Brief Original Article

An eight-year study of *Shigella* species in Beijing, China: serodiversity, virulence genes, and antimicrobial resistance

Mei Qu[1]*, Xin Zhang[1]*, Guirong Liu[1], Ying Huang[1], Lei Jia[1], Weili Liang[2], Xitai Li[1], Xiaona Wu[1], Jie Li[1], Hanqiu Yan[1], Biao Kan[2], Quanyi Wang[1]

* Authors contributed equally to this work.

[1] Beijing Key Laboratory of Diagnostic and Traceability Technologies for Food Poisoning, Beijing Centre for Disease Prevention and Control, Capital Medical University School of Public Health and Family Medicine, Beijing, China
[2] State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Beijing, China

Abstract

Introduction: This study was conducted to determine the prevalence of serotypes, virulence factors, and antimicrobial resistance patterns of *Shigella* spp. in Beijing, China, from 2004 to 2011.

Methodology: Real-time PCR assays were used to detect virulent genes, and the Kirby-Bauer disk diffusion method was used to evaluate antimicrobial resistance.

Results: Among the total of 1,652 *Shigella* isolates, *S. sonnei* (57.1%) was the predominant species, followed by *S. flexneri* (42.3%), *S. dysenteriae* (0.4%), and *S. boydii* (0.2%). Nineteen serotypes were discovered among *S. flexneri* strains. The virulence gene *ipa*H was the most frequent, followed by *sen* and *set*. The presence of *set* showed significant difference in two dominant serogroups, *S. flexneri* and *S. sonnei*. Over 90% of *Shigella* isolates showed resistance to at least three drugs with widened spectrum. High-level antimicrobial resistance to single and multiple antibiotics was more common among *S. sonnei* than *S. flexneri*.

Conclusion: There was an obvious serotype change and a dramatic increase of antibiotic resistance in *Shigella* prevalence in Beijing.

Key words: *Shigella*; serodiversity; prevalence; antimicrobial resistance; diarrhea.

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Introduction

Shigellosis, as a major source of diarrhea, is a global human health problem. Any of four species of *Shigella* (*S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*) can cause shigellosis. *S. sonnei* and *S. boydii*, associated with mild illness of short duration, most often occur in developed countries; *S. flexneri* is the most prevalent species in developing countries, and *S. dysenteriae* is known to cause sporadic outbreaks and epidemics worldwide, with severe complications and very high mortality [1-3]. In China, *Shigella* spp. is the most frequently isolated diarrheal pathogen, accounting for up to 1.7 million episodes of bacillary dysentery annually, with an estimated 200,000 patients admitted to hospitals [4].

Virulence genes responsible for the pathogenesis of shigellosis are often multifactorial and coordinately regulated. *Ipa*H is present in multiple copies on both the invasion plasmid and on the chromosome, and is responsible for the modification of host response to infection. Another two genes encoding enterotoxins, *set* and *sen*, have roles in altering electrolyte and water transport in the small intestine during the initial watery phase of shigellosis [5,6]. Knowledge about the distribution of the three genes is limited, especially for the last two, which has been reported only in *S. flexneri* serotypes in some detail [7,8]. The purpose of this study is to describe the prevalence of various *Shigella* species, the distribution of three virulence genes, and the local antibiotic resistance patterns in Beijing, the capital of China, between 2004 and 2011.

Methodology

Shigella *isolates and bacteriological examination*

A hospital-based active surveillance was conducted in 16 districts of Beijing from 2004 to 2011. The sentinel hospitals collected stool specimens of outpatients, with a monthly enrollment number of 20–

30 patients per district. For isolation *of Shigella*, the fecal swabs were plated directly onto Salmonella-Shigella agar and incubated at 37°C for 16 to 24 hours. After culture and screening, the biochemical identification was confirmed with the VITEK 2 Compact instrument (bioMérieux, Marcy l'Etoile, France). Finally, serologic identification was performed by the slide agglutination test.

Real-time PCR assay

Real-time PCR assays were employed to detect for the presence of *ipa*H, *set* and *sen* genes. For *ipa*H, *ipa*H-P (FAM-CTGTCGAAGCTCCGCAGAGGCAC-TAMRA), *ipa*H-F (5'-CATCAGCAGCAACAGTGAAAGAC-3') and *ipa*H-R (5'-CACGCAATACCTCCGGATTC-3') were used; for *set*, *set*-P (FAM-CCCGGCACCTGTGGAATGGC-TAMRA), *set*-F (5'-GGTTCACGCTACCATCAAAGATTAC-3'), and *set*-R (5'-GCTGACCGGGAATATGGATGT-3') were used; for *sen*, *sen*-P (FAM-CGTCCTCCCATTCCTTCCGCAG-TAMRA), *sen*-F (5'- AGTGCTTGGGATAAACCCGATA-3'), and *sen*-R (5'-ACGGAGAACTCTTGGAAACTTCTG-3') were used. The real-time PCR assay was performed using the TaqMan Universal PCR Master Mix Kit (Applied Biosystems, Foster City, United States).

Antimicrobial resistance testing

Antibiotic resistance of the *Shigella* isolates was tested using the Kirby-Bauer disk diffusion method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2005). *Escherichia coli* (ATCC 25922) was included in the test as a quality control. The disk concentrations of 12 antimicrobial agents (Oxoid, Basingstoke, United Kingdom) were as follows: 10 µg ampicillin (AMP), 5 µg ciprofloxacin (CIP), 25 µg sulphamethoxazole/trimethoprim (SXT), 30 µg nalidixic acid (NAL), 30 µg tetracycline (TET), 30 µg amoxicillin clavulanic acid (AMC), 10 µg gentamicin (GEN), 10 µg norfloxacin (NOR), 5 µg ofloxacin (OFX), 30 µg cefazolin (KZ), 30 µg cephalothin (KF), and 30 µg cefotaxime (CTX). Multidrug resistance was defined as resistance to at least three classes of antibiotics.

Statistical analysis

Statistical analysis was performed with SPSS version 11.5 software. Comparison of proportions and statistical significance were calculated using the two-tailed Chi-square test.

Results

During the eight-year study period, a total of 1,652 isolates received were confirmed as *Shigella* and serotyped. The species distribution over time is shown in Figure 1. *S. sonnei* was the most prevalent serogroup (n = 943, 57.1%), followed by *S. flexneri* (n = 698, 42.3%), *S. dysenteriae* (n = 7, 0.4%), and *S. boydii* (n = 4, 0.2%). The highest number of infections were recorded in 2010, with 322 isolations, whereas the number of isolations decreased to below 300 in other years, with obvious fluctuations. *S. flexneri* was the most common species isolated in 2004 (69.4%), 2005 (73.8%), 2006 (60.7%), and 2008 (50.7%). In 2007, a shift occurred where *S. sonnei* (57.9%) replaced *S. flexneri* (40.4%) as the most prevalent species. Subsequently, the relative importance of *S. sonnei* gradually increased, and it accounted for 85.0% of the dominant species instead of *S. flexneri* (14.5%) in 2011.

The trends in 19 serotypes of *S. flexneri* are described in Figure 2. The seven most frequently isolated serotypes (in order of prevalence: 4c, 2a, 1a, 2b, 5b, var x, 4) were responsible for 94.1% of all *S. flexneri* episodes, whereas any other serotype was

Figure 1. Distribution of Shigella species from 2004 to 2011 in Beijing

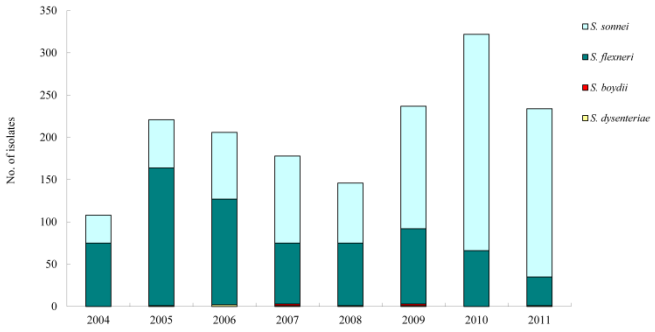
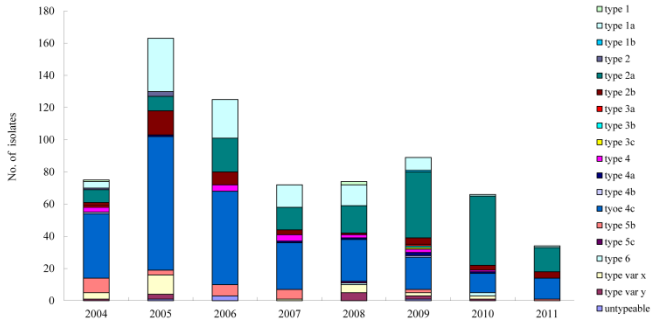


Figure 2. Distribution of Shigella flexneri serotype from 2004 to 2011 in Beijing



present in less than 1.0%. For three major serotypes – 4c, 1a, and 2a – the former two showed a pronounced decrease with occasional inter-annual variations, while the third had an obvious fluctuation over time, with peaks in 2009 and 2010. The other infrequently observed serotypes, such as 2b, 4, 5b, var x and so on, varied in different years. Statistically significant shifts in the relative proportions of *S. flexneri* serotypes were observed during the period.

The detection of the virulence genes from 274 *Shigella* strains revealed that *ipa*H (96.3%) was the most frequent, followed by *sen* (85.8%) and *set* (46.0%) (Supplementary Table 1). Of note, all the strains positive for *set* were observed with the presentence of both *ipa*H and *sen*. Of the 274 strains, 43.1% were found to be positive for *ipa*H+*set*+*sen*+, 85.8% for *ipa*H+*sen*+, 46.0% for *ipa*H+*set*+, and 43.1% for *set*+*sen*+. The prevalence of the three virulence genes among diverse *Shigella* spp. was analyzed. The *ipa*H gene was detected in 97.0% of 135 *S. flexneri* isolates, 99.2% of 133 *S. sonnei* isolates, one of four *S. boydii* isolates, and none of two *S. dysenteriae* isolates. The *set* gene was present in 88.1% of *S. flexneri* isolates and in 5.3% of *S. sonnei* isolates, whereas it was absent in *S. boydii* and *S. dysenteriae* isolates. The *sen* gene was present in 88.9% of *S. flexneri* isolates, 85.7% of *S. sonnei* isolates, one *S. boydii* isolate, and none of the *S. dysenteriae* isolates. The *set* gene showed the difference of distribution in two dominant serogroups, *S. flexneri* (88.1%) and *S. sonne* (5.3%) ($p < 0.001$). As for *ipa*H and *sen*, there were no statistically significant differences in different *Shigella* species ($p > 0.05$).

Antimicrobial drug testing was carried out on 872 *Shigella* isolates, including 495 *S. sonnei*, 369 *S. flexneri*, as well as three *S. dysenteriae* and three *S. boydii* isolates (Supplementary Table 2). Resistance to one or more drugs was observed in 98.9% of the 872 isolates. The highest resistance was detected for NAL (90.7%), followed by TET (89.9%), AMP (86.9 %), and SXT (81.5%). Moderate resistance of *Shigella* isolates to GEN (49.2%) also was detected; however, resistance to NOR or OFX was relatively low (9.9% and 9.7%, respectively).

The resistance to individual antimicrobials varied by species. The majority of *S. sonnei* isolates were resistant to NAL (96.4%), SXT (93.8%), or TET (93.4%), whereas the resistance was lower among *S. flexneri* isolates (83.7%, 65.9%, and 85.9%, respectively). Furthermore, the differences of resistance to these agents was statistically significant ($p < 0.001$). For GEN, 78.5% of *S. flexneri* had high level of resistance, as did only 10.3% of *S. sonnei* isolates. *S. flexneri* showed more resistance to AMC, CIP, NOR, and OFX compared to *S. sonnei* (18.4%–40.7% versus 3.0%–8.0%, $p < 0.001$), but *S. sonnei* tended to demonstrate an intermediate level of resistance to KF, KZ, and CTX, higher than *S. flexneri* did (22.7%–46.5% versus10.8%–16.3%, $p < 0.001$) (Supplementary Table 2).

In addition, multidrug resistance (MDR) is also important to consider. In this study, 90.8% of *Shigella* isolates were resistant to at least three antimicrobial drug subclasses, 82.2% were resistant to four, 70.4% were resistant to five, and 39.7% were resistant to six. Among many different patterns, the five most predominant resistance patterns were AMP/NAL/TET (78.4%), NAL/SXT/TET (76.4%), AMP/SXT/TET (72.2%), AMP/NAL/SXT (71.6%), and AMP/NAL/SXT/TET (70.5%) (Supplementary Table 3). There were also notable differences in MDR phenotypes between *S. sonnei* and *S. flexneri*. The dominant pattern AMP/NAL/TET was more common among *S. sonnei* than among *S. flexneri* (83.1% versus 72.4%, $p < 0.001$). For *S. sonnei*, the most common phenotype was NAL/SXT/TET, which was much higher than for *S. flexneri* (90.5% versus 58.3%, $p < 0.001$).

Discussion

Shigellosis is the most important cause of diarrhea worldwide, especially in underdeveloped and developing nations with substandard hygiene and poor quality water supplies. In this study, we found an unexpectedly complex landscape of circulating *Shigella* strains in Beijing. *S. flexneri* was the most common of the four species from 2004 to 2006, whereas *S. sonnei* has replaced *S. flexneri* as the most prevalent serogroup since 2009. These findings differ from those of studies conducted in developing countries [9] and other provinces in China [10], where *S. flexneri* was still the most frequently isolated species; however, our results match the findings in developed countries [2,11] and Thailand [12], which is rapidly becoming industrialized. The reason for this change is not known, but improvement of environmental conditions, hygiene habits, and water supplies should be considered. The significant increase in percentage of *S. sonnei* accompanied by the obvious reduction in *S. flexneri* may reflect that Beijing has undergone considerable socioeconomic changes during the period, with the infection pattern having

changed from that of a developing city to that of a developed city.

Amongst the *S. flexneri* isolates, there were a surprisingly wide range of 19 serotypes, with 4c and 2a being the most prevalent serotypes. *S. flexneri* 2a is still the most common in many developing countries, including China [1,4,12]. It should be noted that *S. flexneri* serotype 4c has remained in continuous existence over time in Beijing, although it has rarely been reported in other studies. We found statistically significant shifts in *S. flexneri* serotypes between observation years, consistent with previous works in India and Chile [13,14]. The variety in *Shigella* species and serotypes shows high heterogeneous characteristics in temporal distribution, which further emphasizes the importance of identifying isolates to serotype level for the implementation of effective control strategies.

In the present study, the prevalence of *ipa*H was independent of the four different species of *Shigella* tested, confirming previous works that the gene is highly conserved in various serotypes [9]. The *set* and *sen* genes were found to be widely distributed in various *S. flexneri* serotypes, unlike in previous studies where they were almost exclusively found in *S. flexneri* 2a isolates and rarely in other species or serotypes [5-8,15]. Overall, the present study detected *ipa*H, *set*, and *sen* in all the prevalent serotypes of *S. flexneri*; however, the presentence of *set* in *S. sonnei* was much lower than in *sen* and *ipa*H. Working on temporal variations of diverse species, our results enhance knowledge on the prevalence and distribution of these genes, which might assist in developing markers for *Shigella* of different types.

This study demonstrated the drastically increasing spectrum of antimicrobial resistance in *Shigella* isolates. All *Shigella* species also exhibited a very high rate of resistance (80%–90%) to older generation antimicrobials such as NAL, TET, AMP, and SXT. Although a similar pattern of resistance has been reported in our region and in other Asian countries [4,16,17], the frequency of resistance reported here is higher than frequencies observed earlier, and the trend to MDR is more pronounced [3,4]. Cephalosporins and fluoroquinolones were two popular empirical options to treat severe gastrointestinal infections caused by pathogenic bacteria. Our finding of a 17.8% resistance rate to the third-generation cephalosporins (CTX) was in agreement with observations from India and Cameroon, where resistance rates of 12% and 20%, respectively, were reported [16,18]. Additionally, our study found a 10% resistance rate to other fluoroquinolones such as CIP, NOR, and OFX. It has been noted that in other countries with strict antimicrobial controls (*e.g.*, the United States), less than 1% of *Shigella* isolates were resistant to cephalosporins and fluoroquinolones [19]. In China, the use of antimicrobial drugs has been poorly regulated and the indiscriminate overuse of antibiotics remains a serious issue that might be responsible for the increase of multiple antimicrobial resistance at an alarming speed.

In the present study, we found that *S. sonnei* was more frequently resistant to NAL, TET, and SXT, alone or in combination, than was *S. flexneri.* Other studies showed that *S. sonnei* had lower rates of resistance, compared with *S. flexneri,* to most of the antibiotics [19]. A possible explanation is that infections due to *S. sonnei* are becoming more and more common than infections due to the other *Shigella* species in Beijing, making exposure to selective pressure from antibiotics more likely.

In summary, this study provides initial data on the prevalence and distribution of *ipa*H, *set,* and *sen* genes in a wide variety of *Shigella* isolates over an eight-year period. *S. sonnei* has replaced *S. flexneri* as a dominant species with high rates of multidrug resistances in Beijing. Continuous local monitoring of resistance patterns is necessary for effective therapy and control measures against shigellosis.

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Corresponding author

Quanyi Wang
Department of Infectious and Endemic Diseases Control
Beijing Centre for Disease Prevention and Control
No. 16, He Ping Li Middle Street, Beijing, 100013, China
Phone: +86-10-64407109
Fax: +86-10-64407113
Email: bjcdcxm@126.com

Conflict of interests: No conflict of interests is declared.

Supplementary Items

Supplementary Table 1. Prevalence of three virulence genes in *Shigella* strains by species

Genotype	No. of isolates (%) positive to virulence genes					*P**
	Total (n = 274)	*S. dysenteriae* (n = 3)	*S. boydii* (n = 3)	*S. flexneri* (n = 135)	*S. sonnei* (n = 133)	
*ipa*H	264 (96.3)	0	1 (33.3)	131 (97.0)	132 (99.2)	0.181
set	126 (46.0)	0	0	119 (88.1)	7 (5.3)	< 0.001
sen	235 (85.8)	0	1 (33.3)	120 (88.9)	114 (85.7)	0.435
*ipa*H_*set*	126 (46.0)	0	0	119 (88.1)	7 (5.3)	< 0.001
*ipa*H_*sen*	235 (85.8)	0	1 (33.3)	120 (88.9)	114 (85.7)	0.435
set_sen	118 (43.1)	0	0	111 (82.2)	7 (5.3)	< 0.001
*ipa*H_*set_sen*	118 (43.1)	0	0	111 (82.2)	7 (5.3)	< 0.001

*Chi-square test was calculated by two predominant serotypes, *S. flexneri* and *S. sonnei*; *S. dysenteriae* and *S. boydii* were not included because of too few cases.

Supplementary Table 2. Antimicrobial resistance of *Shigella* strains by species

Antimicrobial agent	No. of isolates (%) resistant to various antimicrobial agent					*P**
	Total (n = 872)	*S. dysenteriae* (n = 3)	*S. boydii* (n = 3)	*S. flexneri* (n = 369)	*S. sonnei* (n = 497)	
NAL	791 (90.7)	2 (66.6)	1 (33.3)	309 (83.7)	479 (96.4)	< 0.001
TET	784 (89.9)	1 (33.3)	2 (66.6)	317 (85.9)	464 (93.4)	< 0.001
AMP	758 (86.9)	2 (66.6)	1 (33.3)	324 (87.8)	431 (86.7)	0.637
SXT	711 (81.5)	1 (33.3)	1 (33.3)	243 (65.9)	466 (93.8)	< 0.001
GEN	429 (49.2)	0	1 (33.3)	38 (10.3)	390 (78.5)	< 0.001
KF	294 (33.7)	2 (66.6)	1 (33.3)	60 (16.3)	231 (46.5)	< 0.001
KZ	203 (23.3)	2 (66.6)	1 (33.3)	49 (13.3)	151 (30.4)	< 0.001
AMC	190 (21.8)	0	0	150 (40.7)	40 (8.0)	< 0.001
CTX	155 (17.8)	1 (33.3)	1 (33.3)	40 (10.8)	113 (22.7)	< 0.001
CIP	93 (10.7)	2 (66.6)	0	82 (22.2)	9 (1.8)	< 0.001
NOR	86 (9.9)	2 (66.6)	0	75 (20.3)	9 (1.8)	< 0.001
OFX	85 (9.7)	2 (66.6)	0	68 (18.4)	15 (3.0)	< 0.001

*Chi-square test was calculated by two predominant serotypes, *S. flexneri* and *S. sonnei*; *S. dysenteriae* and *S. boydii* were not included because of too few cases.

Supplementary Table 3. Predominant multidrug resistance patterns of *Shigella* strains by species

Predominant resistance pattern	No. of isolates (%) resistant to multidrug					*P**
	Total (n = 872)	*S. dysenteriae* (n = 3)	*S. boydii* (n = 3)	*S. flexneri* (n = 369)	*S. sonnei* (n = 497)	
3 antibiotics	792 (90.8)	2 (66.7)	1 (33.3)	316 (85.6)	473 (95.2)	< 0.001
AMP,NAL,TET	682 (78.2)	1 (33.3)	1 (33.3)	267 (72.4)	413 (83.1)	< 0.001
NAL,SXT,TET	666 (76.4)	0	1 (33.3)	215 (58.3)	450 (90.5)	< 0.001
AMP,SXT,TET	630 (72.2)	0	1 (33.3)	220 (59.6)	409 (82.3)	< 0.001
AMP,NAL,SXT	624 (71.6)	1 (33.3)	1 (33.3)	213 (57.7)	409 (82.3)	< 0.001
4 antibiotics	717 (82.2)	2 (66.7)	1 (33.3)	282 (76.4)	432 (86.9)	< 0.001
AMP,NAL,SXT,TET	615 (70.5)	0	1 (33.3)	207 (56.1)	407 (81.9)	< 0.001
AMP,GEN,NAL,TET	414 (47.5)	0	1 (33.3)	32 (8.7)	381 (76.7)	< 0.001
5 antibiotics	614 (70.4)	2 (66.7)	1 (33.3)	193 (52.3)	418 (84.1)	< 0.001
AMP,GEN,NAL,SXT,TET	408 (46.8)	0	1 (33.3)	27 (7.3)	380 (76.5)	< 0.001
6 antibiotics	346 (39.7)	2 (66.7)	1 (33.3)	105 (28.5)	238 (47.9)	< 0.001
AMP,KF,GEN,NAL,SXT,TET	201 (23.1)	0	1 (33.3)	12 (3.3)	188 (37.8)	< 0.001

*Chi-square test was calculated by two predominant serotypes, *S. flexneri* and *S. sonnei*; *S. dysenteriae* and *S. boydii* were not included because of too few cases.