Prevalence and Virulence Genes of \textit{Shigella} spp. Isolated from Patients with Diarrhea in Rosario, Argentina

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SUMMARY: The aim of this study was to determine the prevalence and virulence factors of \textit{Shigella} species isolated from patients with diarrhea. \textit{Shigella} species were isolated from 1,022 stool samples collected from different hospitals in Rosario, Argentina. The isolates were characterized using phenotypic tests, serotyping, and detection of virulence genes by PCR. One hundred strains (9.8% of samples collected) of \textit{Shigella} were isolated. \textit{Shigella flexneri} was the most frequently identified species (74%), followed by \textit{S. sonnei} (26%). \textit{S. flexneri} was also the predominant species isolated from children aged 6–14 years. These clinical strains of \textit{Shigella} were then tested for the presence of \textit{ipaH, virA, iai, sen,} and \textit{set} using specific primers. \textit{virA} was present in all strains, whereas \textit{ipaH} was detected in 98% of strains and \textit{iai} in 83%. \textit{sen} was found in 71.6% of \textit{S. flexneri} and 42.3% of \textit{S. sonnei} isolates, and 41.9% of \textit{S. flexneri} isolates were positive for \textit{set}. Furthermore, 32.4% of \textit{S. flexneri} isolates were positive for both \textit{set} and \textit{sen}. This study provides data on the prevalence and distribution of diverse \textit{Shigella} strains.

INTRODUCTION

Shigellosis continues to be a major health problem in many parts of the world, particularly in underdeveloped and developing countries with poor sanitary systems and improper treatment of water supplies and among travelers from industrialized nations (1,2). Annually, more than 140 million diarrheal episodes are caused by \textit{Shigella} worldwide, with 600,000 cases resulting in death. Of these deaths, 60% are in children <5 years of age (3,4). Among the 4 \textit{Shigella} species, \textit{S. flexneri} is the most commonly isolated in the developing world and the most frequent cause of bacterial dysentery. In countries with improved water supplies and sanitation, the predominant species is \textit{S. sonnei}. In Argentina, according to the National Epidemiological Surveillance of Infectious Diseases, there were 471 shigellosis notifications in 2003, 597 in 2004, and 560 in 2005, for a total of 1,628 cases through 6 February 2006.

All members of the genus \textit{Shigella} are human-restricted pathogens that exert their effects on the gastrointestinal mucosa via the production of various virulence factors, including enterotoxins and effector proteins (5,6). The infective dose varies from 10 to 100 bacterial cells, and transmission of the pathogen is mainly fecal-oral, passed from the stools of sick or convalescing people or by asymptomatic carriers (7). This low infectious dose can at least partially be attrib-


enuted to the acid resistance of \textit{S. flexneri}, which enables it to survive in the stomach (8–10).

Infection with this invasive pathogen is a multistep process (11,12) that includes invasion of epithelial cells and intercellular dissemination (11). \textit{Shigella} spp. exhibit several characteristics associated with virulence, the most common being the ability to invade and colonize intestinal cells (13).

The invasion-associated locus (\textit{ial}) gene is carried on a plasmid of 120–140 MDa (13), and the invasion plasmid antigen H (\textit{ipaH}) gene is present in multiple copies both on plasmids and in the chromosomes of these organisms. \textit{virA} has been found on the virulence plasmid of \textit{S. flexneri} 2a; the virulence factor encoded by this gene has been implicated in invasion and intercellular spreading. VirA facilitates the formation of entry structures, suggesting that several effectors may act synergistically to promote internalization of the bacterium (14).

Another virulence factor of \textit{Shigella dysenteriae} is an exotoxin called Shiga toxin (Stx); it is not excreted by the bacterium, but is released only during cell lysis (15). Despite its clear toxigenicity, the role of Stx in shigellosis is not clear, because Stx is not essential for invasion or cellular lysis.

Two new enterotoxins have recently been described in \textit{S. flexneri} 2a. The first toxin is \textit{Shigella} enterotoxin 1 (ShET-1), which is encoded by the \textit{setI} chromosomal gene. The active toxin of ShET-1 is configured in one A subunit and several B subunits (A1-Bn) (11,16,17). The second enterotoxin, \textit{Shigella} enterotoxin 2 (ShET-2), is encoded by \textit{sen}. This gene is located on a plasmid of 140 MDa that is associated with invasion in this pathogen. These enterotoxins play an important role in pathogenesis by altering electrolytes and water transport in the intestine during the initial watery phase of the disease (16).

The aim of this study was to determine the preva-
MATERIALS AND METHODS

Patients: From December 2011 to June 2012, 1,022 stool samples from diarrheic patients were obtained in 5 different hospitals of Rosario, Argentina.

Microbiological tests: To isolate Shigella species, stool samples were inoculated onto MacConkey agar and Salmonella-Shigella agar (Becton Dickinson, Heidelberg, Germany), and the resulting colonies that exhibited characteristics of Shigella spp. were identified to the species level by standard biochemical and serological (Bio-Rad Laboratories, Hercules, CA, USA) tests.

Detection of virulence genes by PCR: A suspension of 200 μl of 24-h bacterial culture was treated at 100°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, hybridization at the appropriate annealing temperatures (Ta), set, virA, ipaH, and iai. The primers used are presented in Table 1. Amplification of the genes encoding virulence factors were carried out in a reaction mixture of 25 μl, with 1 × buffer (Thermo Fisher, Waltham, MA, USA), 200 μM each deoxynucleotide triphosphate (dNTP), 20 pmol of each primer, and 5 U/μl of Taq DNA polymerase (Thermo Fisher). PCR assays were performed in a DNA thermal cycler (IVEMA, Buenos Aires, Argentina). The amplification program consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, hybridization at the appropriate annealing temperatures (Ta, Table 1) for 45 s, and an elongation phase at 72°C for 1 min, and ending with a final extension at 72°C for 5 min.

Proper negative controls for all components of the PCR mix, except for the DNA template, were included in each PCR run. Amplification products were visualized on a 1.5% agarose gel with 0.5 μg/ml of ethidium bromide. Amplicon sizes were estimated by comparing them with the bands of a molecular weight marker (Thermo Scientific, Waltham, MA, USA).

RESULTS

Prevalence of Shigella species: During the study period, 100 Shigella strains were recovered from 1,022 stool specimens. The prevalence of Shigella in the patients was 9.8%, and the isolation frequency was 74% for S. flexneri, the predominant species, and 26% for S. sonnei.

Table 2 shows the distribution of Shigella by age group. Shigella was most frequently isolated from the 6–14 year age group. Almost 92.5% of the 40 isolates were recovered from stool specimens of this age group; the predominant species was S. flexneri, which accounted for 57.5% (n = 23) of the 100 isolates. In the 0–5-year age group, S. flexneri was again the most frequent species recovered.

Prevalence of virulence genes: The genes ipaH, virA, set, sen, and iai, which encode virulence factors, were detected by PCR. The PCR product of ipaH was 661 bp, and that of iai was 320 bp. Amplification of virA produced a PCR product of 215 bp. In the amplification reaction with set, which encodes ShET-1, a band of 309 bp was observed. The product amplified from sen, which encodes ShET-2, was 799 bp (image not shown).

The virulence gene distribution among Shigella isolates is presented in Table 3. All strains of Shigella were

Table 1. Nucleotidic sequences of the primers used and size of the amplicons

| Virulence factor | Primer       | Oligonucleotide sequence                  | Ta (°C) | Amplicon size (bp) | Reference |
|-----------------|--------------|------------------------------------------|---------|--------------------|-----------|
| iai             | iai-R        | CTGGATGGTAGATGCGAG                      | 56      | 320                | (18)      |
|                 | iai-F        | GAGAGCCCAATATTCA                         |         |                    |           |
| ipaH            | ipaH-F       | GTCCCCTGACCGCCCTTGCACCCCTGAC           | 60      | 661                | (19)      |
|                 | ipaH-R       | GCGGCTGACCCCTGAGG                       |         |                    |           |
| virA            | virA-F       | CTGCATTCTGCAATCTCCACCTCC                | 65      | 215                | (20)      |
|                 | virA-R       | TCGAGGCCTATCGCACCGAGGC                 |         |                    |           |
| set             | set-F        | TACGCTCATTCAACATCCAGG                   | 50      | 309                | (21)      |
|                 | set-R        | TATCCCCCTTTGTTGTA                      |         |                    |           |
| sen             | sen-F        | ATGGCGCTGGCATATTATAT                   | 55      | 799                | (21)      |
|                 | sen-R        | CATATAATAAAGCGTCAG                     |         |                    |           |

1: Gene encoding Virulence factor.
2: Annealing temperature.
3: Bases pair.
virA positive, whereas only 98 (98%) were ipaH positive. ipaH was detected in 98.6% of S. flexneri strains and 96.2% of S. sonnei strains. ial was observed in isolates from 83 (83%) patients with shigellosis, and was found in 68/74 (91.9%) S. flexneri and 15/26 (57.7%) S. sonnei isolates.

Among the 100 strains of Shigella studied, 31 and 64 were positive for set and sen, respectively, whereas 24 isolates were positive for both genes. Among the toxin producers, 7 (9.8%) S. flexneri strains produced only ShET-1, 29 (40.8%) S. flexneri strains produced only ShET-2, and 24 (33.8%) S. flexneri strains produced both enterotoxins, whereas 11 (15.5%) S. sonnei strains produced ShET-2 alone. The enterotoxin gene distributions in all isolates studied are shown in Table 4.

### DISCUSSION

Acute diarrheal diseases, a major public health problem in developing countries, are associated with significant morbidity and mortality, especially among children aged 1–4 years of age. Of pathogens causing diarrhea, Shigella continues to be the major etiologic agent of inflammatory diarrhea and dysentery, thus presenting a serious challenge to health authorities worldwide (17,22).

In the present study, Shigella was recovered from 9.8% of acute infectious diarrhea cases during the study period. The isolation rate of Shigella species in our study was comparable to those of studies conducted in Ethiopia (2009) (7.5%) (23), northwestern Ethiopia (2006) (8.7%) (24), South Africa (2009) (8.5%) (25), and in countries of South America (2008) (8%) (26).

The species of Shigella responsible for the majority of shigellosis cases are S. flexneri, S. boydii, S. sonnei, and S. dysenteriae and the geographical distribution differs among these species. S. flexneri is the most common cause of morbidity and mortality; in contrast, illness caused by S. sonnei is generally less severe. Of the species, S. dysenteriae and S. flexneri are predominant in developing countries, whereas S. sonnei accounts for most of the reported cases of shigellosis in developed countries, and S. boydii is predominant in Asia, mainly in India (2,27,28). The frequencies of S. flexneri, S. sonnei, S. boydii, and S. dysenteriae were found to be 16%, 77%, 2%, and 1% in developed countries and 60%, 15%, 6%, and 6% in developing countries, respectively (27,28).

In a study conducted in India, S. flexneri was the most common species detected (57.6%), followed by S. sonnei (31%) (29). Another study conducted by Srinivasa et al. found an isolation rate of Shigella species of 4.6%. Of the isolates, S. flexneri strains accounted for 64.9% of Shigella-positive cultures, whereas S. sonnei accounted for 21.6%, S. boydii for 8.2%, and S. dysenteriae for 3.7% of cultures (30).

Information reported in Argentina by the Laboratorial Surveillance System indicated that the most common species in Argentina are S. flexneri and S. sonnei, with S. flexneri being the predominant species. Neighboring countries such as Uruguay, Peru, Colombia, and Venezuela show a similar distribution of species (31–33).

In our study, S. flexneri (74%) was most common, followed by S. sonnei (26%). No isolates of S. boydii and S. dysenteriae were identified. These results are in keeping with reports of previous studies in which S. flexneri was responsible for more than 50% of shigellosis cases (34–36).

In both developed and developing countries, endemic shigellosis is primarily a childhood disease, whereas epidemic shigellosis affects all age groups (26). Worldwide, morbidity and mortality due to shigellosis are highest among children aged 1–5 years and the elderly. Srinivasa et al., for example, isolated Shigella species primarily from children aged 0–14 years (30). Children within this age group are most susceptible to shigellosis because of inadequate resistance, lack of previous exposure, poor personal hygiene, and greater exposure to contaminated environments owing to play-related activities (37,38). In this study, shigellosis was observed in all age groups, but it was the highest in the 6–14 year age group.

Shigella can harbor several virulence factors, including factors that encode toxins or that are associated with invasion of the colonic epithelium and dissemination from cell to cell (16,39).

In our study, PCR was used to investigate various genes (ial, ipaH, set, sen, and virA) associated with Shigella pathogenicity. The product of ial assists Shigella in penetration of epithelial cells, and that of ipaH facilitates cell-to-cell spread (40). Another virulence factor, VirA, is involved in the uptake, motility, and cell-to-cell transmission of Shigella within a human host, and it is considered an essential factor in Shigella pathogenesis. The genes detected in this study, especially ipaH and virA, were present in the isolates, with a frequency of 100% for virA and 98% for ipaH, values consistent with previous reports by Bassa and Villalobos (18,20). On the other hand, ial, located exclusively on a plasmid, was detected in 83% of Shigella isolates in this study. ial has been less frequently examined in previous studies (17,41).

In a considerable number of patients, watery diarrhea is observed prior to the onset of dysentery. This is likely explained by the synthesis of 2 enterotoxins, ShET-1 and ShET-2.
ShET-1 is found almost exclusively in *S. flexneri* 2a (42), whereas plasmid-encoded ShET-2 has been reported in different species of *Shigella*, although its distribution among different serotypes of *S. flexneri* has not been studied (21,43).

In our study, although no serotyping was performed, *set* was detected in 41.9% (31/74) of *S. flexneri* strains, but in none of the *S. sonnei* isolates, which is in agreement with previous results (17,44).

Previous studies found *sen* in 49–98% of *Shigella* isolates, including members of all 4 *Shigella* species (21,27). In the present work, *sen* was found in 71.6% (53/74) of *S. flexneri* and 42.3% (11/26) of *S. sonnei* isolates. In 32.4% (24/74) of *S. flexneri* strains, both *sen* and *set* were detected. No *S. sonnei* isolates were positive for both genes.

In conclusion, this study showed a high prevalence of *Shigella* infection in children aged 6–14 years in this region of Argentina. This work also determined the distribution of virulence genes in *Shigella* spp. isolated from clinical cases, which may contribute to enhanced clinico-epidemiological monitoring.

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Conflict of interest None to declare.

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