBCD*+ (χ^2_{2t} 8.55 p = 0.0043) (Table).

All 20 *Shigella* isolates from subjects associated with colitis were able to invade HeLa cells and five *Shigella* isolates from patients without colitis could not invade HeLa cells (χ^2_{2t} 19.14, p = 0.000018) (Table).

PCR detection of *ipa* virulence genes represents an excellent tool for diagnosis of shigellosis (Thiem et al. 2004). Our data revealed an association between colitis enteric and children with shigellosis.

TABLE

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

	Colitis[a]	Without colitis
IpaH		
Positive	19	2
Negative	1	3
	χ^2_{2t} = 5.38	p = 0.016
Ipa BCD		
Positive	20	2
Negative	0	3
	χ^2_{2t} = 8.55	p = 0.0043
Invasion		
Positive	20	0
Negative	0	5
	χ^2_{2t} =19.14	p = 0.000018

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 too 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 detaches the presence of *Shigella* within our region.

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