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Genotypes and antimicrobial profiles of *Shigella sonnei* isolates from diarrheal patients circulating in Beijing between 2002 and 2007☆

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ABSTRACT

Shigella sonnei has become the dominant serotype causing shigellosis in Asian countries in recent years. In this study, we characterize the increasing trend of antibiotic resistance profiles and genotypes of *S. sonnei* isolates in the Beijing area. From January 2002 to December 2007, a total of 1108 *Shigella* isolates including 362 *S. sonnei* were recovered from diarrhea patients at the 302nd Hospital in Beijing. While the frequency of *S. flexneri* gradually decreased, *S. sonnei* gradually increased and became the dominant species. A total of 362 *S. sonnei* isolates were further analyzed for their antimicrobial profiles and 272 revived isolates were selected for genotyping analysis, respectively. High-level antimicrobial resistances were observed in sulfamethoxazole/trimethoprim (94.5%), ampicillin (40.3%), piperacillin (36.5%), and ceftriaxone (12.8%) with significant single- and multiple-drug resistance increase trends from 2002 to 2007 ($P = 0.0000$). Pulsed-field gel electrophoresis analysis indicated that 263 (96.7%) *S. sonnei* belonged to 1 clonal genotype A, which were further divided into A1–A6 subtypes. While subtype A2 was dominant in the early stage of study years, subtype A4 started to emerge and increased significantly in later years. Antimicrobial resistance rates are statistically different among the 6 subtypes ($P = 0.0000$), and A4 possessed the highest resistance rates to ampicillin (83.7%) and piperacillin (81.4%). Subtype A3 was highly clustered in inpatients compared to other subtypes ($P = 0.0145$). This study indicates that a clonal *S. sonnei* strain has become dominant in the Beijing area, and subtype A4 is responsible for increased antibiotic resistance.

1. Introduction

Shigellosis is one of the most common causes of diarrhea in humans worldwide. The annual number of *Shigella* episodes throughout the world has been estimated to be 164.7 millions, of which 163.2 million have occurred in developing nations resulting in 1.1 million deaths (Kotloff et al., 1999). Humans and other primates are the only natural reservoirs for *Shigella* species (Niyogi, 2005). Epidemics usually occur in crowded areas with poor sanitary conditions where transmission from person to person is common, or when food or water is contaminated by the organism. Despite economic and public health improvements, outbreaks of shigellosis are still reported regularly (Anonymous, 1999a, b; Gaynor et al., 2009; Kuo et al., 2009; Marcus et al., 2004; Morgan et al., 2006; Wei et al., 2007). A definitive diagnosis of shigellosis can be made by isolating the organism from a stool sample. In China, shigellosis has been ranked third in morbidity, following tuberculosis and hepatitis, and has become the number 1 cause of disease-related death in children (CDC, 2007; Mathers et al., 2009). Historically, there have been 4 subgroups of *Shigella* that have been described: subgroup A as *S. dysenteriae*, subgroup B as *S. flexneri*, subgroup C as *S. boydii*, and subgroup D as *S. sonnei*. Among these subgroups, the epidemic subgroup of diarrhea was typically *S. sonnei* in industrialized countries and *S. flexneri* in developing countries including the Beijing area for many years (Gupta et al., 2004; Kotloff et al., 1999; Qu et al., 2008; von Seidlein et al., 2006). However, cases of *S. sonnei* have noticeably increased and *S. sonnei* is becoming the dominant subgroup in Asian countries in recent years (Bangtrakulnonth et al., 2008; Filliol-Toutain et al., 2011; Kotloff et al., 1999; Mamishi et al., 2009; Orrett, 2008; Qu et al., 2008; Salmanzadeh-Ahrabi et al., 2007; Wei et al., 2007). It is necessary to explore the clinical importance of *S. sonnei*–related infections because it can cause systematic infections such as bacteriemia and meningitis in immunocompromised individuals (Chapel et al., 2005). In addition, *S. sonnei* is often associated with international food-borne infection outbreaks by airline passengers, imported food, travelers, animals, and insect vectors. *S. sonnei* can also be sexually transmitted among homosexual males (Anonymous,

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1999a, 1999b; Gaynor et al., 2009; Kuo et al., 2009; Marcus et al., 2004; Morgan et al., 2006; Okeke and Edelman, 2001; Salam and Bennish, 1991). *S. sonnei* remains a public health concern and has become a threat in both developed and developing nations.

Prompt treatment with effective antimicrobial agents may shorten the duration of clinical symptoms and excretion of the pathogens as well as decrease the carriage, severity, and transmission of dysentery by *S. sonnei* (Salam and Bennish, 1991). However, the progressive increase in antimicrobial resistance, especially multidrug resistance among *Shigella* to β-lactam antibiotics due to the overuse of antibiotics in developing countries, is becoming a critical problem (Salmanzadeh-Ahrabi et al., 2007). In addition, the subgroup distribution and antimicrobial resistance patterns among *Shigella* spp. varies from region to region and even within the same region (Wong et al., 2010).

The correct treatment and prompt control of shigellosis depend on the recognition of dominant prevalent *Shigella* subgroup and serotype as well as on related antimicrobial resistance profiles in a local region. The epidemic trends, resistance, and clonal transmission of *S. sonnei* isolates studied in China have not been well reported. In this study, we evaluated the epidemiology of *Shigella* serogroups, antibiotic resistance patterns, prevalence of clonal transmissions, and related risk factors of *S. sonnei* strains circulating in the Beijing region for 6 years between 2002 and 2007.

2. Materials and methods

2.1. Bacterial strains

From January 2002 to December 2007, fresh stool specimens were collected from diarrhea patients with clinically suspected dysentery and submitted to the Microbiology Laboratory of the 302nd Hospital of the People's Liberation Army, Beijing, China, a 1300-bed infectious disease teaching hospital. Samples were cultured for *Shigella* by streaking directly onto *Salmonella–Shigella* (SS) agar (Tian Tan Biologic Technology Company, China) and incubated overnight at 37 °C. Colorless, semitransparent, smooth, and moist circular colonies were screened for on SS agar and streaked out on Kligler iron agar (Ye et al., 2006). *Shigella* strains were identified according to their biochemical characteristics (Nataro et al., 2011). Serotypes of *Shigella* isolates were further determined with commercially variable polyclonal and monoclonal antisera against *Shigella* serotypes by the slide agglutination method according to the manufacturer's instructions (Japan Institute of Health Diagnostic Serum Shigella, Japan). Only 1 *Shigella* isolate per patient per diarrhea episode was included in the analysis.

2.2. Antimicrobial susceptibility testing

In vitro activities of ampicillin (AMP, 10 μg), piperacillin (PIP, 100 μg), ceftriaxone (CRO, 30 μg), cefepime (FEP, 30 μg), ciprofloxacin (CIP, 5 μg), norfloxacin (NOR, 10 μg), ofloxacin (OFX, 5 μg), levofloxacin (LVX, 5 μg), cefmetazole (CMZ, 30 μg), chloramphenicol (CHL, 30 μg), sulfamethoxazole/trimethoprim (SXT, 23.75/1.25 μg), and fosfomycin (FOS, 200 μg) were determined by the Kirby–Bauer disc-diffusion method in accordance with the guidelines of the Performance Standards for Antimicrobial Susceptibility Testing as recommended by Clinical and Laboratory Standards Institute (CLSI, 2006). An *Escherichia coli* (ATCC 25922) strain was used as the quality control strain. Results were interpreted as either sensitive, intermediate, or resistant. In our study, we considered both intermediate and resistant results as resistant.

2.3. Molecular typing

Clonality and transmission patterns were determined by pulsed-field gel electrophoresis (PFGE) as described previously (Qu et al., 2010). Briefly, genomic DNA was extracted from logarithmic-phase cultures and prepared in low-melting-point agarose plugs and digested with the restriction enzyme *Xba*I (Takara, Shiga, Japan) according to a standard procedure. Electrophoresis was performed with the Bio-Rad CHEF DR II system (Bio-Rad, Hercules, CA, USA). PFGE run conditions were 200 V with a switch from 10 to 50 s for 15 h at 14 °C. Along with the specimens, DNA from *Salmonella* serotype Braenderup strain H9812 was digested with *Xba*I and used as molecular weight standard. After gel electrophoresis, gels were stained with ethidium bromide, rinsed, and photographed under UV light. The banding patterns of all isolates evaluated with PFGE were compared by visual inspection, and the isolates were grouped as suggested by Na-Ubol et al. (2006). Isolates were defined as epidemic ones when belonging to a genotype that was identified in at least 2 individuals.

2.4. Statistical analysis

Statistical comparisons were performed with the Epi Info software (version 3.5.1; Centers for Disease Control and Prevention, Atlanta, GA, USA). Categorical data were expressed as percentages and calculated using a chi-squared test. *P* values were calculated, and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Strain distribution

During the 6-year study period, a total of 1108 *Shigella* strains were isolated from diarrhea patients. Among them, 362 (32.7%) were *S. sonnei* isolates which came from 197 males (54.4%) and 165 females (45.6%) with ages ranging from 4 months to 88 years old (mean ± SD = 18.8 ± 17.8 years). Children less than 14 years old accounted for 46.7% of the study patients with no death revealed. There were 59 inpatients and 303 outpatients. The *S. sonnei* isolates

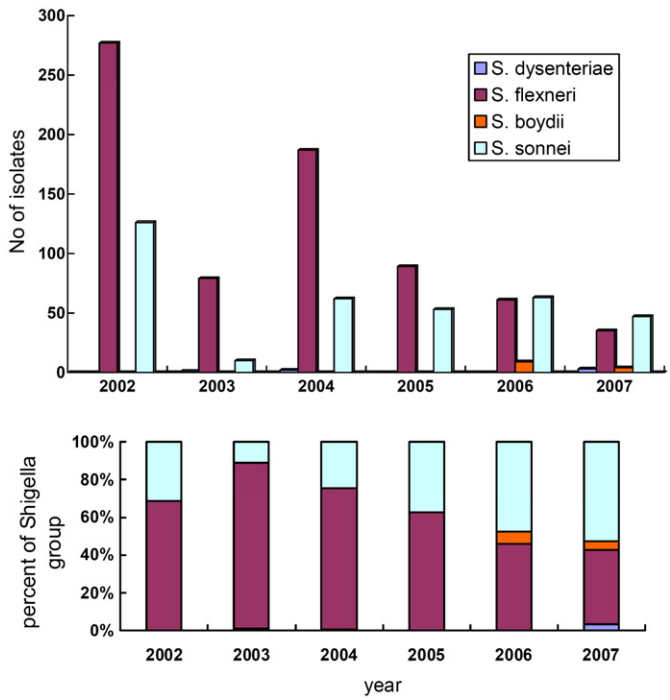

Fig. 1. (A) *Shigella diarrhoea* species distribution from 2002 to 2007 in the Beijing area. (B) *Shigella diarrhoea* species proportions from 2002 to 2007 in the Beijing area.

Table 1
Antibiotic resistance trends during the 6-year study period.

Antibiotics	No. (%) of resistant *S. sonnei* cases for each year						χ^2	*P* value
	2002 ($n = 124$)	2003 ($n = 10$)	2004 ($n = 62$)	2005 ($n = 53$)	2006 ($n = 66$)	2007 ($n = 47$)		
AMP	22 (17.7)	1 (10)	18 (29)	21 (39.6)	52 (78.8)	36 (76.6)	114.19	0.0000
PIP	16 (12.9)	1 (10)	12 (19.4)	21 (39.6)	50 (75.7)	37 (78.7)	134.25	0.0000
CRO	4 (3.2)	0	2 (3.2)	7 (13.2)	21 (32.2)	15 (32)	66.80	0.0000
FEP	3 (2.4)	0	0	4 (7.6)	12 (18.2)	1 (2.1)	44.57	0.0000
CMZ	0	0	0	0	1 (1.5)	0	4.50	0.4802
SXT	119 (96)	8 (80)	56 (90.3)	53 (100)	60 (90.7)	47 (100)	30.16	0.0008
OFX	5 (4.0)	0	2 (3.2)	1 (1.9)	2 (3.0)	2 (4.3)	1.03	0.9603
CIP	2 (1.6)	0	0	1 (1.9)	2 (3.0)	2 (4.3)	3.24	0.6627
NOR	5 (4.0)	0	1 (1.6)	1 (1.9)	2 (3.0)	2 (4.3)	1.63	0.8974
LEV	2 (1.6)	0	1 (1.6)	1 (1.9)	2 (3.0)	2 (4.3)	1.67	0.8922
CHL	3 (2.4)	0	1 (1.6)	1 (1.9)	1 (1.5)	1 (2.1)	0.46	0.9936
FOS	0	0	4 (6.4)	1 (1.9)	0	0	15.26	0.0093

were recovered dominantly in summer seasons with 3 (0.8%), 4 (1.1%), 248 (68.5%), and 107 (29.6%) in winter (December, January, and February), spring (March, April, and May), summer (June, July, and August), and fall (September, October, and November), respectively. The *S. sonnei* subgroup was responsible for 31.26%, 11.11%, 24.70%, 37.32%, 47.36%, and 52.81% of shigellosis cases from the years 2002 to 2007, respectively. In contrast, the *S. flexneri* group was responsible for 68.73%, 87.78%, 74.51%, 62.68%, 45.86% and 39.33%, respectively. Starting from 2006, *S. sonnei* has replaced *S. flexneri* to become the dominant subgroup in the Beijing area (Fig. 1A and B).

3.2. Antibiotic resistance

The resistance rates of 362 *S. sonnei* isolates to AMP, PIP, CRO, FEP, CIP, NOR, OFX, LVX, CMZ, CHL, SXT, and FOS were 40.3%, 36.5%, 12.8% , 5.5%, 5.5%, 3.9%, 3.3%, 3.0%, 0.3%, 1.9%, 94.5%, and 1.4%, respectively. The resistance rates of *S. sonnei* to AMP, PIP, CRO, FEP ($P = 0.0000$), SXT ($P = 0.0008$), and FOS ($P = 0.0093$) had changed significantly during the research period ($P = 0.0000$) (Table 1). In addition, multidrug-resistant *S. sonnei* isolates, which were defined as resistant to 3 or more antibiotic agent subclasses, were determined with 35.6% to AMP, PIP, and SXT; 13.3% to AMP, PIP, and CRO; and 5.3% to AMP, PIP, CRO, and FEP. None of the antimicrobial agents was effective against all strains.

3.3. Genotyping profiles

Among 272 *S. sonnei* isolates, 266 (97.8%) and 6 (2.1%) were phase I and phase II isolates, respectively. Based on the PFGE analysis, 263 (96.7%) were determined to be epidemic clone A, and these were further divided into A1–A6 subtypes (Fig. 2). Subtype A2 was the most prevalent genotype covering 86.6%, 82.1%, 53.8%, 30.9%, and 42.9% of isolates from 2002 to 2007 (except for 2003), respectively, with clone A4 being the next most common subtype. Subtype A2 prevalence decreased gradually from 2002 to 2006 but increased again in 2007. Subtype A4 increased since 2005 and remains constant, which indicates that there are different majorities of clonal transmission at different times (Fig. 3). The year 2003 was unusual due to the severe acute respiratory syndrome (SARS) outbreak in the region.

3.4. Factors in relation to S. sonnei subtypes

The antibiotic resistance rates of *S. sonnei* isolates varied among different PFGE subtypes, especially β-lactam antibiotics. As shown in Table 2, the AMP and PIP resistance rates in subtype groups A4 ($P = 0.0000$), A5 ($P = 0.0052$), A3 ($P = 0.01$), and A1 ($P = 0.017$) were statistically higher than those in A2. The resistant rate to CRO was the highest in clone A3, which was statistically higher than those in clone A2 ($P = 0.0000$), clone A4 ($P = 0.0131$), and clone A5 ($P = 0.0077$). Even for fourth-generation cephalosporin FEP, the resistance in clone A3 and A1 was statistically higher than that in clone A2 ($P = 0.0000$) and clone A4 ($P = 0.0032$) (Table 2). Among the factors of the original department source, sex, and age of diarrheal patients, subtype A3 was

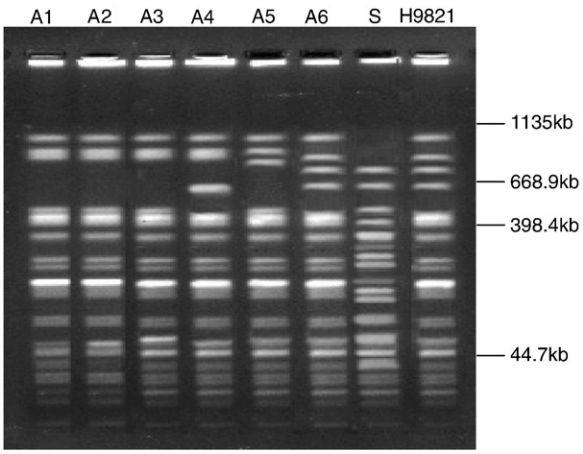

Fig. 2. PFGE of *Xba*I-digested genomic DNA from *S. sonnei* isolates; lanes A1–A6 are epidemic and lane S is sporadic. Lane M is *Xba*I-digested genomic DNA from the *Salmonella* Braenderup strain H9812 which served as a molecular size marker.

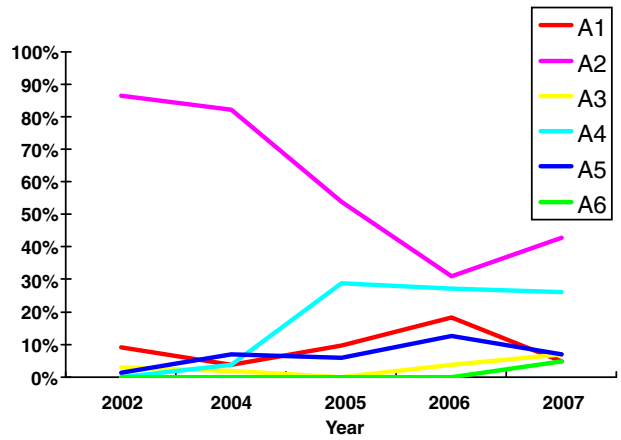

Fig. 3. Changing trend of *S. sonnei* subtypes between 2002 and 2007 ($\chi^2 = 80.21$, $P = 0.0000$).

Table 2
Relationship between PFGE subtypes and antibiotic resistant rates in 272 *S. sonnei*.

Antibiotic	No. (%) of subjects for each case type						χ^2	*P* value
	Epidemic PFGE subtype							
	A1 ($n = 25$)	A2 ($n = 167$)	A3 ($n = 8$)	A4 ($n = 43$)	A5 ($n = 18$)	A6 ($n = 2$)		
AMP	13 (52.0)	44 (26.3)	6 (75.0)	36 (83.7)	11 (61.1)	1 (50.0)	54.8	0.0000
PIP	12 (48.0)	41 (24.6)	6 (75.0)	35 (81.4)	11 (61.1)	1 (50.0)	55.3	0.0000
CRO	11 (44.0)	12 (7.2)	6 (75.0)	10 (23.3)	3 (16.7)	0 (0.0)	46.4	0.0000
FEP	7 (28.0)	5 (3.0)	3 (37.5)	3 (7.0)	0 (0.0)	0 (0.0)	34.3	0.0000

observed in significantly higher frequencies in inpatients than other PFGE subtypes ($P = 0.0145$) (Table 3).

4. Discussion

Our study presented the antibiotic resistance and genotyping profiles of clinical *S. sonnei* isolates circulating for a 6-year period from 2002 to 2007 in the Beijing area. Our data showed a decreased frequency of *Shigella* infection cases from 2002 to 2007 (with the exception of 2003) due to improved economic situations, environmental conditions, hygiene habits, and quality of water supplies, which had a similar trend of decrease to other intestinal pathogens during the same period (Qu et al., 2008). In 2003, during the epidemic of SARS, few people ate out and fewer *S. sonnei* cases occurred compared to other years, which indicated that eating out has been the main cause of *S. sonnei* infections. Another related reason might be that much fewer patients with diarrhea desired to go to the hospital, which decreased the chances of acquiring lethal SARS infections. Another important finding was that *S. sonnei* replaced *S. flexneri* to become the predominant subgroup causing shigellosis in the Beijing area, which was similar to the trends and patterns reported in industrialized countries (Bonfiglio et al., 2002; Ekdahl and Andersson, 2005; Filliol-Toutain et al., 2011; Gupta et al., 2004; Ozmert et al., 2011).

The emergence of drug-resistant and multidrug-resistant *S. sonnei* strains has become an important issue and has complicated the selection of empirical agents for the treatment of shigellosis. Identifying and monitoring the local resistance patterns of *S. sonnei* can provide effective empiric treatment regimens. The resistance rates and single- and multidrug resistance of *S. sonnei* isolates in Beijing were higher than those reported in other developed and developing countries (Ozmert et al., 2011; Pourakbari et al., 2010; Talukder et al., 2006; Wong et al., 2010; Wu et al., 2009). Our study results revealed that drug resistance occurred in all antibiotics tested with *S. sonnei*. As in other countries (Huang et al., 2005; Jain et al., 2005; Lartigue et al., 2005; Nagano et al., 2009; Vinh et al., 2009; Vrints et al., 2009), multidrug-resistant *S. sonnei* isolates and the resistant rate of *S. sonnei* to AMP, PIP, CRO, and FEP have increased significantly over the study period years, which will significantly limit the empiric therapy capacity of shigellosis in this area. As resistance to antimicrobial agents increases constantly, it is important to survey and monitor local resistance in order to formulate policies for the rational use of antimicrobial agents.

Our study indicates that current resistance patterns should limit the use of sulfonamides and β-lactam antibiotics even though SXT, AMP, and PIP are currently considered acceptable empirical agents for therapy of shigellosis in developed countries. According to the resistant results, fluoroquinolones are an effective alternative for treating adult shigellosis but are not approved by the Food and Drug Administration for shigellosis treatment in children aged less than 18 years (Stahlmann, 2002). In comparison, fosfomycin with good antibacterial activities in vitro and low incidence of adverse events can be used as an alternative treatment for diarrhea infection including in pediatric patients (Fukuyama et al., 2000; Michalopoulos et al., 2011), but chloramphenicol with lower level of resistance is rarely used in diarrhea patients in clinical settings because of its more adverse effects (Arjyal et al., 2011).

We have used PFGE to characterize the diversity of *S. sonnei* isolates and to determine the clonality of these isolates in subtype levels as other studies have previously reported (DeLappe et al., 2003; Huang et al., 2005). In our study, the PFGE genotype analysis of 272 *S. sonnei* strains indicated that 96.7% were determined to be 1 epidemic clonal genotype A. At the subtype level, subtype A2 was the most dominant one in the early stages of our study period, while subtype A4 started to emerge and increase significantly in later years, indicating that clonal transmission of *S. sonnei* remains at the genotype level, while alternation starts at the subtype level in Beijing area.

So far, limited data are available on the relationship of genotyping and antimicrobial resistance profiles of *S. sonnei* isolates recovered in China. The PFGE subtype of *S. sonnei* isolates has changed during the 6-year study period. Antimicrobial resistance rates were statistically different among the 6 subtypes. The resistance rates of different PFGE subtypes to antibiotics varied, especially β-lactam antibiotics. Subtypes A4 and A3 were associated with resistance to AMP and PIP with the highest resistance rate to CRO found in A3. The higher levels of AMP, PIP, CRO, and FEP resistance in *S. sonnei* isolates play an important role in the majority of clonal transmission in Beijing. Other risk factors related to *S. sonnei* circulation were department besides sex and age.

S. sonnei has become the dominant *Shigella* subgroup causing gastroenteritis in the Beijing area. Studies on *S. sonnei* have

Table 3
PFGE Subtype distribution and related factors in 272 *S. sonnei* isolates.

Factor	Group	No. (%) of subjects for each case type						χ^2	*P*
		Epidemic PFGE subtype							
		A1 ($n = 25$)	A2 ($n = 167$)	A3 ($n = 8$)	A4 ($n = 43$)	A5 ($n = 18$)	A6 ($n = 2$)		
Department	Inpatients	8	27	5	7	3	0	14.18	0.0145
	Outpatients	17	140	3	36	15	2		
Sex	Male	15	85	4	21	14	0	7.80	0.1675
	Female	10	82	4	22	4	2		
Age	≤14 years	14	83	7	23	11	1	7.14	0.7117
	15–59 years	10	78	1	17	7	1		
	≥60 years	1	6	0	3	0	0		

become very important due to the increase of epidemic frequency, multiresistant strain emergence, and clonal transmission. Furthermore, continuous monitoring of subgroups, resistance patterns, and prevalence of *S. sonnei* is mandatory for the appropriate selection of empiric antimicrobial drugs in the therapy and prevention of the emergence of resistant strains and of the dissemination of resistance genes.

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