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Epidemiological characterization of resistance and PCR typing of *Shigella flexneri* and *Shigella sonnei* strains isolated from bacillary dysentery cases in Southeast Brazil

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Abstract

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Shigella spp are Gram-negative, anaerobic facultative, non-motile, and non-sporulated bacilli of the Enterobacteriaceae family responsible for “Shigellosis” or bacillary dysentery, an important cause of worldwide morbidity and mortality. However, despite this, there are very few epidemiological studies about this bacterium in Brazil. We studied the antibiotic resistance profiles and the clonal structure of 60 *Shigella* strains (30 *S. flexneri* and 30 *S. sonnei*) isolated from shigellosis cases in different cities within the metropolitan area of Campinas, State of São Paulo, Brazil. We used the following well-characterized molecular techniques: enterobacterial repetitive intergenic consensus, repetitive extragenic palindromic, and double-repetitive element-polymerase chain reaction to characterize the bacteria. Also, the antibiotic resistance of the strains was determined by the diffusion disk method. Many strains of *S. flexneri* and *S. sonnei* were found to be multi-resistant. *S. flexneri* strains were resistant to ampicillin in 83.3% of cases, chloramphenicol in 70.0%, streptomycin in 86.7%, sulfamethoxazole in 80.0%, and tetracycline in 80.0%, while a smaller number of strains were resistant to cephalothin (3.3%) and sulfazotrim (10.0%). *S. sonnei* strains were mainly resistant to sulfamethoxazole (100.0%) and tetracycline (96.7%) and, to a lesser extent, to ampicillin (6.7%) and streptomycin (26.7%). Polymerase chain reaction-based typing supported the existence of specific clones responsible for the shigellosis cases in the different cities and there was evidence of transmission between cities. This clonal structure would probably be the result of selection for virulence and resistance phenotypes. These data indicate that the human sanitary conditions of the cities investigated should be improved.

Key words

- *Shigella flexneri*
- *Shigella sonnei*
- Antimicrobial resistance
- Enterobacterial repetitive intergenic consensus-polymerase chain reaction
- Repetitive extragenic palindromic-polymerase chain reaction
- Double-repetitive element-polymerase chain reaction

Introduction

Shigella spp are Gram-negative, facultative anaerobic, non-motile, and non-sporulated bacilli belonging to the Enterobacteriaceae family. Additionally, they are cytochrome-oxidase negative, an important feature that differentiates Enterobacteriaceae bacilli from other fermentative and non-fermentative bacilli. They are also able to ferment glucose (1), reduce nitrates to nitrites, and produce acids from carbohydrates. However, they are unable to produce H₂S, urease, desaminase, or lysine decarboxylase (2).

Shigella spp are divided into four groups (or species), namely *Shigella flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae*. They are responsible for shigellosis or bacillary dysentery, a disease that causes high fever, neurological disturbances, and mucus-pyohemorrhagic dysentery (3). These bacteria are responsible for morbidity and mortality in high risk populations such as children under five years of age, senior citizens, toddlers in day-care centers, patients in custodial institutions, homosexual men, and those affected by war and famine (4). Shigellosis is characterized by destruction of the colonic epithelium due to the inflammatory response induced upon bacterial invasion of the mucosa (5). The worldwide burden of this disease has been estimated to be 150 million cases, with 1 million deaths per year in the developing world (5).

Virulent *Shigella* species are characterized by the presence of a virulence plasmid of ≈220 kb and by the ability to invade the epithelial cells of the intestinal mucosa, with further dissemination to other cells. Bacterial entry is a consequence of bacterial factors that induce internalization of *Shigella* by the host cells (5). The invasion process is complex and involves three chromosomal and five plasmid loci (3). The virulence plasmid related to the invasion process was first described in *S. flexneri* strains (6), and was later found in other serogroups of *Shigella*,

namely *S. dysenteriae*, *S. boydii*, as well as enteroinvasive *Escherichia coli* (7).

Although genomic analyses have demonstrated that there are no different species among the shigellas (8-11), in the present study for consistency we will use the traditional classification scheme for the genus. Differentiation of *Shigella* isolates is classically based on serotyping and biochemical assays. However, molecular techniques such as multilocus enzyme electrophoresis (12), restriction of amplified O-antigen gene cluster loci (9), pulsed field gel electrophoresis (PFGE), polymerase chain reaction (PCR) (13,14), DNA hybridization (15-17), ribotyping (8), and multilocus enzyme electrophoresis-*mdh* gene sequencing (18,19), or even a combination of these techniques (20, 21) have also been used.

To our knowledge, only two reports regarding the epidemiology of shigellosis in Brazil have been published to date (22,23). The first describes the resistance profile of strains, while the second uses plasmid, PFGE, and antibiotic resistance profiles to demonstrate the persistence of certain PFGE patterns in the populations studied, as well as the presence of only three plasmid profiles in the strains. A third study (24), which does not involve *Shigella* strains only, has contributed significantly to the epidemiology of this genus because the authors described relationships among strains on the basis of the DNA sequencing of specific chromosomal and plasmid loci.

The present study was carried out to determine the genomic variability of 60 strains of *Shigella* spp (30 *S. flexneri* and 30 *S. sonnei* strains) isolated from different cases in the Southeast region of Brazil and belonging to the bacterial collection of the Adolfo Lutz Institute (the reference Institute in the State of São Paulo). Additionally, the possible origin and transmission of strains among cities located in the metropolitan area of Campinas, State of São Paulo, Brazil, were investigated. A combination of the entero-

bacterial repetitive intergenic consensus-PCR (ERIC-PCR) (25), repetitive extragenic palindromic-PCR (REP-PCR) (25), and double-repetitive element-PCR (DRE-PCR) (26) techniques was used to characterize the strains. The relationship of these results with antibiotic resistance and plasmid profiles was determined in order to determine the possible origin of the cases.

Material and Methods

Bacterial strains

The 30 *S. flexneri* and 30 *S. sonnei* strains analyzed in this study were provided by the Adolfo Lutz Institute, São Paulo, SP, Brazil. All available epidemiological data such as patient age, date and city of isolation are described in Table 1. Patient ages below 1 year are reported in months.

Determination of antibiotic resistance

Ampicillin, cephalothin, chloramphenicol, streptomycin, trimethoprim-sulfamethoxazole, sulfamethoxazole, and tetracycline were used to determine the resistance of all *Shigella* strains studied by the diffusion disk method according to the Manual of the Clinical and Laboratory Standards Institute (<https://www.anvisa.gov.br/servicosaude/manuais/clsai.asp>).

Extraction of genomic and plasmid DNA

Bacterial genomic DNA was extracted as described by Ausubel et al. (27). DNA integrity was determined by electrophoresis on 0.7% agarose gel. Plasmid DNA was extracted by the alkaline lysis method and separated by electrophoresis on agarose gels as described by Sambrook et al. (28).

Polymerase chain reaction

Genomic DNA (20-40 ng), extracted as

described by Ausubel et al. (27) and resuspended in sterile deionized water, was used to perform ERIC-PCR (25), REP-PCR (25), and DRE-PCR (26). The following primers were used: ERIC-PCR (ERIC-1R 5'ATG TAAGCTCCTGGGGATTAC3'); (ERIC-2 5'AAGTAAGTGACTGGGGTGAGC G3'); REP-PCR (REP1R 5'IIIICGICGICATCIGGC3'), (REP2 5'ICGICITATCIG GCCTAC3'); DRE-PCR (PNTB1 5'CCG TTGCCGTACAGCTG3'), (PNTB2 5'CCT AGCCGAACCCTTTG3') (Ris1 5'GGC TGAGGTCTCAGATCAG3'), (Ris2 5'ACC CCATCCTTTCCAAGAAC3') under the PCR conditions described previously (25,26). PCR-amplified DNA fragments were separated by electrophoresis on agarose gel as described by Sambrook et al. (28).

Polymerase chain reaction fingerprint analyses

The PCR fingerprints of amplified DNA fragments obtained by agarose gel electrophoresis were recorded. The presence of a given band was coded as 1, and the absence of a given band was coded as 0 in a data matrix. The matrix was analyzed with the POPGENE software, version 1.31 (29), which employs the unweighted pair group method using arithmetic averages. Dendrograms of similarity between the isolates of each *Shigella* species were constructed.

Results

We determined the molecular epidemiology of *Shigella* spp strains (30 *S. flexneri* and 30 *S. sonnei*) isolated from shigellosis cases that occurred in different cities in the metropolitan area of Campinas city, State of São Paulo, Southeast Brazil. Each strain was analyzed using specific primer pairs in three different PCR procedures and antibiotic resistance and plasmid profiles were also determined.

Patient age was known in 29 of the 30

cases reported to be caused by *S. flexneri* and in 22 of the 30 cases reported to be caused by *S. sonnei* (Table 1). *S. flexneri* strains were detected in 60% of the patients under 6 years of age and in 36.6% of the patients over 5 years of age. Isolates from patients older than 60 years represented only 3.3% (N = 1) of the cases. Regarding *S. sonnei*, the cases occurred in 68.8 and 36.3% of the patients under 6 years of age and over 5 years of age, respectively. There was only one case (4.5%) involving a patient older than 60 years (an 87-year-old patient) and another case involving a 42-year-old patient.

Most of the *S. flexneri* strains demon-

strated multiple drug resistance profiles, with 90% of them being resistant to at least 3 drugs (Table 1, Figure 1). Of these, 16.7% were resistant to 3, 20% were resistant to 4, 50% were resistant to 5, and 3.3% were resistant to 6 antimicrobial agents. Only 1 strain (3.3%) was resistant to only one antimicrobial agent and two strains (6.6%) were resistant to two different antimicrobial agents (Table 1). The resistance profiles indicated that most of the strains either isolated in the same city or isolated on close dates exhibited a very similar resistance pattern (Table 1); however, there were exceptions, as can be seen for strains Sf3, Sf7,

Table 1. *Shigella flexneri* and *Shigella sonnei* strains isolated from cases of shigellosis from the region of the city of Campinas, State of São Paulo.

<i>S. flexneri</i> strains	Isolation date	City	Age	Antibiotic resistance	<i>S. sonnei</i> strains	Isolation date	City	Age	Antibiotic resistance
Sf1A	11/02/87	SJBV	44 years	AP, ET, SUT	Ss1	02/14/97	MG	23 years	ET, SUT, TT
Sf1B	12/02/87	SJBV	12 years	AP, ET, SUT	Ss2	05/14/97	BP	15 years	AP, ET, SUT
Sf2	10/03/89	C	2 years	CO, ET, SUT, TT	Ss3	10/15/98	L	11 years	ET, SUT, TT
Sf3	04/23/90	J	35 years	ET, SUT	Ss4	06/24/99	C	10 years	SUT, TT
Sf4	11/24/97	C	8 months	AP, CO, ET, SFT, TT	Ss5	03/10/00	C	2 years	AP, SUT, TT
Sf5	12/30/97	C	6 months	AP, CO, ET, SFT, TT	Ss6	05/23/00	C	7 months	ET, SUT, TT
Sf6	11/02/98	C	2 years	AP, CO, ET, SFT, TT	Ss7	05/31/00	C	87 years	ET, SUT, TT
Sf7	09/16/98	C	4 years	AP, ET, SUT, TT	Ss8	06/02/00	C	2 years	ET, SUT, TT
Sf8	01/21/99	C	1 year	AP, CO, ET, SUT, TT	Ss9	01/25/01	COS	2 years	SUT, TT
Sf9	02/17/99	C	70 years	AP, CO, ET, SUT, TT	Ss10	03/02/01	C	9 years	ET, SUT, TT
Sf10	03/29/99	C	29 years	CO, ET, SUT, TT	Ss11	03/02/01	C	3 years	SUT, TT
Sf11	09/30/99	C	11 months	AP, CO, ET, SUT, TT	Ss12	03/08/01	C	3 years	ET, SUT, TT
Sf12A	11/12/99	I	50 years	AP, CO, ET, SUT, TT	Ss13	05/10/01	C	1 year	SUT, TT
Sf12B	11/12/99	I	40 years	AP, CO, ET, SUT, TT	Ss14	06/29/01	C	-	SUT, TT
Sf12C	11/16/99	I	4 years	AP, CO, ET, SUT, TT	Ss15	07/05/01	C	-	SUT, TT
Sf12D	11/30/99	I	19 years	AP, CO, ET, SUT, TT	Ss16A	02/28/02	C	4 years	SUT, TT
Sf13	02/14/00	C	8 months	AP, CO, ET, SUT, TT	Ss16B	02/28/02	C	3 years	SUT, TT
Sf14	05/02/00	C	5 years	AP, CO, ET, SUT, TT	Ss16C	02/28/02	C	5 years	SUT, TT
Sf15	05/15/00	C	3 years	AP, CF, CO, ET, SUT, TT	Ss16D	02/28/02	C	4 years	SUT, TT
Sf16	06/05/00	C	4 years	AP, ET, SUT, TT	Ss16E	02/28/02	C	5 years	SUT, TT
Sf17	07/10/00	C	6 months	AP, ET, SUT, TT	Ss16F	02/28/02	C	-	SUT, TT
Sf18	07/10/00	C	-	AP, ET, TT	Ss16G	03/07/02	C	6 years	SUT, TT
Sf19	08/15/00	C	1.5 years	AP, CO, ET, SUT, TT	Ss16H	03/11/02	C	42 years	SUT, TT
Sf20	11/10/00	C	5 years	AP, CO, TT	Ss16I	03/26/02	C	6 years	SUT, TT
Sf21	11/14/00	C	50 years	AP, CO, SUT, TT	Ss17	03/06/02	C	5 years	SUT, TT
Sf22	12/21/00	C	5 years	AP, CO, ET, SUT, TT	Ss18	03/15/02	C	-	SUT, TT
Sf23	01/16/01	C	40 years	AP, CO, ET, SUT, TT	Ss19A	04/18/02	VIN	-	SUT, TT
Sf24	01/22/01	C	10 months	ET, TT	Ss19B	04/18/02	VIN	-	SUT, TT
Sf25	06/18/01	MG	49 years	SUT	Ss19C	04/18/02	VIN	-	SUT, TT
Sf26	12/27/01	C	3 years	AP, CO, TT	Ss20	05/03/02	C	-	SUT, TT

SJBV = São João da Boa Vista; C = Campinas; J = Jundiaí; I = Itapira; MG = Mogi Guaçu; BP = Bragança Paulista; L = Limeira; COS = Cosmópolis; VIN = Vinhedo; AP = ampicillin; CF = cephalothin; CO = chloramphenicol; ET = streptomycin; SFT = trimethoprim-sulfamethoxazole; SUT = sulfamethoxazole; TT = tetracycline.

Sf10, Sf15, Sf18, Sf20, Sf24, Sf25, and Sf26. Resistance to ampicillin (83.3%), chloramphenicol (70.0%), streptomycin (86.7%), sulfamethoxazole (80.0%), and tetracycline (80.0%) was the most prevalent, whereas resistance to cephalothin (3.3%) and sulfazotrim (10.0%) was the least prevalent (Table 1 and Figure 1).

S. sonnei strains presented resistance to either 2 (70.0%) or 3 (30.0%) antimicrobial agents (Table 1). Resistance to sulfamethoxazole and tetracycline was present in 100.0 and 96.7% of the strains, respectively, and resistance to ampicillin (6.7%) and streptomycin (26.7%) was also detected in the strains studied (Table 1, Figure 1).

Plasmid analyses showed that *S. flexneri* and *S. sonnei* strains harbored high- and/or low-molecular weight plasmids (data not shown). On the basis of the existence and size of these plasmids, the strains of the two species were classified into 11 and 9 groups, respectively, but an association between strains belonging to a specific group and the place of isolation and antimicrobial drug resistance was not evident.

The recording of DNA fragments amplified by the three (ERIC-PCR, DRE-PCR, REP-PCR) techniques (data not shown) permitted the classification of all *S. flexneri* strains into three main groups (A, B, and C; Figure 2). Group A contained 14 strains (Sf1A, Sf1B, Sf2-Sf8, Sf13-Sf15, Sf19, and Sf26). Among these, Sf1A and Sf1B were identical, with partial similarity with Sf5 (approximately 94%); Sf2, Sf6, and Sf7 were identical, with partial similarity (approximately 97%) with Sf8, Sf13-Sf15, and Sf26, which were identical to each another. Strains Sf3 and Sf4 were identical, with a similarity coefficient of approximately 91% with strains from groups containing strains Sf2 and Sf8. Group B contained 9 strains (Sf9, Sf10, Sf12A-Sf12D, Sf16-Sf18). Strains Sf12A-Sf12D were identical and had a similarity coefficient of approximately 89% with strain Sf9. Strains Sf10 and Sf16-Sf18 were identical, with a similarity

coefficient of approximately 80% with groups containing strains Sf9 and Sf12. Group C comprised 7 strains (Sf11 and Sf20-Sf25). Strains Sf21-Sf23 and Sf25 were identical, with a similarity coefficient of approximately 81% with strains Sf11, Sf20, and Sf24, which were identical. Groups A and B had a similarity coefficient of approximately 76% with one another and of 62% with group C.

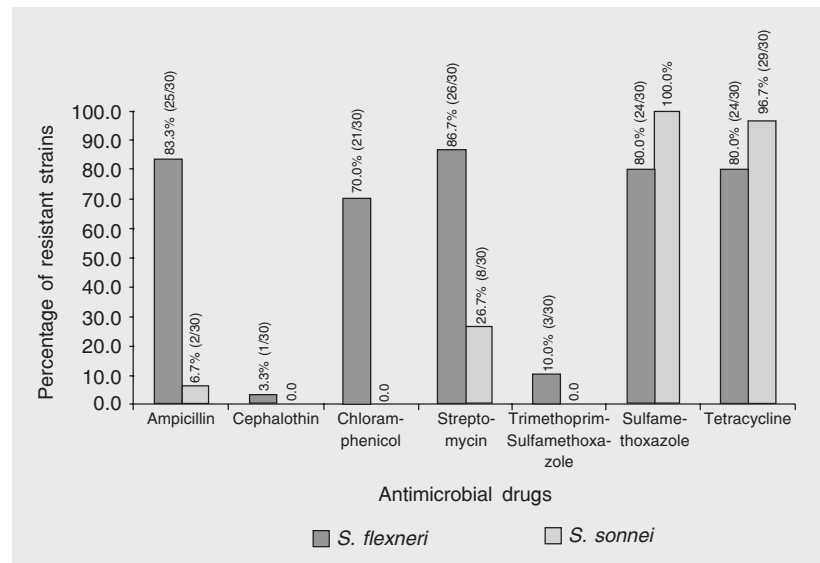


Figure 1. Percentage of *Shigella* spp strains resistant to different antimicrobial agents.

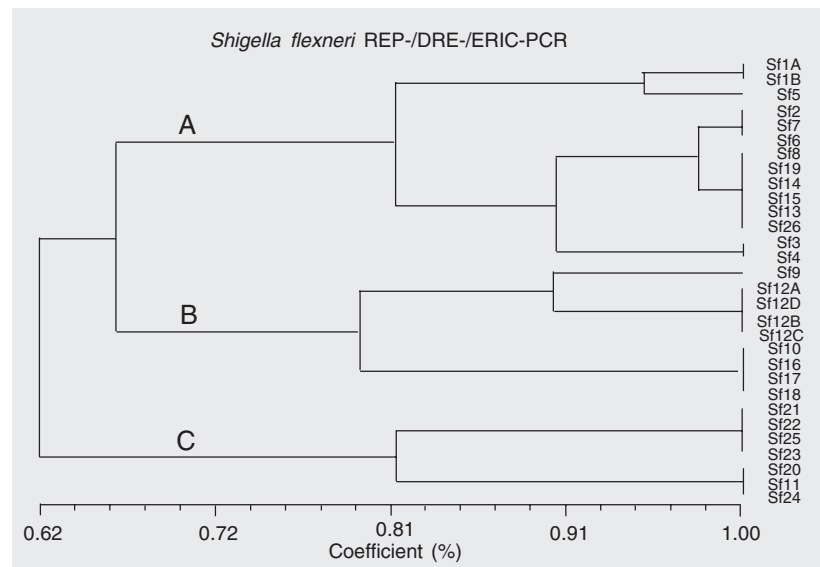


Figure 2. Similarity dendrogram generated for *Shigella flexneri* strains using the different (ERIC-PCR, REP-PCR, DRE-PCR) techniques. Group classification is indicated by the letters A, B and C on the dendrogram. See text.

S. sonnei strains were also grouped into three main groups: A, B, and C (Figure 3). Group A contained 11 strains (Ss2, Ss4, Ss5, and Ss8-Ss15) and group B contained 14 strains (Ss1, Ss3, Ss6, Ss7, Ss16A-Ss16I, and Ss20). All strains numbered Ss16 were identical, with a similarity coefficient of approximately 85% with strains Ss6, Ss1, Ss3,

Ss7, and Ss20. Strains Ss1 and Ss3 were identical, with a similarity coefficient of 96% with strains Ss7 and Ss20, which were identical. Group C comprised 5 strains (Ss17, Ss18, and Ss19A-Ss19C). Strains Ss17 and Ss18 were identical, with a similarity coefficient of approximately 92% with strains from group Ss19, which were identical. Groups B and C were more similar to each other (79%) than to group A (77%).

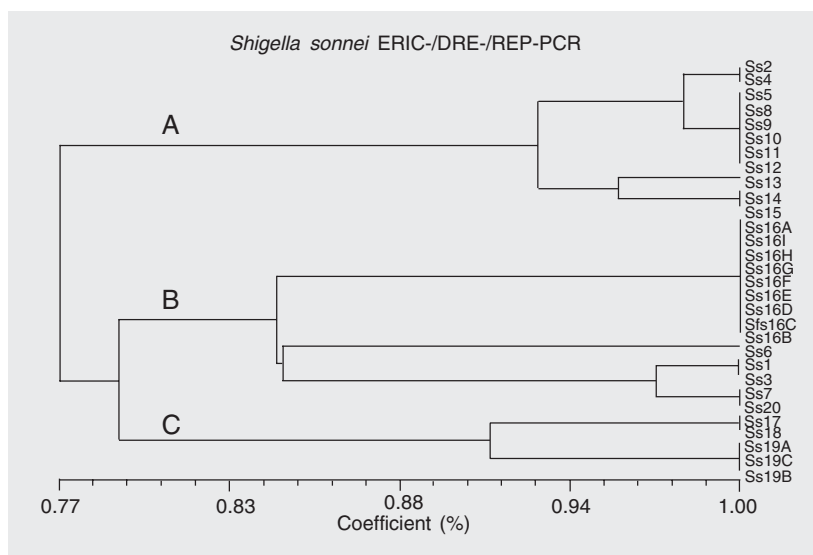
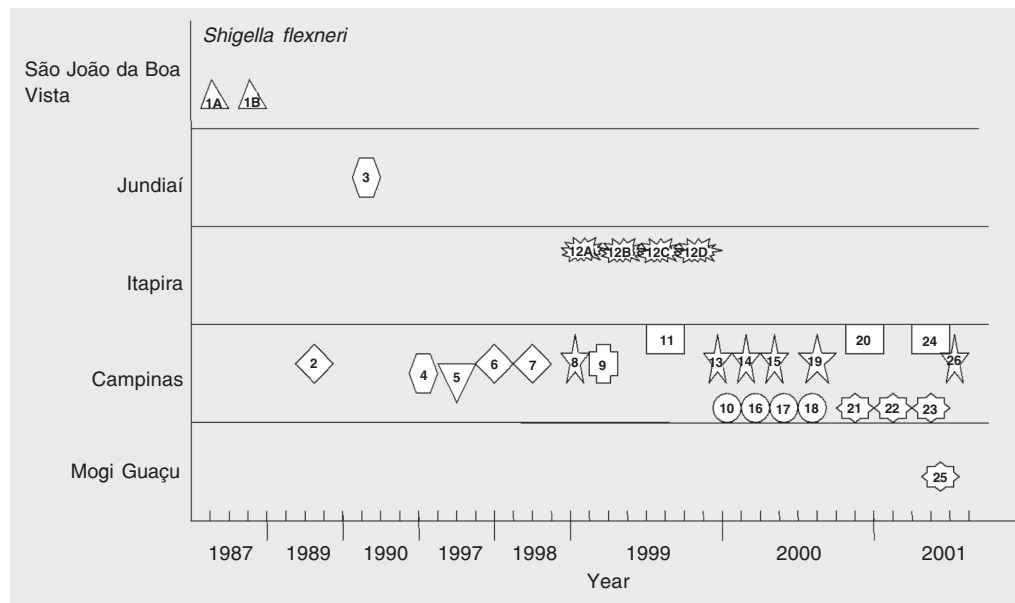


Figure 3. Similarity dendrogram generated for *Shigella sonnei* strains using the different (ERIC-PCR, REP-PCR, DRE-PCR) techniques. Group classification is indicated by the letters A, B and C on the dendrogram. See text.

S. flexneri strains were isolated in 5 cities in the region of Campinas (Table 1, Figure 4): São João da Boa Vista (2), Jundiaí (1), Itapira (4), Campinas (22), and Mogi Guaçu (1). The number of strains is given between parentheses. Strains isolated in São João da Boa Vista were identical and were not detected in any other city. Strains isolated in Itapira were also identical and unique. Strain 3, isolated in Jundiaí (1990), was identical to strain 4, which was later (1997) isolated in Campinas. The 22 strains isolated in the city of Campinas could be sub-classified into eight patterns (or types), within the three groups observed (A, B, C) according to the amplified DNA fragments generated by PCR (Figure 2). Of these eight patterns, five in-

Figure 4. Time of isolation (per year) of the 30 *Shigella flexneri* strains isolated from patients in the different cities examined in the present study. Strains encircled by the same symbols belong to the same PCR type. Note that the time scale (years) is not linear.



cluded most of the strains. Just one pattern was found for each one of strains 4, 5, and 9. Strain 25, which was isolated in Mogi Guaçu (06/18/01), was identical to strains 21, 22, and 23, which had been earlier isolated in Campinas (Figure 4).

A similar picture was obtained for *S. sonnei*, which was isolated in 6 cities in the region of Campinas. Most of the strains (23) were isolated in Campinas (Table 1, Figure 5). In Cosmópolis, only 1 strain (9) was isolated, exhibiting a pattern identical to that observed for strains 5 and 8, and 10, 11, and 12, isolated in Campinas in 2000 and 2001, respectively. Strain 1, which was isolated in Mogi Guaçu (1997), displayed a pattern identical to that of strain 3, isolated in Limeira (1998). Strain 2 isolated in Bragança Paulista (1998), also displayed a pattern identical to that of strain 4, later (1999) found in Campinas. Strains 19A, 19B, and 19C, isolated in Vinhedo, were identical to one another, but were different from the strains isolated in the other cities. The 22 strains obtained in Campinas could be classified into seven patterns respectively containing 9

strains (16A, 16B, 16C, 16D, 16E, 16F, 16G, 16H, and 16I), 5 strains (5, 8, 10, 11, and 12), 3 strains (13, 14, and 15), 2 strains (7 and 20), 2 strains (17 and 18), and one strain each, namely 4 and 6.

Discussion

Approximately 1.2 million people live in the city of Campinas, a major industrialized, technological, and educational center in the State of São Paulo, Southeast Brazil. Many other smaller cities are located in the region of Campinas and, although Campinas and these cities are considered to be developed cities with good living standards, a few slums have arisen because of migration from other Brazilian regions, a phenomenon that has worsened over the last decade. Also, many people from other cities commute every day for professional, educational, and leisure purposes. Taken together, these factors should facilitate the spread of infectious diseases, which would be very difficult to detect, eradicate, or control. Therefore, we decided to study all *Shigella* spp isolates ob-

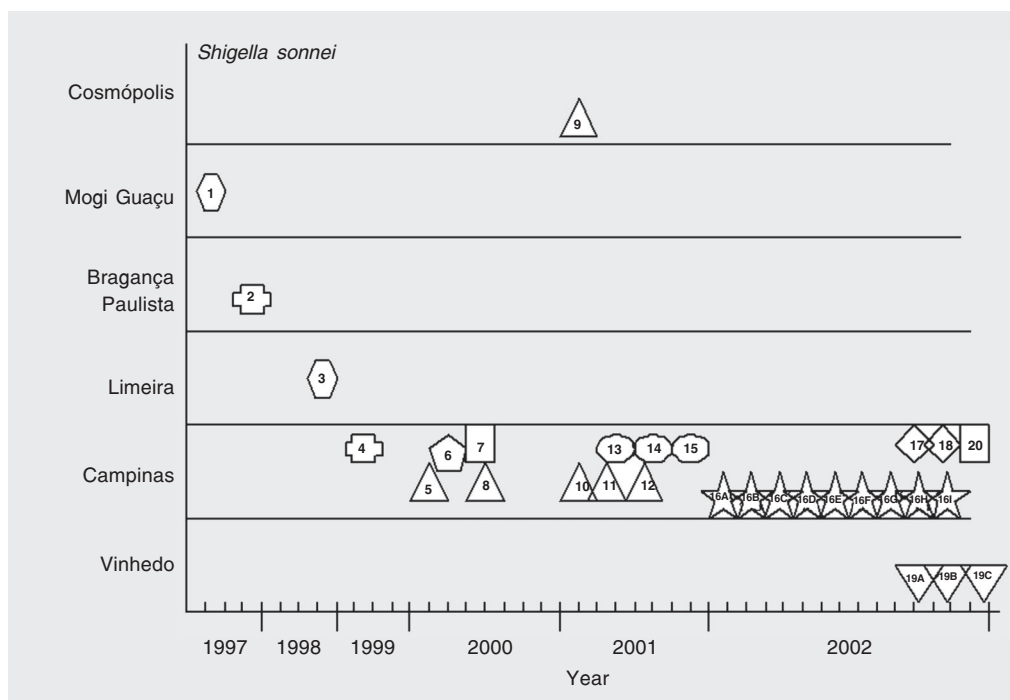


Figure 5. Isolation time (per year) of the 30 *Shigella sonnei* strains isolated from patients in the different cities examined in this study. Strains encircled by the same symbol belong to the same PCR type. Note that the time scale (years) is not linear.

tained from different cases that occurred in the region of Campinas from 1987 to 2002, and which belong to the collection of the Adolfo Lutz Institute (the infectious disease reference Institute in the State of São Paulo). The present study has attempted to determine the genomic profile of these isolates and trace the possible origin of the cases. Since most molecular techniques presently available do not demonstrate a real difference between isolates in a definitive manner, we decided to use a combination of three separate but similar PCR techniques (ERIC-PCR, REP-PCR, DRE-PCR). Also, we associated these data with antibiotic resistance and plasmid profiles to help identify isolates and rule out a possible, but not real, positive identity. We also compared these strains with others previously isolated in Brazil.

In previous studies conducted in the Southeast region of Brazil (30-37), it was clearly demonstrated that *Shigella* infections are an important cause of morbidity and mortality. Fagundes-Neto and de Andrade (32) and Medeiros et al. (35) demonstrated that age can be a risk factor for *Shigella* infections, with most of the cases occurring among children under 6 and 11 months of age, respectively. In the present study, most of the strains of both *S. flexneri* and *S. sonnei* were isolated from patients under 6 years of age, indicating that age could be a risk factor. The isolation data for each strain (Table 1) allowed us to observe a possible seasonal bacterial distribution. This characteristic is extremely common in endemic areas (38) since most bacterial strains (70%) were isolated during the warmest months of the year (January, February, March, April, October, November, and December) in Brazil. These data suggest a relationship between environmental conditions and pathogen dissemination.

Also in Brazil, Lima et al. (22) observed that almost 50% of the *S. flexneri* strains were resistant to ampicillin or trimethoprim-

sulfamethoxazole, or both. Over 64% were resistant to streptomycin, chloramphenicol, and tetracycline, with 82% of all the strains being resistant to four or more of the antimicrobial agents tested. In a subsequent investigation, Lima et al. (23) studied the molecular epidemiology of the *S. flexneri* strains and concluded that they displayed only three plasmid profile patterns, and the strains were allocated to six pattern groups as determined by PFGE.

In a more recent paper describing the Brazilian Surveillance Program that used *Shigella* spp strains isolated by several centers, Oplustil et al. (39) observed that 80% of the strains exhibited a high level of resistance to trimethoprim-sulfamethoxazole. Resistance to ampicillin (33.3%) and chloramphenicol (10.9%) was also found among strains isolated from outpatients. None of the strains were resistant to ceftriaxone.

The pattern of resistance of the strains studied here resembles that reported by Lima et al. (22), but in our case the strains were also resistant to other antimicrobial agents such as chloramphenicol (70%), streptomycin (86.7%), and tetracycline (80%), indicating that the chromosomal genes encoding ampicillin, chloramphenicol, streptomycin, and tetracycline resistance described by Casalino et al. (40) in Somalian strains could also have been selected in the region of Campinas. In addition, since most of the *S. sonnei* strains were resistant mainly to sulfamethoxazole and tetracycline, this may indicate that strains harboring specific gene loci were selected.

The determination of the plasmid profiles of all of these strains did not permit us to characterize the strains in a specific manner, although some plasmid patterns could be observed. Since plasmids are genetically mobile elements with a possible horizontal transfer capacity, it would not be surprising to find different plasmid patterns even among chromosomally identical strains. This would cast doubts on our results if we used them for

characterization purposes, as was done by Lima et al. (23). Moreover, since the strains studied were isolated from cases of human infection, the pINV plasmid(s) would be expected to be present in all of the strains studied, tending to homogenize the results. Since plasmid profiling alone is not sufficient to characterize strains as identical or not, we decided to rely only on the results obtained by the PCR-based techniques.

Figures 2 and 3 show the genotypic profile as determined by combined analysis with the three PCR techniques used in the present study, and the isolation time of each strain in each city, for *S. flexneri* and *S. sonnei*, respectively. These data clearly demonstrate that there was some transmission of strains between cities, as was the case for strains 3 and 4, and 23 and 25 of *S. flexneri*, transmitted between Jundiá and Campinas, and between Campinas and Mogi Guaçu, respectively. Transmission between cities was also observed in the case of *S. sonnei* strains 1 (Mogi Guaçu) and 3 (Limeira), 2 (Bragança Paulista) and 4 (Campinas), strain 9 (Cosmópolis) and others isolated in Campinas (strains 5, 8, 10, 11, and 12).

Given the possibility that shigellosis manifests in an endemic manner (38), with

cases occurring sporadically mainly in the warmest months of the year, and because most of the cases studied here were caused by very similar or identical strains, we may infer that these strains could survive in the environment either in contaminated water and food or in carriers such as humans and other animals. This hypothesis requires further study to confirm a role for environmental sources of infection.

We can conclude from the data discussed above that transmission of infectious *Shigella* spp strains occurred among individuals and between cities, with some of the strains being responsible for most of the cases. This evidence requires well-designed interventional strategies to control transmission of infectious diseases, in this case multiple antimicrobial-resistant *Shigella* strains. This control is more complex when the cases occur in highly populated areas, since there are many other risk-associated factors such as disadvantaged populations without a good standard of public health (treated water supplies, treated sewage, ingestion of inappropriate food) and the added risk factors of a high level of commuting and the presence of infected hosts that could serve as reservoirs of the pathogen.

References

- Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. *Medical Microbiology*. 3rd edn. St. Louis: Mosby-Year Book; 1998.
- Jawetz E, Melnick JL, Adelber EA. *Microbiologia Médica*. Rio de Janeiro: Guanabara Koogan; 2000.
- Sansonetti PJ. Rupture, invasion and inflammatory destruction of the intestinal barrier by *Shigella*, making sense of prokaryote-eukaryote cross-talks. *FEMS Microbiol Rev* 2001; 25: 3-14.
- Thong KL, Hoe SL, Puthuchery SD, Yasin RM. Detection of virulence genes in Malaysian *Shigella* species by multiplex PCR assay. *BMC Infect Dis* 2005; 5: 8.
- Parsot C. *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. *FEMS Microbiol Lett* 2005; 252: 11-18.
- Kopecko DJ, Holcombe J, Formal SB. Molecular characterization of plasmids from virulent and spontaneously occurring avirulent colonial variants of *Shigella flexneri*. *Infect Immun* 1979; 24: 580-582.
- Hale TL. Genetic basis of virulence in *Shigella* species. *Microbiol Rev* 1991; 55: 206-224.
- Rolland K, Lambert-Zechovsky N, Picard B, Denamur E. *Shigella* and enteroinvasive *Escherichia coli* strains are derived from distinct ancestral strains of *E. coli*. *Microbiology* 1998; 144 (Pt 9): 2667-2672.
- Coimbra RS, Grimont F, Grimont PA. Identification of *Shigella* serotypes by restriction of amplified O-antigen gene cluster. *Res Microbiol* 1999; 150: 543-553.
- Joklik WK, Willet HP, Amos DB, Wilfert CM. *Microbiologia*. Zinsser (Editor), Buenos Aires: Editora Médica Panamericana; 1994.
- Shears P. *Shigella* infections. *Ann Trop Med Parasitol* 1996; 90: 105-114.
- Ochman H, Whittam TS, Caugant DA, Selander RK. Enzyme polymorphism and genetic population structure in *Escherichia coli* and *Shigella*. *J Gen Microbiol* 1983; 129: 2715-2726.
- Navia MM, Capitano L, Ruiz J, Vargas M, Urassa H, Schelleberg D, et al. Typing and characterization of mechanisms of resistance of *Shigella* spp. isolated from feces of children under 5 years of age

- from Ifakara, Tanzania. *J Clin Microbiol* 1999; 37: 3113-3117.
14. Surdeanu M, Ciudin L, Pencu E, Straut M. Comparative study of three different DNA fingerprint techniques for molecular typing of *Shigella flexneri* strains isolated in Romania. *Eur J Epidemiol* 2003; 18: 703-710.
 15. Brenner DJ, Fanning GR, Miklos GV, Steigerwalt AG. Polynucleotide sequence relatedness among *Shigella* species. *Int J Syst Bacteriol* 1973; 23: 1-7.
 16. Brenner DJ, Fanning GR, Skerman FJ, Falkow S. Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. *J Bacteriol* 1972; 109: 953-965.
 17. Brenner DJ, Steigerwalt AG, Wathen HG, Gross RJ, Rowe B. Confirmation of aerogenic strains of *Shigella boydii* 13 and further study of *Shigella* serotypes by DNA relatedness. *J Clin Microbiol* 1982; 16: 432-436.
 18. Pupo GM, Karaolis DK, Lan R, Reeves PR. Evolutionary relationships among pathogenic and nonpathogenic *Escherichia coli* strains inferred from multilocus enzyme electrophoresis and *mdh* sequence studies. *Infect Immun* 1997; 65: 2685-2692.
 19. Pupo GM, Lan R, Reeves PR. Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. *Proc Natl Acad Sci U S A* 2000; 97: 10567-10572.
 20. Lee TM, Chang CY, Chang LL, Chen WM, Wang TK, Chang SF. One predominant type of genetically closely related *Shigella sonnei* prevalent in four sequential outbreaks in school children. *Diagn Microbiol Infect Dis* 2003; 45: 173-181.
 21. Liu PY, Lau YJ, Hu BS, Shyr JM, Shi ZY, Tsai WS, et al. Analysis of clonal relationships among isolates of *Shigella sonnei* by different molecular typing methods. *J Clin Microbiol* 1995; 33: 1779-1783.
 22. Lima AA, Lima NL, Pinho MC, Barros Junior EA, Teixeira MJ, Martins MC, et al. High frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, streptomycin, chloramphenicol, and tetracycline isolated from patients with shigellosis in northeastern Brazil during the period 1988 to 1993. *Antimicrob Agents Chemother* 1995; 39: 256-259.
 23. Lima AA, Sidrim JJ, Lima NL, Titlow W, Evans ME, Greenberg RN. Molecular epidemiology of multiply antibiotic-resistant *Shigella flexneri* in Fortaleza, Brazil. *J Clin Microbiol* 1997; 35: 1061-1065.
 24. Lan R, Alles MC, Donohoe K, Martinez MB, Reeves PR. Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. *Infect Immun* 2004; 72: 5080-5088.
 25. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 1991; 19: 6823-6831.
 26. Friedman CR, Stoeckle MY, Johnson WD Jr, Riley LW. Double-repetitive-element PCR method for subtyping *Mycobacterium tuberculosis* clinical isolates. *J Clin Microbiol* 1995; 33: 1383-1384.
 27. Ausubel FM, Brent R, Kingston RE, Struhl K, Moore DD, Smith JÁ, et al. *Current protocols in molecular biology*. Brooklyn: Greene Publishing Associates; 1988.
 28. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. 2nd edn. New York: Cold Spring Harbor Laboratory Press; 1989.
 29. Yeh FC, Yang RC, Boyle T. Popgene, version 1.31. Microsoft Windows-based freeware for population genetic analysis. <http://www.ualberta.ca/~fyeh/>.
 30. Almeida MT, Silva RM, Donaire LM, Moreira LE, Martinez MB. Enteropathogens associated with acute diarrheal disease in children. *J Pediatr* 1998; 74: 291-298.
 31. de Andrade JA, de Oliveira JO, Fagundes NU. Lethality in hospitalized infants with acute diarrhea: risk factors associated with death. *Rev Assoc Med Bras* 1999; 45: 121-127.
 32. Fagundes-Neto U, de Andrade JA. Acute diarrhea and malnutrition: lethality risk in hospitalized infants. *J Am Coll Nutr* 1999; 18: 303-308.
 33. Fernandes MR, Trabulsi LR. Infectious resistance in pathogenic enteric organisms isolated in São Paulo, Brasil (Preliminary report). *Rev Inst Med Trop São Paulo* 1968; 10: 52-53.
 34. Gomes TA, Rassi V, MacDonald KL, Ramos SR, Trabulsi LR, Vieira MA, et al. Enteropathogens associated with acute diarrheal disease in urban infants in São Paulo, Brazil. *J Infect Dis* 1991; 164: 331-337.
 35. Medeiros LC, Hillers VN, Kendall PA, Mason A. Food safety education: what should we be teaching to consumers? *J Nutr Educ* 2001; 33: 108-113.
 36. Piechaud D, Szturm-Rubinsten S, Pessoa G. Diversity of resistance types among *Shigella* in São Paulo (Brasil) (author's transl). *Ann Microbiol* 1974; 125: 581-584.
 37. Zuliani ME, Trabulsi LR. *In vitro* sensitivity of 166 *Shigella* strains isolated in São Paulo, Brazil, to sulphadiazine and five antibiotics. *Rev Inst Med Trop São Paulo* 1968; 10: 70-77.
 38. Sears CL, Kaper JB. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol Rev* 1996; 60: 167-215.
 39. Oplustil CP, Nunes R, Mendes C. Multicenter evaluation of resistance patterns of *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* spp and *Shigella* spp isolated from clinical specimens in Brazil: RESISTNET Surveillance Program. *Braz J Infect Dis* 2001; 5: 8-12.
 40. Casalino M, Nicoletti M, Salvia A, Colonna B, Pazzani C, Calconi A, et al. Characterization of endemic *Shigella flexneri* strains in Somalia: antimicrobial resistance, plasmid profiles, and serotype correlation. *J Clin Microbiol* 1994; 32: 1179-1183.