

MOLECULAR EPIDEMIOLOGY OF *SHIGELLA* SPP STRAINS ISOLATED IN TWO DIFFERENT METROPOLITAN AREAS OF SOUTHEAST BRAZIL

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ABSTRACT

Shigella spp., the human pathogen responsible for shigellosis, is highly infectious even at low levels. The incidence rate of shigellosis varies with geographical distribution, location human development index, and age groups, being higher among children aged under 5 years. In Brazil, a few works indicate that shigellosis cases are underestimated, with *S. flexneri* and *S. sonnei* strains being the major agents responsible for the shigellosis cases. The present study used pulsed field gel electrophoresis (PFGE) to investigate the molecular epidemiology of 119 strains of *S. sonnei* and *S. flexneri* isolated from shigellosis cases that occurred in the metropolitan areas of Ribeirão Preto and Campinas Cities, São Paulo State, southeast Brazil. The results indicated (i) the existence of just a few strain clusters for both species, but with genotype variability with either a high speed of genetic change or constant introduction of several genotypes, considering the intense migration to these two metropolitan areas, and (ii) the prevalence of specific genotypes in each geographical area, which suggests the successful adaptation of some genotypes to the local environmental conditions. Our results indicate the need of more efficacious sanitary barriers to prevent *Shigella spp.* outbreaks and epidemics.

Key words: *Shigella sonnei*, *Shigella flexneri*, Epidemiology, Characterization, PFGE

INTRODUCTION

Shigella spp., the human pathogen responsible for shigellosis, is highly infectious, even at low counts (17). The clinical signs of shigellosis range from mild diarrhea to

severe dysentery, with extra intestinal manifestations composed of fever, vomiting, abdominal discomfort, nausea, and neurological symptoms (headache, lethargy, confusion, nuchal rigidity or hallucinations). The worldwide annual

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incidence of shigelloses is approximately 164.7 million cases, with 163.2 million of the cases occurring in developing countries (6). The incidence rate varies according to age, geographical distribution, and local human development index (HDI). The isolation rate of *Shigella spp.* from diarrheagenic stools is higher among children under 5 years old (5), particularly in children younger than six months living in low HDI countries (14).

Traditionally, the *Shigella* genus is classified as being formed by four species. Shigelloses are caused by *S. dysenteriae*, *S. flexneri*, and *S. sonnei*, which are the species of major epidemiological interest (7). Among the pathogenic *Shigella* subgroups, *S. flexneri* is the most commonly detected strain (57%) in infectious processes, followed by *S. sonnei* strains. *S. sonnei* strains tend to cause milder illness than *S. flexneri*, persist in both developed and developing countries and tend to cause sporadic diarrhea and occasional outbreaks in specific epidemiological niches, such as day-care centers, where personal hygiene may be suboptimal (8, 14). In Brazil, a few studies (4, 9, 11) indicate that shigellosis cases are underestimated, with *S. flexneri* and *S. sonnei* strains being the major pathogenic agents (11).

Molecular techniques, combined with traditional techniques, are used to differentiate species and strains (2, 10, 12, 13) of pathogenic bacteria. Pulsed field gel electrophoresis (PFGE) is considered one of the most reliable, discriminatory, and reproducible typing method, allowing the detection of a high degree of DNA polymorphism (18) for epidemiological purposes (15, 18, 19, 21) and in routine typing of *Shigella* species (3).

In our study, PFGE was applied to the genotyping of 119 *Shigella* strains (61 *S. flexneri* and 58 *S. sonnei*) isolated from shigellosis cases that had occurred in two metropolitan areas located 250 km apart (Campinas and Ribeirão Preto Cities, São Paulo State, southeast Brazil). The purpose of the study

was to analyze the genetic relationship among strains and to determine the geographically predominant clones, if any.

MATERIAL and METHODS

Bacterial Strains

From 1999 to 2000, 59 *Shigella* strains (31 *S. flexneri* and 28 *S. sonnei*) were isolated from stool samples of different patients assisted at the São Paulo State University Hospital in Ribeirão Preto (FMRP-USP). From 1987 to 2001, 60 strains (30 *S. flexneri* and 30 *S. sonnei*) were isolated from stool samples of different patients residing in metropolitan Campinas (12) by Adolfo Lutz Institute. The isolates were stored at -70 °C in Luria Bertani (LB) medium (16) with 15% glycerol and grown in LB medium (37 °C; 18-24 h) for routine assays.

PFGE

For genomic DNA extraction, strains were grown overnight in LB medium at 37°C and total DNA extracted as described by Ausubel *et al.* (1). The PFGE procedure was performed as described by Yatsuyanagi *et al.* (20), with some modifications. Briefly, 2 mL of culture containing 10⁹ colony-forming units (CFU)/mL was harvested, suspended in a suspension buffer (100 mM pH 7.2 Tris, 20 mM NaCl, 100 mM EDTA), mixed with an equal volume of 2% low-melting-point agarose (GIBCO BRL, Grand Island, N.Y. 14072 USA), dispensed in a plug mold (Bio Rad Laboratories), and allowed to solidify. For lysis, the plugs were incubated for 2 h at 37°C in the cell suspension buffer with 1 mg/mL of lysozyme (Sigma-Aldrich CO. Box 14508, St. Louis, MO 63178 USA). The plugs were washed with sterilized-distilled water and incubated overnight at 50°C in a protein removing buffer (0.25 M pH 9 EDTA, 0.5% sarcosil, 0.5 mg/mL proteinase K) and washed 4 times for 30 min in TE buffer (10mM pH 8 Tris-HCl, 1 mM EDTA)

afterwards. One plug was washed for 1 h with 300 μ L of the enzyme buffer and incubated overnight at 37°C with 20 U of XbaI in the reaction conditions recommended by the manufacturer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). A plug slice was washed for 30 min in TE buffer, loaded into a well of 1% Ultra Pure™ Agarose gel (Invitrogen, Auckland 1135, New Zealand) in 0.5X TBE (Tris-Borate EDTA) buffer. Electrophoresis was performed in 0.5X TBE buffer at 14°C for 22 h using a CHEF DR-III apparatus (Bio-Rad, 2000 Alfred Nobel Drive Hercules, CA94547, USA) with an electric field of 6 V/cm and pulse time from 5 to 35 s. A lambda ladder (New England Biolabs) was used as a molecular size marker. The PFGE banding patterns were fingerprinted and clustered using GelCompar II software (version 2.0, Applied Maths, Saint-Martens-Latem, Belgium). The similarity dendrogram was obtained using the Dice similarity index by the unweighted pair group method with arithmetic averages (UPGMA) using 1% tolerance coefficient.

RESULTS

The genotypes obtained by PFGE were analyzed separately according to *Shigella* species *S. sonnei* (Figure 1) and *S. flexneri* (Figure 2). A similarity dendrogram was constructed for each species.

S. sonnei genotypes resulted in a dendrogram constituted by two main clusters (A and B; Figure 1), both with less than 35% similarity and strains with bands that had molecular weights between 580 Kb and 25 Kb, with an average of 14 bands for cluster A2 and 18 for cluster B1. Cluster A presented two strain subgroups (A1 and A2), with 20 (34.5%) and 10 (17.2%) strains, respectively. Subgroup A1 was composed of 15 (25.8%) and 5 (8.6%) of strains isolated in the metropolitan areas of Campinas and Ribeirão Preto,

respectively. Subgroup A2 was composed of 10 (17.2%) strains, all isolated in Ribeirão Preto. Cluster B also presented two strain subgroups (B1 and B2), with 16 (27.6%) and 12 (20.6%) strains, respectively. In subgroup B1, most of the strains (13; 22.4%) were isolated in Campinas, while only 3 strains were isolated in Ribeirão Preto. Subgroup B2 was composed of 10 (17.2%) strains isolated in Ribeirão Preto and 2 strains isolated in Campinas.

The analysis of the *S. flexneri* genotypes resulted in a dendrogram constituted by two main clusters (C and D; Figure 2) with less than 40% similarity and strains with bands with molecular weights between 560 Kbp and 30 Kbp, with an average of 17 bands for cluster C2 and 18 for cluster C3. Cluster D had 7 (11.5%) strains, all isolated in Ribeirão Preto. Cluster C included most of the strains (54; 88.5%) and comprised strains from both metropolitan areas. Cluster C had three strain subgroups (C1, C2, and C3), with subgroup similarity ranging from 40% to 45%. Subgroup C1 contained 13 (21.3%) strains with 8 (13.1%) and 5 (8.2%) strains isolated in Ribeirão Preto and Campinas, respectively. Subgroup C2 contained 31 (51%) strains with 7 (11.5%) strains isolated in Ribeirão Preto and 24 (39.4%) in Campinas. Subgroup C3 contained 10 strains (16.5%) with 9 (14.75%) strains isolated in Ribeirão Preto.

DISCUSSION

This work analyzed 119 *Shigella spp* strains isolated from shigellosis cases that occurred in two major metropolitan areas located 250 km apart in southeast Brazil. These two areas are important industrial, technological, and agricultural regions, tourism, educational and business destinations and migration poles of workers and students from other regions of Brazil. Despite their economic importance, these metropolitan areas have different HDI and

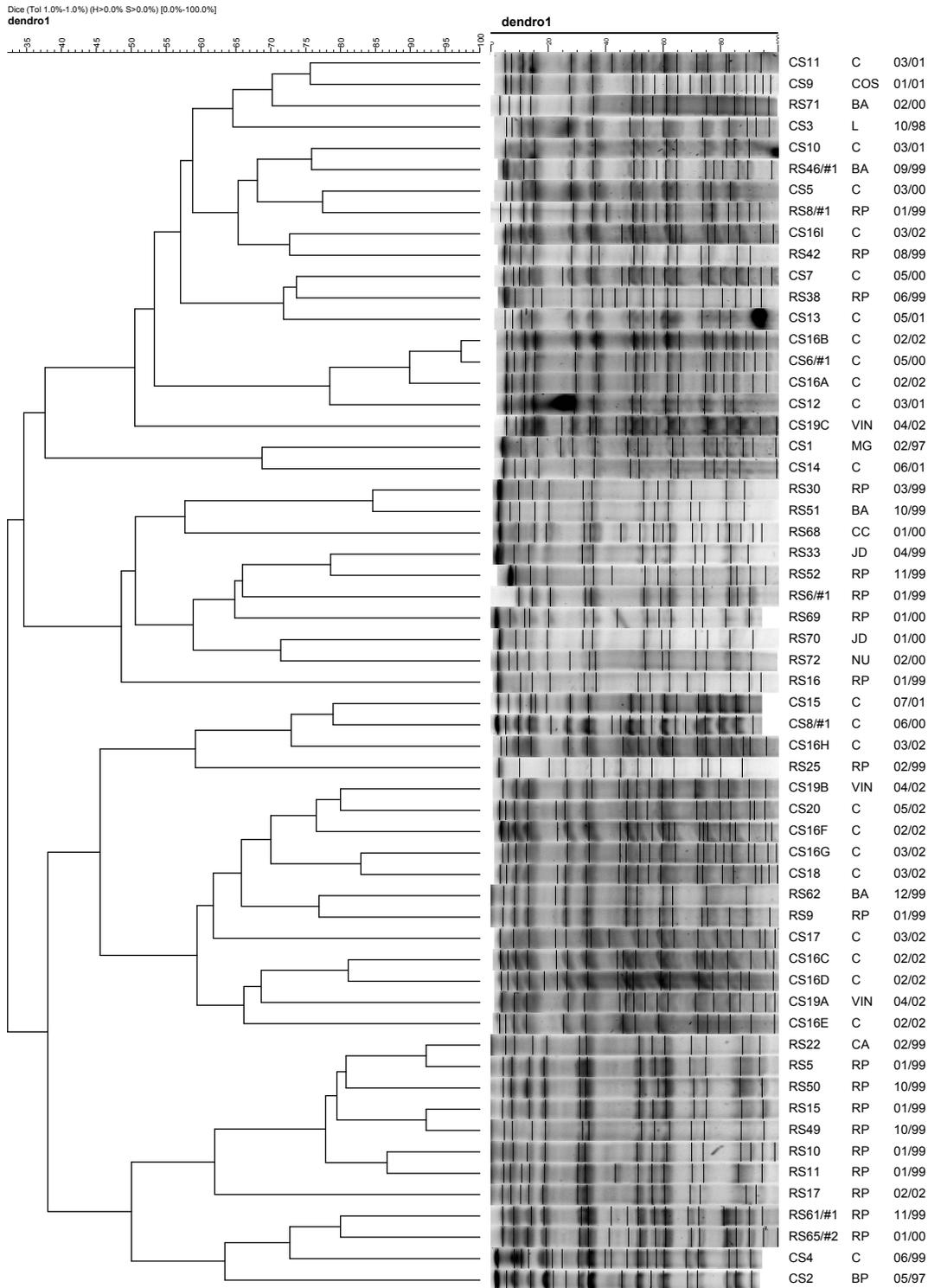


Figure 1. Similarity dendrogram of fingerprinting patterns obtained for *S. sonnei* strains isolated in Campinas (CS) and Ribeirão Preto (RS) metropolitan regions. The two clusters were designated as A and B and divided into two subgroups (A1 and A2, and B1 and B2, respectively). The name of the strains are followed by the site* abbreviation and the isolation date. The Ribeirão Preto strains were isolated in Jardinópolis (JD), Barrinha (BA), Cambirá (CA), Cássia dos Coqueiros (CC), Nuporanga (NU), and Ribeirão Preto (RP). The Campinas region strains were isolated in Campinas (C), Mogi Guaçu (MG), Bragança Paulista (BP), Limeira (L), Cosmópolis (COS), and Vinhedo (VIN).

have slums, which favor the spread of infectious illnesses. The study and characterization of the genotypes of pathogenic agents that cause potentially fatal diseases, such as shigellosis, would help to track these agents during outbreaks and epidemics and determine better control and eradication measures.

The results herein obtained indicate the existence of two main groups of genetic similarity between the *S. sonnei* and the *S. flexneri* strains isolated in both areas. These two groups were constituted by strains isolated in both metropolitan areas, as happened for most strains, or in just one metropolitan area, such as subgroup A2 of *S. sonnei* and group D of *S. flexneri*, whose strains were all isolated in metropolitan Ribeirão Preto.

The strains of *S. sonnei* from the Campinas region were majority in group A1 and group B1 while the strains from Ribeirão Preto region were majority in the groups A2 and B2 indicating that although being from different regions, there is a genetic similarity between strains in each strains group and the predominance of closely related genotypes in specific geographical regions. These data are also corroborated by the

findings in the *S. flexneri* dendrogram where group C1 is mainly composed by strains isolated in Ribeirão Preto region, the group C2 is mainly composed by strains from Campinas region and group C3 is mainly composed by strains (90%) isolated in the Ribeirão Preto region, agreeing with a previous study accomplished in towns located in the metropolitan Campinas area (12).

In the *S. sonnei* dendrogram the cluster A2 is composed only by strains isolated in the years of 1999 and 2000 with the group B1 being composed only by strains isolated after the year of 1999. In the dendrogram of *S. flexneri*, all strains of group C3, but strain CF24 that was isolated in the region of Campinas in the year of 2001, were isolated in 1999 in the region of Ribeirão Preto. In group D the majority (85.7%) of the strains were isolated in 1999. These data, similarly to those observed in the previous paragraph indicate that specific strains with related genotypes are responsible for causing the disease outbreaks.

Also, it is possible to observe that 77.5% of the shigellosis cases caused by *S. sonnei* and 71.7% of those caused by *S. flexneri* affect children under 6 years old, confirming previous works that show that these are infectious processes that attack mainly very young malnourished-children (5). In Brazil, a few works indicate that shigellosis cases are underestimated, since is not considered a compulsory notifiable disease (11).

The isolation dates of all the strains indicate that most of the cases occurred during the summer, i.e, the rain season, strengthening the hypothesis that the probable existence of the lack of basic sanitation conditions together with the excess of contaminated floodwater would facilitate the transmission of pathogens (14).

In conclusion, the existence of just a few *Shigella* strain clusters for both species with genotypic variability in the two studied areas could be explained either by the constant

Dice (Tot 1.0%-1.0%) (H>0.0% S=0.0%) [0.0%-100.0%]
dendro1

dendro1

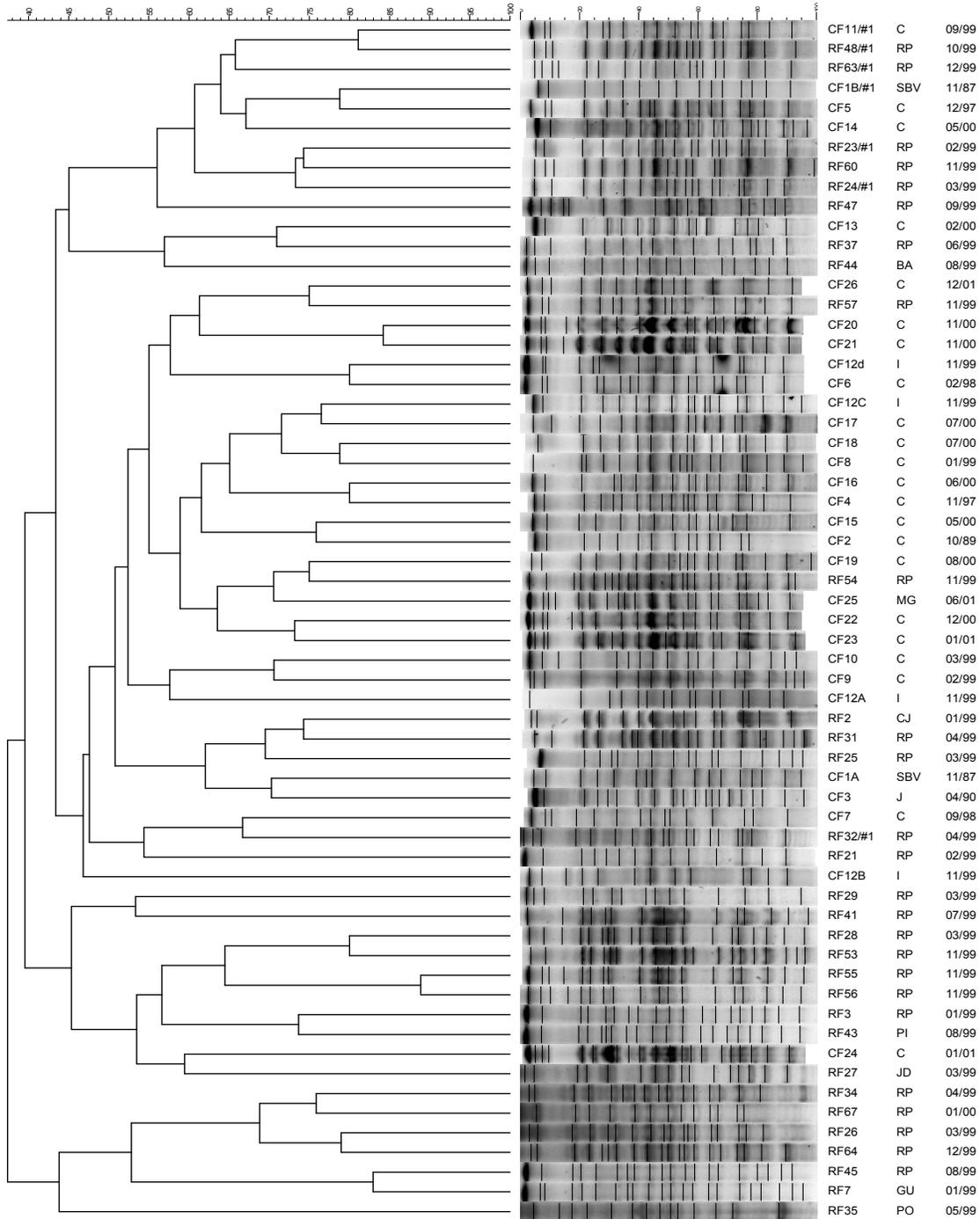


Figure 2. Similarity dendrogram of the fingerprinting patterns obtained for *S. flexneri* strains isolated in the Campinas (CF) and Ribeirão Preto (RF) metropolitan regions. The two clusters were designated as C and D. Cluster C was divided into three subgroups (C1, C2, and C3). The names of the strains are followed by the site abbreviation and isolation date. The Ribeirão Preto region strains were isolated in Cajuru (CJ), Guatapar (GU), Jardinpolis (JD), Pontal (PO), Pitangueiras (PI), Barrinha (BA), and Ribeiro Preto (RP). The Campinas region strains were isolated in So Joo da Boa Vista (SBV), Campinas (C), Jundi (J), Itapira (I), and Mogi Gua (MG).

introduction of new genotypes, considering the intense migration to these two regions, or a high rate of genetic change and the prevalence of specific genotypes in each geographical area. This might suggest that some genotypes have adapted to the local environmental conditions successfully.

Our findings urge health officers to take measures for more efficacious sanitary barriers to prevent shigellosis outbreaks and epidemics.

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RESUMO

EPIDEMIOLOGIA MOLECULAR DE LINHAGENS DE *SHIGELLA* SPP ISOLADAS EM DIFERENTES REAS METROPOLITANAS DO SUDESTE DO BRASIL

Shigella spp., o patgeno humano responsvel pela shigelose, apresenta grande poder infeccioso, mesmo em pequenas doses. A incidncia de shigelose varia de acordo com a distribuio geogrfica, o ndice de desenvolvimento humano local e a faixa de idade, sendo alto entre crianas com menos de 5 anos de idade. No Brasil, alguns trabalhos indicam que os casos de shigelose so subnotificados sendo, *S. flexneri* e *S. sonnei* os principais agentes responsveis pelos casos ocorridos. O presente estudo usou a tcnica de eletroforese em campo pulsado (PFGE) para investigar a epidemiologia molecular de 119 linhagens de *S. flexneri* e *S. sonnei*, isoladas de casos de shigelose que ocorreram nas regies metropolitanas das cidades de Ribeiro Preto e Campinas, estado de So Paulo, na regio Sudeste do Brasil. Os resultados indicam (i) a existncia de apenas alguns grupos clonais para ambas as espcies, mas com variabilidade de gentipos indicando ou um alto ndice de variao gentica, ou a constante introduo de vrios gentipos, considerando o movimento migratrio intenso de pessoas nestas duas reas metropolitanas, e (ii) a prevalncia de gentipos especficos em cada rea geogrfica, o que sugere a existncia de gentipos com maior capacidade de adaptao s condies ambientais locais. Os resultados indicam a necessidade de barreiras sanitrias mais eficientes para prevenir surtos e epidemias de *Shigella spp.*

Palavras Chave: *Shigella sonnei*, *Shigella flexneri*, Epidemiologia, Caracterizao, PFGE.

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