Shigella in Brazilian children with acute diarrhoea: prevalence, antimicrobial resistance and virulence genes

Mireille Ângela Bernardes Sousa[1], Edilberto Nogueira Mendes[2], Guilherme Birchal Collares[1], Luciano Amedée Péret-Filho[3], Francisco José Penna[3], Paula Prazeres Magalhães[1]/+

[1]Departamento de Microbiologia, Instituto de Ciências Biológicas [2]Departamento de Propedêutica Complementar
[3]Departamento de Pediatria, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Diarrhoeal disease is still considered a major cause of morbidity and mortality among children. Among diarrhoeagenic agents, Shigella *should be highlighted due to its prevalence and the severity of the associated disease. Here, we assessed* Shigella *prevalence, drug susceptibility and virulence factors. Faeces from 157 children with diarrhoea who sought treatment at the Children's Hospital João Paulo II, a reference children's hospital in Belo Horizonte, state of Minas Gerais, Brazil, were cultured and drug susceptibility of the* Shigella *isolates was determined by the disk diffusion technique.* Shigella *virulence markers were identified by polymerase chain reaction. The bacterium was recovered from 10.8% of the children (88.2%* Shigella sonnei*). The* ipaH*,* iuc*,* sen *and* ial *genes were detected in strains isolated from all shigellosis patients;* set1A *was only detected in* Shigella flexneri*. Additionally, patients were infected by* Shigella *strains of different* ial*,* sat*,* sen *and* set1A *genotypes. Compared to previous studies, we observed a marked shift in the distribution of species from* S. flexneri *to* S. sonnei *and high rates of trimethoprim/sulfamethoxazole resistance.*

Key words: *Shigella* - dysentery - diarrhoea - virulence - drug resistance

Diarrhoeal disease constitutes one of the major causes of morbidity and mortality among infants and young children worldwide. It has been estimated that two-four billion episodes of infectious diarrhoea annually occur in developing countries, resulting in three-five million deaths, with the highest incidence and case-fatality rates in children below the age of five years (Sánchez & Holmgren 2005, Boschi-Pinto et al. 2008, Navaneethan & Gianella 2008).

The etiological agents of diarrhoea include a wide array of viruses, bacteria and parasites, many of which have only been recognised in the last two decades. *Shigella* has long been considered an important human pathogen throughout the world, especially in developing countries with substandard hygiene and unsafe water supplies (Ina et al. 2003, Niyogi 2005, Navaneethan & Gianella 2008, Bhattacharya et al. 2012, Shakoor et al. 2012).

Shigellosis is an invasive infection of the human colon that leads to a spectrum of clinical presentations, from brief watery diarrhoea to severe colitis. Clinical disease generally begins within 24-48 h of ingestion of 10-100 organisms. Watery diarrhoea typically precedes dysentery and is often the sole clinical manifestation of mild infection. However, progression to obvious dysentery may occur within hours to days. Although in most cases supportive anti-dehydration treatment is the cornerstone of diarrhoea therapy, antimicrobial treatment for shigellosis reduces the duration and severity of the disease. According to the World Health Organization (WHO 2005) guidelines, the choice of therapy scheme should be decided by the antimicrobial susceptibility pattern of locally circulating *Shigella* strains (Niyogi 2005). There are four major subgroups of *Shigella*, which are classified by biochemical and antigenic characteristics: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*. *S. dysenteriae* and *S. flexneri* are the predominant species in developing countries, while *S. sonnei* accounts for most of the reported cases of shigellosis in developed countries (Nataro et al. 1995, Ina et al. 2003, Niyogi 2005, Nyachuba 2010).

Several virulence factors are associated with *Shigella* spp. The ability to colonise and invade intestinal cells, resulting in an intense acute inflammatory response, is mediated by plasmidial and chromosomal sequences, such as the invasion-associated *locus* (*ial*) (Li-Yan et al. 1993) and the invasion plasmid antigen H gene (*ipaH*) (Lüscher & Altwegg 1994). It has been suggested that *ial* is responsible for epithelial cell penetration by the bacterium and *ipaH* also for dissemination from cell to cell (Wei et al. 2003, Parsot 2005).

There are other virulence factors associated with the genus *Shigella*, such as toxins and iron acquisition systems. *Shigella* enterotoxin 1 (ShET-1) is a chromosomally encoded, iron-regulated, 55-kDa complex protein that is produced almost exclusively by *S. flexneri* 2a and only rarely by other serotypes. ShET-1 consists of two types of subunits: one A subunit, encoded by *set1A* and five B subunits, encoded by *set1B*. *Shigella* enterotoxin 2 (ShET-2) is a 62.8-kDa protein encoded by *sen*, located on the 140-MDa virulence plasmid. ShET-2 was originally discovered in enteroinvasive *Escherichia coli,* but

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+ Corresponding author: ppmagalhaes@ufmg.br
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is produced by many *Shigella* serotypes (Fasano et al. 1995, Nataro et al. 1995, Noriega et al. 1995, Vargas et al. 1999). The secreted autotransporter toxin Sat is a 107-kDa protein that is chromosomally encoded by the *sat* gene and was first described in uropathogenic *E. coli* (Guyer et al. 2000). The aerobactin gene *iuc* is chromosomally located on a pathogenicity island and codes for siderophores, which are compounds associated with iron acquisition (Lawlor & Payne 1984, Vokes et al. 1999).

Despite the profusion of reports from different parts of the world concerning the aetiology of acute infectious diarrhoea, few data addressing *Shigella*-associated diarrhoea and organism susceptibility profiles are available in Brazil, especially for our region, where the last population-based study of diarrhoeagenic bacteria was conducted 25 years ago (Queiroz et al. 1987). Furthermore, investigations into *Shigella* spp virulence markers are still scarce worldwide and we are not aware of any paper on this subject in Brazil. Therefore, we evaluated the prevalence of *Shigella* and characterised the strains' antimicrobial susceptibility patterns and virulence factors.

SUBJECTS, MATERIALS AND METHODS

Patients - The study group consisted of 157 children (83 male and 74 female, age range 1-48 months, mean age 11.7 months, median 8 months) presenting with acute diarrhoea who sought treatment at the Children's Hospital João Paulo II/Hospital Foundation of the State of Minas Gerais, Belo Horizonte, state of Minas Gerais (MG), Brazil. All patients were from low socioeconomic strata and had no history of hospitalisation or antimicrobial therapy in the 15 days prior to sample collection. Between March 2004-March 2005, passed stool specimens were prospectively collected and transferred to two sterile leak-proof screw-cap collectors (J Prolab, São José dos Pinhais, state of Paraná, Brazil), one of which contained a transport medium of equal parts glycerol and 0.033 M phosphate buffer. The specimens were transported to the laboratory in an ice bath within 1 h of collection.

Faecal leukocyte detection - To detect leukocytes, faecal smears were stained with May-Grünwald-Giemsa and examined under bright field microscopy at 400X and 1,000X magnification.

Shigella identification - The specimens transported in buffered glycerol were used to isolate *Shigella* on MacConkey agar and SS agar media (Difco, Sparks, MD, USA). Faecal specimens were also inoculated into tetrathionate broth (Acumedia, Baltimore, MD, USA) and, after incubation for approximately 18 h at 35ºC, were subcultured on SS agar. All agar cultures were incubated for up to 24 h at 35ºC. Approximately five lactose-negative colonies from each agar medium were picked and inoculated into triple sugar iron agar (Acumedia), EPM (Toledo et al. 1982a), MILi (Toledo et al. 1982b) and citrate media (Biobrás, Montes Claros, MG, Brazil). Whenever possible, morphologically different colonies were selected. Following identification, *Shigella* isolates were antigenically characterised with polyvalent antisera, as recommended by the manufacturer [Probac, São Paulo, state of São Paulo (SP), Brazil].

Antimicrobial susceptibility test - The antimicrobial susceptibility profiles of *Shigella* strains were investigated using a standard agar diffusion method with ampicillin (10 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg) and trimethoprim/sulfamethoxazole (25 µg) (Cecon, São Paulo, SP, Brazil). Screening for organisms producing extended-spectrum β-lactamases (ESBLs) was performed with ceftriaxone (30 µg), ceftazidime (30 µg), aztreonam (30 µg) and cefotaxime (30 µg) (Cecon). *E. coli* ATCC 25922 was included as a reference strain for antimicrobial susceptibility testing quality control (CLSI 2005).

Virulence factor detection - After overnight cultivation on tryptic soy agar (Difco) plates at 35ºC, bacterial cells were suspended in 500 µL sterile distilled water and pelleted for 15 min at 6,000 *g*. DNA was isolated using phenol-chloroform extraction (Fox et al. 1994). The DNA concentration was estimated with an RNA/DNA calculator (Pharmacia Biotech, Cambridge, England) and adjusted to 20 ng/µL. All polymerase chain reaction (PCR) mixtures were prepared in a dedicated PCR chamber (Plaslabs, Lansing, MI, USA). The reaction mixtures were cycled in an MJ Research thermocycler (Watertown, MA, USA). Amplicons were assessed in a separate area to prevent contamination. All amplified PCR products were resolved on 8% polyacrylamide gels stained with ethidium bromide and visualised under ultraviolet light (Cole-Parmer, Vernon Hills, IL, USA). A 100 bp ladder (Life Technologies, Gaithersburg, MD, USA) was used as a molecular size marker. Positive and negative controls, as well as an internal negative control (sterile water) (LiChrosolv, Merck, Darmstadt, Germany), were included in each batch of reactions.

The *ipaH*, *ial*, *set1A*, *sen*, *sat* and *iuc* virulence markers were amplified as previously reported, with slight modifications (Lüscher & Altwegg 1994, Vargas et al. 1999, Ruiz et al. 2002, Kingombe et al. 2005). Approximately 20 ng of bacterial DNA was used as the template for amplification reactions. Details of the protocols are given in Table I. *Taq* DNA polymerase from Invitrogen (São Paulo, SP, Brazil) was used for *sat* and *set1A* amplification; polymerase from Phoneutria (Belo Horizonte, MG, Brazil) was used for *ial*, *iuc*, *ipaH* and *sen* amplification.

To ascertain the sensitivity and specificity of the amplification reactions, the following reference strains were used: *S. flexneri* (ATCC 12022), *S. sonnei* (ATCC 25931), *S. dysenteriae* (ATCC 13313), *S. enterica* Enteritidis (ATCC 13076), *S. enterica* Typhimurium (ATCC 14028), enteropathogenic *E. coli* (INCQS 00184), enteroinvasive *E. coli* (ATCC 43893), enterohaemorrhagic *E. coli* (ATCC 43895), *E. coli* (ATCC 25922) and *Aeromonas hydrophila* (IOC/FDA 110-36).

Ethics - This study was approved by the Ethical Committee of the Federal University of Minas Gerais (protocol ETIC 047/03). Written consent to participate was obtained from the parents or guardians of all children included in the study.

RESULTS

Shigella species were isolated from stool specimens of 17/157 (10.8%) patients: *S. sonnei* from 15/17 (88.2%) and *S. flexneri* from two/17 (11.8%). A total of 135 *Shigella* colonies were obtained (2-18 isolates/patient, average 7.9). Of the colonies, 104 were *S. sonnei* (2-18 isolates/patient, average 6.9) and 31 were *S. flexneri* (15-16 isolates/patient, average 15.5) strains. Each bacterial isolate was submitted to serotyping and antimicrobial susceptibility profile determination, as well as virulence factor identification.

The mean age of *Shigella*-positive patients (27.7 months, ranging from 3-48 months) was higher than that of *Shigella*-negative patients (9.7 months, ranging from 1-47 months; $p < 10^{-3}$). Most cases of shigellosis (approximately 90%) were detected in children older than one year [$p < 10^{-6}$, odds ratio (OR) = 22.3, confidence interval (CI) = 4.71-205.8]. More cases were observed in the warm and rainy months (October-March), although this difference was not statistically significant. *Shigella* positivity was not associated with gender.

Faecal leukocytes were found in 40 of the 152 (26.3%) stool specimens analysed. The test was more frequently positive for shigellosis patients (15/17, 88.2%; $p < 10^{-7}$, OR = 33.0, CI = 6.8-305.9); 25 of the 135 (18.5%) *Shigella*-negative children gave positive results for this assay.

All *Shigella* isolates were sensitive to ceftriaxone, ciprofloxacin and nalidixic acid. Resistance rates to ampicillin and trimethoprim/sulfamethoxazole are given in Table II. Multiple drug susceptibility patterns among isolates obtained from the same patient were not observed. The test for ESBL production was negative for all *Shigella* isolates.

The *ipaH*, *iuc* and *sen* genes, as well as the *ial locus*, were detected in *Shigella* strains isolated from all *Shigella*-positive patients. The *sat* gene was observed in bacterial isolates from stool specimens obtained from five of 17 (29.4%) children with shigellosis: two/two (100%) and three/15 (20.0%) patients from whom *S. flexneri* and *S. sonnei* were recovered, respectively. The *set1A* gene was only detected in *S. flexneri* isolates.

All *Shigella* isolates exhibited the *ipaH*$^{+}$*iuc*$^{+}$ genotype. Concerning the *ial*, *sat* and *sen* markers, diversity among isolates from the same child was observed in eight patients infected with *S. sonnei* (Table III). Different isolates were obtained from the two children infected with *S. flexneri*. A total of 16 isolates were obtained from one of these patients, all of which were *sat*$^{+}$*set1A*$^{+}$, 13 were *ial*$^{+}$ and three were *ial*$^{-}$. Fifteen isolates were obtained from the other *S. flexneri*-positive child: nine were *ial*$^{+}$, 14 *sat*$^{+}$ and seven *set1A*$^{+}$.

TABLE II

Resistance rates of *Shigella* strains to ampicillin and trimethoprim/sulfamethoxazole as evaluated by patient

Antimicrobial drug	*Shigella sonnei* (n = 15) n (%)	*Shigella flexneri* (n = 2) n (%)	Total (n = 17) n (%)
Ampicillin	2/13.3	2/100	4/23.5
Trimethoprim/sulfamethoxazole	13/86.7	1/50	14/82.4

TABLE III

Distribution of *Shigella sonnei* strains in children infected by bacterium isolates harbouring different virulence markers

	Virulence marker						
	ial		*sat*		*sen*		Isolates
Patient	+	-	+	-	+	-	(n)
1	2	1	0	3	3	0	3
2	13	0	0	13	12	1	13
3	7	2	0	9	9	0	9
4	7	0	0	7	6	1	7
5	4	0	0	4	3	1	4
6	2	1	0	3	3	0	3
7	3	3	6	0	6	0	6
8	5	1	0	6	6	0	6

TABLE I

Polymerase chain reaction conditions employed for the detection of virulence markers of *Shigella*

Gene	[*primer*] (μM)	[$MgCl_2$] (mM)	[*Taq*] (U)	Annealing temperature (ºC)	Reference
ial	0.6	2.5	0.5	60	Kingombe et al. (2005)
ipaH	0.125	1.2	0.25	62	Lüscher and Altwegg (1994)
iuc	0.4	2.5	0.5	70	Kingombe et al. (2005)
sat	0.25	1.5	1.0	64	Ruiz et al. (2002)
sen	0.125	1.5	0.5	56	Vargas et al. (1999)
set1A	0.25	1.5	0.5	58	Vargas et al. (1999)

DISCUSSION

Recognition of the importance of *Shigella* as an enteric pathogen with a global impact has increased in recent years. Its importance is particularly visible in developing countries, where, as a consequence of vigorous use of oral rehydration therapy in recent decades, mortality from diarrhoeal dehydration has substantially decreased. However, because this intervention provides little benefit to patients with dysentery caused by invasive bacteria, the relative importance of shigellosis as a clinical problem has increased (Niyogi 2005, Bhattacharya et al. 2012, Shakoor et al. 2012).

Compared to a local data reported by Queiroz et al. (1987) at the end of the 1980s, we observed a decrease in the prevalence of *Shigella*. Although Brazil is a developing country, the vast majority of isolates we obtained were *S. sonnei* (≅ 90%). This finding is similar to recent results from El Salvador, but in contrast to previously reported data for our region, states of Rondônia, Pará (PA) and Piauí (Queiroz et al. 1987, Diniz-Santos et al. 2005, Silva et al. 2008, Bastos & Loureiro 2011, Nunes et al. 2012). In fact, *S. sonnei* is the most common *Shigella* species isolated in industrialised countries (Ina et al. 2003, Navia et al. 2005, Niyogi 2005). It is plausible that improvements to local hygiene and sanitation conditions in the intervening decades have reduced the prevalence of shigellosis and altered the local distribution pattern of *Shigella* species.

Shigellosis was more frequently observed in the summer (47.1%) and autumn (29.4%) than in the winter and spring, though no significant difference was found. Indeed, the incidence of shigellosis in our region peaked (≅ 65%) in the warmer and more humid weather.

The faecal leukocyte test has long been considered a rapid and simple tool for screening patients with inflammatory diarrhoea associated with invasive, cytotoxic agents, especially *Shigella* spp. Despite the limited evidence supporting the efficacy of this test, it is still considered valuable by many clinicians. We found leukocytes in the faeces of nearly 90% of the patients with shigellosis. Additionally, as expected, faecal leukocytes were much more commonly detected in children with shigellosis than in *Shigella*-negative patients (Huicho et al. 1996).

Acute infectious diarrhoeal disease, whether inflammatory or non-inflammatory, is usually self-limited. However, antimicrobial therapy is recommended in specific conditions, such as shigellosis, to limit both the clinical course of the illness and the duration of faecal excretion of the causative organism (Lima et al. 1995, Cheasty et al. 2004, Diniz-Santos et al. 2005, Nguyen et al. 2005, Peirano et al. 2005, Christopher et al. 2010). There has been an alarming increase in bacterial resistance to antimicrobials, mainly in developing countries, where the use of these drugs is relatively unrestricted. Indeed, over the last half century, *Shigella* has demonstrated extraordinary prowess in acquiring plasmid-encoded resistance to the antimicrobial drugs previously considered first-line therapies (Niyogi 2005).

The vast majority of our *Shigella* isolates were resistant to trimethoprim/sulfamethoxazole (82.4%). In contrast, we found low ampicillin resistance rates (23.5%), despite the fact that all *S. flexneri* isolates were resistant to this drug. Some studies report multidrug resistance in *Shigella* species, especially to trimethoprim/sulfamethoxazole and ampicillin, which are commonly used to treat shigellosis. Resistance rates to ampicillin and trimethoprim/sulfamethoxazole in the ranges of approximately 75-85% and 85-98%, respectively, have been reported (Lima et al. 1995, Nguyen et al. 2005, Meng et al. 2011). Similar to our findings, high susceptibility rates to ceftriaxone (100%), ciprofloxacin (96.4-100%) and nalidixic acid (93-97.1%) have already been reported by several authors (Oplustil et al. 2001, Torres et al. 2001, Cheasty et al. 2004, Nguyen et al. 2005).

Regarding recently published data for Brazil, 63.1% of *Shigella* strains isolated in PA displayed resistance to trimethoprim/sulfamethoxazole (62.5% and 64.2% of *S. sonnei* and *S. flexneri*, respectively) and 43.4% to ampicillin (17.5% and 56.8% of *S. sonnei* and *S. flexneri*, respectively). All isolates were sensitive to ciprofloxacin and nalidixic acid (Bastos & Loureiro 2011). The *S. flexneri* and *S. sonnei* strains studied by Nunes et al. (2012) in an investigation conducted in Northeast Brazil were all susceptible to nalidixic acid, ceftriaxone and ciprofloxacin. Ampicillin resistance was reported in 66.7% of *S. flexneri* isolates. Even higher rates of resistance to trimethoprim/sulfamethoxazole (71.4% for *S. flexneri* and 100% for *S. sonnei*) were observed.

Despite the fact that *Shigella* species are considered a leading cause of inflammatory diarrhoea, little is known about their genetic diversity worldwide. In Brazil, we are aware of no such study. *Shigella* may harbour several virulence factors, including those encoding toxins or associated with invasion of the colonic epithelium and dissemination from cell to cell (Fasano et al. 1995, Rudel 2012).

Historically, typing of *Shigella* has been performed using both phenotypic and genotypic methods. As with other bacteria, the methodology was initially based on conventional phenotypic tests (e.g., serotyping) or antimicrobial susceptibility testing, which are both inexpensive and technically simple, although their discriminatory powers are low. However, the development of molecular biology techniques has introduced genotyping methods with higher discriminatory power and better reproducibility (Navia et al. 2005). Methods such as pulsed-field gel electrophoresis and multilocus variable number tandem-repeat analysis have been successfully employed to evaluate genetic diversity among *Shigella* populations (Filliol-Toutain et al. 2011, Koh et al. 2012). In this study, PCR was used to investigate virulence markers in *Shigella* isolates.

All bacterial isolates were *ipaH*$^{+}$, as expected because this gene has been found in multiple copies on both the chromosome and the virulence plasmid of *Shigella* (Kingombe et al. 2005, Thong et al. 2005). Conversely, the *ial locus* is exclusively observed on the virulence plasmid and was only detected in some *Shigella* isolates. Indeed, less frequent detection of this marker has been reported by several authors (Lüscher & Altwegg 1994, Kingombe et al. 2005, Thong et al. 2005). The absence of this *locus* may be explained by the in-

trinsic genetic instability of this microorganism, which has hundreds of IS-elements capable of causing many types of DNA rearrangements (Yang et al. 2005). Additionally, the lack of *ial* could be due to the occurrence of mutations or lateral gene transfer (Navia et al. 2005). Furthermore, the possibility of multiple-strain infection cannot be ruled out.

In addition to their capacity for invasion, the expression of toxins is frequently observed in certain *Shigella* strains. These toxins include enterotoxins, such as Shet1 and Shet2 and Sat, a toxin first described in uropathogenic *E. coli*. We found *set1A*, which codes for Shet1, exclusively in *S. flexneri* strains, in agreement with previous results (Vargas et al. 1999, Guyer et al. 2000, Niyogi et al. 2004, Thong et al. 2005). The *sen* gene, which codes for Shet2, is located on the large virulence plasmid of *Shigella* spp that also carries sequences required for the invasion and spread of the bacterium, such as the *ipa* gene and the *ial locus* (Fasano et al. 1995, Noriega et al. 1995). The *sen* gene was detected in the vast majority of our *Shigella* isolates. Only three (≅ 2%) of the 135 isolates studied were sen^-, which is possibly a consequence of the same events mentioned for the *ial locus*. In 2005, a *sen* gene was described on the chromosome of an *S. sonnei* strain (Yang et al. 2005). The *sat* gene was detected in almost all *S. flexneri* strains and in a low proportion of *S. sonnei* isolates, as previously reported (Ruiz et al. 2002, Niyogi et al. 2004). Strains that were sat^+ were isolated from five children and only one isolate obtained from one of the five children was sat^-. The occurrence of a mutation in the gene may explain this finding.

The *iuc* gene was detected in all *Shigella* isolates, in agreement with previously reported results (Kingombe et al. 2005). The *iuc* gene is chromosomally located and stable in *Shigella* spp. Its detection could therefore be included in strategies designed to identify this bacterium (Lawlor & Payne 1984, Kingombe et al. 2005).

In conclusion, this study found a high prevalence of *Shigella* infection and a marked shift in the distribution of species, from *S. flexneri* to *S. sonnei*, among children less than four years of age in our region. Our data show a large proportion of trimethoprim/sulfamethoxazole-resistant *Shigella* isolates, a phenomenon that requires special attention because trimethoprim/sulfamethoxazole is considered the drug of choice to treat patients with inflammatory diarrhoea. We also described, for the first time, an evaluation of virulence markers of *Shigella* isolated from Brazilian patients, which may provide clues about the genetic diversity of this organism. Some of these markers were found in isolates from all *Shigella*-positive patients, but not in all bacterial isolates, which raises the hypothesis of infection of the same child by different clones of the bacterium or, alternatively, the hypothesis that this result is associated with the plasticity of the *Shigella* genome due to the large number of IS-elements. Our study provides useful information about the prevalence, antimicrobial susceptibility patterns and virulence markers of *Shigella*, a threat to public health and an agent of infectious diarrhoea that continuously challenges health care professionals worldwide.

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