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ORIGINAL RESEARCH

# Virulence-Related Gene Distribution Among *Shigella* Isolates in Anhui, China: The Association with Antimicrobial Resistance

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**Objective:** The aim of this study was to investigate the antimicrobial resistance profiles and distribution of virulence-related genes (VRGs) among *Shigella* isolates in Anhui, China, and to identify the correlation between the VRGs and antimicrobial resistance.

**Materials and Methods:** A total of 525 non-duplicate *Shigella* isolates (449 *S. flexneri*, 68 *S. sonnei*, 3 *S. boydii*, and 5 *S. dysenteriae*) were collected in Anhui Province, China between September 2011 and September 2015. The antimicrobial resistance of the strains was determined by the agar dilution method according to CLSI guidelines. The presence of 16 VRGs, including *ipaH, ipaA-D, ial, virB, virF, set, sen, icsA, icsB, sigA, sat, pic*, and *sepA*, was evaluated using PCR amplification and sequencing.

**Results:** *Shigella flexneri* was the most abundant (85.5%), followed by *S. sonnei* (13.0%). The proportion of males with *S. flexneri* was higher than that of females (57% *vs* 43%; $P<0.0001$). The most common resistance pattern was the combination of ampicillin, nalidixic acid, and tetracycline for *S. flexneri* (90.2%) and *S. sonnei* (94.1%). Resistance to ciprofloxacin and levofloxacin was more common among *S. flexneri* than among *S. sonnei* (49.7% *vs*.19.1%, $P<0.0001$; 30.5% *vs* 10.3%, $P=0.001$, respectively). All the isolates were positive for the *ipaH* gene, while the *set, sat, pic*, and *sepA* genes were not detected among the *S. sonnei* isolates. Except for *sigA* and *sen*, resistance to chloramphenicol and ciprofloxacin was more common among VRG-positive *S. flexneri* than among VRG-negative *S. flexneri* ($P<0.05$). Furthermore, resistance to ceftriaxone and ceftazidime was more frequently detected among *sat*- and *set*-positive *S. flexneri* than among *sat*- and *set*-negative *S. flexneri* ($P<0.05$). However, gentamicin resistance was more prevalent among VRG-negative (*ial, virF, set, sat, pic*, and *sepA*) *S. flexneri* than among VRG-positive *S. flexneri* ($P<0.05$).

**Conclusion:** *Shigella flexneri* remains the predominant species in Anhui, China, and the resistance to fluoroquinolones was more widespread among *S. flexneri* than among *S. sonnei*. *Shigella flexneri* strains harboring specific VRGs were associated with antimicrobial resistance. To the best of our knowledge, this is the first report of the correlation between the VRGs and antimicrobial resistance in Anhui, China.

**Keywords:** antimicrobial resistance, *Shigella flexneri*, *Shigella sonnei*, virulence-related gene, type III secretion system

## Introduction

Shigellosis is one of the most important causes of diarrhea worldwide.[1] The annual number of deaths due to shigellosis has been estimated at 1.1 million, and shigellosis-associated mortality is particularly prevalent among children in developing

countries.[2] Shigellosis can be caused by four *Shigella* species: *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei. Shigella sonnei* is the predominant serogroup in developed countries, while *S. flexneri* is the main serogroup found in developing countries.[3] However, the frequency of *S. sonnei* has recently increased in several areas that have undergone rapid socioeconomic improvements.[4,5]

Antimicrobial agents have been increasingly effective in alleviating the dysenteric syndrome associated with shigellosis.[1] However, the effectiveness of antimicrobial agents has become limited due to the emergence of multidrug resistance (MDR).[3] Antimicrobial resistance among *Shigella* species, including resistance to fluoroquinolones and third-generation cephalosporins, is prevalent in China.[6–8] Moreover, antimicrobial resistance differs among regions.[9] Consequently, it is important to understand the nature of the local antimicrobial resistance when choosing an appropriate antibiotic for the treatment of shigellosis.

The type III secretion system (T3SS) of *Shigella* has been reported to be associated with the pathogenesis of shigellosis, and is regulated by multiple virulence-related genes (VRGs). The differential distribution of VRGs among *Shigella* isolates can lead to different clinical manifestations.[10,11] Invasion plasmid antigen (*ipa*) genes and invasion associated locus (*ial*) are important mediators of *Shigella* cell invasion in the intestinal epithelium.[12] *Shigella* also produces distinct enterotoxins such as *Shigella* enterotoxin 1 (ShET-1), encoded by the *set* gene, and *Shigella* enterotoxin 2 (ShET-2), encoded by the *sen* gene.[10] VirF and VirB (InvE) are transcriptional activators of plasmid-bourne genes that control the expression of invasion-related genes.[13] The *icsA* gene is the major determinant of *Shigella* actin-based motility, and has been shown to interact with *icsB* through the prevention of autophagic recognition of *icsA*.[14] Finally, the genes encoding serine protease autotransporters of Enterobacteriaceae (SPATE) are phylogenetically classified into two main classes: class 1 SPATE genes, comprising the *Shigella* IgA-like protease homolog (*sigA*) and secreted autotransporter toxin (*sat*); and class 2 SPATE genes, comprising *Shigella* extracellular protein A (*sepA*) and protease involved in intestinal colonization (*pic*).[15] Overall, *Shigella* isolates harboring these VRGs can induce inflammation and extensive mucosal damage during intestinal infections, especially when the isolates encode more than one VRG. Furthermore, the detection of additional VRGs in *Shigella spp*. together with the concomitant increase in resistance against more antimicrobials suggests that there is a link between virulence and antimicrobial resistance of shigellosis.[11] Hence, understanding the distribution of VRGs in *Shigella* isolates is useful for designing new antimicrobial therapies based on gene targets.

To the best of our knowledge, this is the first report of the VRG profiles of *Shigella* isolates in Anhui, China. The aim of this study was to investigate the antimicrobial resistance profiles and VRG distribution among *Shigella* isolates in Anhui, China, and identify the correlation between the VRGs and antimicrobial resistance to help guide the treatment of shigellosis.

# Materials and Methods

## Bacterial Isolates and Serotyping

A total of 525 non-duplicate *Shigella* isolates (449 *S. flexneri*, 68 *S. sonnei*, 3 *S. boydii*, and 5 *S. dysenteriae*) were collected from the stool samples of patients in Anhui Province, China between September 2011 and September 2015. Individual isolates were identified through standard microbiological and biochemical methods. All *Shigella* isolates were confirmed *via* the VITEK 2 Compact system (bioMérieux, Marcy l' Étoile, France) and serotyped using commercially available antisera (Denka Seiken Co., Ltd, Tokyo, Japan). *Escherichia coli* ATCC 25922 was stored at the Anhui Center for the Surveillance of Bacterial Resistance (Hefei, Anhui, China). All clinical samples were part of the routine hospital laboratory procedure.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki, the principles of Good Clinical Practice, and Chinese regulatory requirements, and was approved by the local Ethics Committees of the First Affiliated Hospital of Anhui Medical University (Hefei, China). All patients gave written informed consent, and parental/legal guardian informed consent was obtained for patients under the age of 18.

## Antimicrobial Susceptibility Testing

Bacteria were cultured to the log-phase (approximately $1.5 \times 10^8$ CFU/mL). The minimum inhibitory concentrations (MICs) of the following 17 antimicrobial agents were determined by the agar dilution method using Mueller-Hinton agar (Oxoid Ltd, Basingstoke, UK) containing a series of twofold diluted antibiotics: ampicillin (AMP), nalidixic acid (NAL), tetracycline (TCY), chloramphenicol (CHL), trimethoprim/sulfamethoxazole (SXT), cefotaxime (CTX),

ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), cefoxitin (FOX), ciprofloxacin (CIP), levofloxacin (LEV), gentamicin (GEN), amikacin (AMK), piperacillin–tazobactam (TZP), fosfomycin (FOS), and imipenem (IMP) (all from Sigma–Aldrich, Shanghai, China). The plates containing the bacterial colonies and the antibiotics were incubated at 37 °C for 18–20 h. The results were interpreted according to CLSI breakpoints published in 2019. *Escherichia coli* ATCC 25922 cells were used as the quality control strain. The antimicrobial susceptibility of the *Shigella* isolates was accepted only if the MICs of the quality control strains that were tested in parallel were within the acceptable ranges set by the CLSI guidelines (2019). MDR was defined as resistance to three or more antimicrobial agents.

## DNA Extraction and VRG Detection

Bacterial DNA was extracted using the boiling method and plasmid DNA was extracted using the Qiagen Plasmid Purification Kit (QIAGEN, Hilden, Germany). The 16 VRGs (*ipaH, ipaA-D, ial, virB, virF, set, sen, icsA, icsB, sigA*, sat, *pic*, and *sepA*) of the *Shigella* isolates were identified by polymerase chain reaction (PCR). The primer sequences are shown in Table 1. The cycling conditions included polymerase activation, then an initial denaturation at 95 °C for 5min, followed by 30 cycles of denaturation at 95 °C for 45s, annealing for 45 s (annealing temperatures are shown in Table 1), and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. All the PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) and directly sequenced. Sequences were compared with those of the GenBank nucleotide database to identify the VRGs.

## Statistical Analysis

Statistical analysis focused on the relationships between *Shigella* serotype and antimicrobial resistance patterns, and the relationships between VRG profiles and antimicrobial resistance. Data were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Univariate analysis was performed by the chi-square test or Fisher's exact test when appropriate. *P*-values were based on two-tailed test results, and *P*-values <0.05 were considered statistically significant.

# Results

## Distribution of *Shigella* Isolates

A total of 525 non-duplicate *Shigella* isolates were collected between September 2011 and September 2015. *Shigella flexneri* was the most abundant (*n*=449, 85.5%), followed by *S. sonnei* (*n*=68, 13.0%). *Shigella dysenteriae* (*n*=5) and *S. boydii* (*n*=3) were relatively uncommon. Figure 1 shows the yearly distribution of the *Shigella* isolates. *Shigella flexneri* was the most frequently isolated species each year. The proportion of *S. sonnei* isolates increased yearly. Two *S. dysenteriae* were isolated in 2013 and one in each of 2011, 2012, and 2014. One *S. boydii* was identified in each of 2012, 2014, and 2015.

## Age and Gender

*Shigella* isolates were collected from specimens from all age groups that presented bacillary dysentery. The median age was 1 year (range: 4 months to 97 years). Infants and children between 1 and 4 years of age accounted for the highest proportion of *Shigella* isolates (*n*=331, 63.1%), followed by 5–19-year-old patients (*n*=72, 13.7%), patients >60 years of age (*n*=64, 12.2%), and patients between 20 and 59 years of age (*n*=58, 11.0%) (Figure 2A). *Shigella* species showed the greatest male predominance. The proportion of *S. flexneri* was higher in males than in females (57% *vs* 43%; *P*<0.0001). There was no significant difference in the proportion of *S. sonnei* between male and female patients (47.1% *vs* 53.9%; *P*=0.49) (Figure 2B).

## Antimicrobial Resistance

A comparison of the resistance rates among the *Shigella* isolates is shown in Table 2. *Shigella flexneri* was most commonly resistant to AMP (97.3%), followed by NAL (96.9%), TCY (93.8%), and CHL (86.6%). *Shigella sonnei* was most commonly resistant to AMP (100%), followed by NAL (98.5%), TCY (94.1%), SXT (92.6%), and GEN (92.6%). *Shigella flexneri* was most sensitive to TZP (99.3%), followed by IPM (98.2%) and AMK (98%). *Shigella sonnei* was most sensitive to TZP (100%) and AMK (100%), followed by IPM (98.5%) and FEP (98.5%).

Among the 449 *S. flexneri* isolates, 417 (92.9%) displayed MDR, and the most common resistance pattern was AMP–NAL–TCY (90.2%). Among the 68 *S. sonnei* isolates, 66 (94.1%) exhibited MDR, and the most common resistance pattern was AMP–NAL–TCY (92.6%).

Resistance to CHL, CIP, and LVX was more common among *S. flexneri* isolates than among *S. sonnei* isolates (86.6% *vs* 8.8%, *P*<0.0001; 49.7% *vs*.19.1%, *P*<0.0001; 30.5% *vs* 10.3%, *P*=0.001, respectively). However, resistance to STX and GEN was more common among *S. sonnei* isolates than among *S. flexneri*

**Table 1** Sequences of the Primers Used for PCR Amplification

| Target Gene | Primer | Sequence (5′–3′) | Annealing Temperature | Size (bp) |
|---|---|---|---|---|
| *ipaH* | *ipaH*-F<br>*ipaH*-R | TGGAAAAACTCAGTGCCTCT<br>CCAGTCCGTAAATTCATTCT | 57 °C | 423 |
| *ipaA* | *ipaA*-F<br>*ipaA*-R | CCTGTGTCCCCGAGAAAGAGA<br>TGACGCACAGGCAAAACTTG | 60 °C | 628 |
| *ipaB* | *ipaB-F*<br>*ipaB-R* | CAAGCCCTGAATCCGATCAT<br>TGCTGCTGCCTGTTTACCAA | 60 °C | 204 |
| *ipaC* | *ipaC*-F<br>*ipaC*-R | CCTCACCACAAACTAACTCTAGCA<br>GAGAAGTTTTATGTTCAGTTGACAGGGATA | 60 °C | 93 |
| *ipaD* | *ipaD*-F<br>*ipaD*-R | AAGAAGCCGAGCTTGATGGAG<br>CCTCGCCATTTCCACCTAGA | 60 °C | 450 |
| *ial* | *ial*-F<br>*ial*-R | CTGGATGGTATGGTGAGG<br>GGAGGCCAACAATTATTTCC | 57 °C | 320 |
| *virB* | *virB*-F<br>*virB*-R | CGCGCGAGACAGATTCTCTT<br>TGGTGGATTTGTGCAACGAC | 60 °C | 488 |
| *virF* | *virF*-F<br>*virF*-R | AGCTCAGGCAATGAAACTTTGAC<br>TGGGCTTGATATTCCGATAAGTC | 57 °C | 618 |
| *set* | *set*-F<br>*set*-R | TCCCTTCATACTGGCTCCTG<br>AACACTCTGGGGGGAACAG | 57 °C | 553 |
| *sen* | *sen*-F<br>*sen*-R | ATCTCCTTGAGGCCAGCAAA<br>GGAAGGAATGGGAGGACGAA | 58 °C | 296 |
| *icsA* | *icsA*-F<br>*icsA*-R | CCAACCCCTCTCATGCAT<br>ATCACCAGCACCACCATGAC | 60 °C | 83 |
| *icsB* | *icsB*-F<br>*icsB*-R | GGCCTGCATCAAGTCTTTCG<br>GGCATCGGTACAGCCAAAAA | 60 °C | 280 |
| *sigA* | *sigA*-F<br>*sigA*-R | ACCGACTTCTCACTTTCTCCCG<br>CCATCCAGCTGCATAGTGTTTG | 58 °C | 430 |
| *sat* | *sat*-F<br>*sat*-R | TCAGAAGCTCAGCGAATCATTG<br>CCATTATCACCAGTAAAACGCACC | 59 °C | 930 |
| *pic* | *pic*-F<br>*pic*-R | ACTGGATCTTAAGGCTCAGGAT<br>GACTTAATGTCACTGTTCAGCG | 58 °C | 570 |
| *sepA* | *sepA*-F<br>*sepA*-R | GCAGTGGAAATATGATGCGGC<br>TTGTTCAGATCGGAGAAGAACG | 58 °C | 789 |

isolates (76.2% *vs* 92.6%, $P<0.0001$; 28.7% *vs* 92.6%, $P<0.0001$, respectively).

## Prevalence of VRG

### Invasion-Associated Genes

The detection of invasion-associated genes among the 449 *S. flexneri* isolates showed that *ipaH* had the highest frequency (100%), followed by *ipaB* (99.8%), *ipaC* (98.7%), *ipaD* (98.7%), *ipaA* (98.4%), and *ial* (96.0%) (Table 3). The detection of invasion-associated genes among the 68 *S. sonnei* isolates showed that *ipaH* had the highest frequency (100%), followed by *ipaB* (98.5%), *ipaC* (97.1%), *ipaD* (95.6%), *ipaA* (95.6%), and *ial* (75.0%) (Table 3).

### Regulatory Genes

Among the 449 *S. flexneri* isolates, 439 (97.8%) were positive for the *virB* gene, while 431 (96.0%) were positive for the *virF* gene (Table 3). Moreover, a total of 413

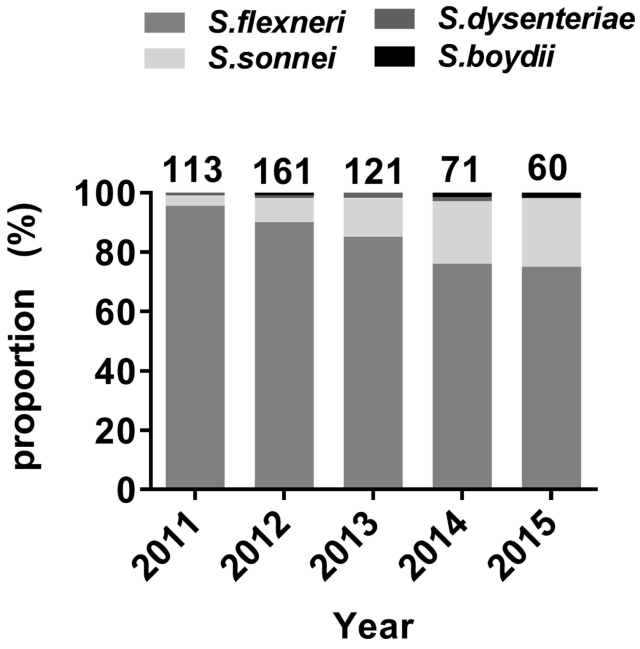


**Figure 1** The yearly distribution of the proportions of the *Shigella* isolates.

isolates (92.0%) were positive for both genes. Among the 68 *S. sonnei* isolates, 66 (97.1%) were positive for the *virB* gene, while 51 (75.0%) were positive for the *virF* gene (Table 3). Fifty (73.5%) isolates were positive for both genes.

Among the 449 *S. flexneri* isolates, 448 (99.8%) were positive for the *icsA* gene, while 439 (97.8%) were positive for the *icsB* gene (Table 3). A total of 437 (97.3%) isolates were positive for both genes. Among the 68 *S. sonnei* isolates, 67 (98.5%) were positive for the *icsA* gene and 66 (97.1%) for the *icsB* gene (Table 3). Moreover, a total of 63 (91.3%) isolates were positive for both genes.

### SPATEs

Among the 449 *S. flexneri* isolates tested, 421 (93.8%) contained class I (*sigA, sat*) and/or class II (*sepA, pic*) SPATE genes (Table 3). Moreover, a total of 415 (92.4%) had two class I SPATE genes, and 394 (87.8%) had both class II SPATE genes. The most common SPATE gene among *S. flexneri* isolates was *sat* (93.8%), followed by *sigA* (92.4%), *pic* (88.2%), and *sepA* (87.8%). Among the 68 *S. sonnei* isolates, 64 (94.1%) harbored only one class I SPATE gene (*sigA*) (Table 3). The *sat, pic*, and *sepA* genes were not detected among the *S. sonnei* isolates in this study.

### Enterotoxin Genes

The *set* gene was present in 420 of the 449 (93.5%) *S. flexneri* isolates, while the *sen* gene was present in 413 (92.0%) of the *S. flexneri* isolates and 60 of the 68 (88.2%) *S. sonnei* isolates. The *set* gene was not detected among the *S. sonnei* isolates in this study.

## VRGs and Antimicrobial Resistance

Resistance to CHL and CIP was more common among VRG-positive (*ial, ipaA, ipaC, ipaD, virB, virF, set, icsB, sat, pic*, and *sepA*) *S. flexneri* isolates than among VRG-negative *S. flexneri* isolates ($P<0.05$) (Figures 3 and 4). Furthermore, resistance to CRO and CAZ was more common among *sat*- and *set*-positive *S. flexneri* isolates than among *sat*- and *set*-negative *S. flexneri* isolates ($P<0.05$) (Figure 3E, F). However, CEN resistance was more frequent

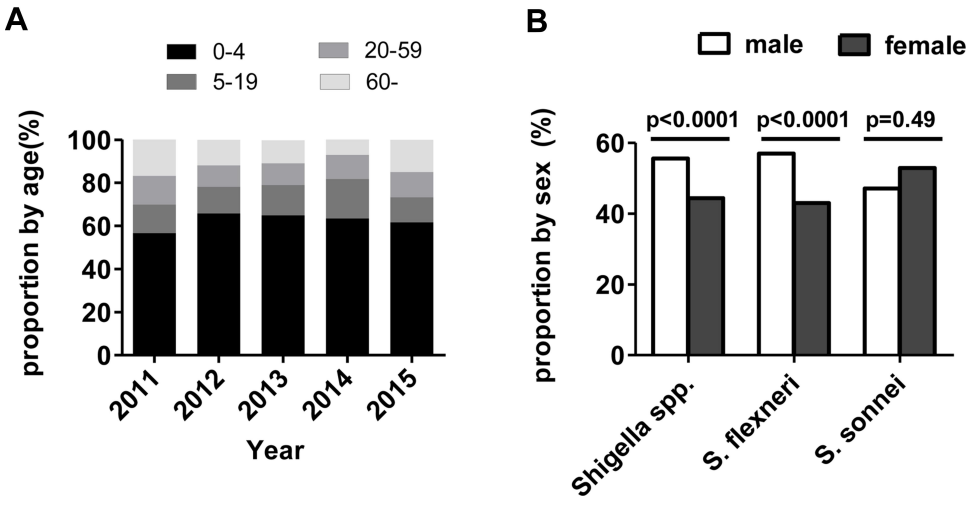


**Figure 2** Age and gender distribution of *Shigella* isolates in China from 2011 to 2015. (**A**) The distribution of the proportions of *Shigella* isolates by age group. (**B**) Comparison of the proportion of *S. flexneri* and *S. sonnei* by gender group.

**Table 2** $MIC_{50}$ and $MIC_{90}$ of 17 Antimicrobial Agents Against *S. Flexneri* and *S. Sonnei*

| Antimicrobial Agent | *S. flexneri* (*n*=449) | | | R | *S. sonnei* (*n*=68) | | | R | $x^2$ | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC Range | $MIC_{50}$ | $MIC_{90}$ | | MIC Range | $MIC_{50}$ | $MIC_{90}$ | | | |
| | (μg/mL) | (μg/mL) | (μg/mL) | (%) | (μg/mL) | (μg/mL) | (μg/mL) | (%) | | |
| AMP | 0.5 – >256 | >256 | >256 | 97.3 | 32 – >256 | >256 | >256 | 100 | | |
| NAL | 1 – >256 | >256 | >256 | 96.9 | 1 – >256 | 128 | >256 | 98.5 | | |
| TCY | 0.25 – >128 | 128 | >128 | 93.8 | 1 – >128 | 64 | >128 | 94.1 | | |
| CHL | 0.5 – >256 | 64 | 128 | 86.6 | 1 – >256 | 4 | 16 | 8.8 | 198.334 | <0.0001* |
| SXT | 0.125 – >32 | 16 | 32 | 76.2 | 0.125–32 | 16 | 32 | 92.6 | 9.449 | 0.002* |
| CTX | 0.0625 – >32 | 16 | 64 | 53 | 0.0625 – >32 | 16 | >32 | 52.9 | | |
| CIP | 0.0625 – >32 | 4 | 16 | 49.7 | 0.125 – >32 | 0.25 | 16 | 19.1 | 22.213 | <0.0001* |
| CRO | 0.0625 – >32 | 0.125 | 32 | 45.7 | 0.0625 – >32 | 0.0625 | 16 | 33.8 | | |
| LVX | 0.125–64 | 4 | 16 | 30.5 | 0.125 – >64 | 0.5 | 8 | 10.3 | 12.013 | 0.001* |
| GEN | 0.25 – >128 | 1 | >128 | 28.7 | 0.5 – >128 | 64 | 128 | 92.6 | 103.345 | <0.0001* |
| FOS | 4 – >2048 | 64 | >2048 | 24.5 | 16 – >2048 | 128 | 256 | 26.5 | | |
| FEP | 0.5–256 | 1 | 32 | 21.6 | 0.5–128 | 0.5 | 32 | 19.1 | | |
| CAZ | 0.25–128 | 0.5 | 16 | 21.2 | 0.25–128 | 0.25 | 16 | 17.6 | | |
| FOX | 0.5 – >256 | 4 | 8 | 3.3 | 0.5 – >256 | 4 | 16 | 1.5 | | |
| AMK | 1 – >512 | 4 | 8 | 2 | 1–16 | 4 | 8 | 0 | | |
| IPM | 0.0625–8 | 1 | 2 | 1.8 | 0.25–16 | 0.5 | 1 | 1.5 | | |
| TZP | 1–128 | 8 | 16 | 0.7 | 2–32 | 8 | 16 | 0 | | |

**Abbreviations:** AMP, ampicillin; NAL, nalidixic acid; TCY, tetracycline; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; CIP, ciprofloxacin; LEV, levofloxacin; GEN, gentamicin; AMK, amikacin; TZP, piperacillin–tazobactam; FOS, fosfomycin; IMP, imipenem; MIC, minimal inhibitory concentration; R, resistance.
**Note:** *Chi-square test.

**Table 3** Distribution of Virulence-Related Genes Among *Shigella* Isolates in Anhui, China

| Gene | Total | | |
|---|---|---|---|
| | *S. flexneri* | *S. sonnei* | *P*-value |
| | *n*=449 | *n*=68 | |
| *ipaH* | 449 (100%) | 68 (100%) | |
| *ial* | 431 (96.0%) | 51 (75.0%) | <0.0001[#] |
| *ipaA* | 442 (98.4%) | 65 (95.6%) | |
| *ipaB* | 448 (99.8%) | 67 (98.5%) | |
| *ipaC* | 443 (98.7%) | 66 (97.1%) | |
| *ipaD* | 443 (98.7%) | 65 (95.6%) | |
| *virB* | 439 (97.8%) | 66 (97.1%) | |
| *virF* | 431 (96.0%) | 51 (75.0%) | <0.0001[#] |
| *set* | 420 (93.5%) | 0 | |
| *sen* | 413 (92.0%) | 60 (88.2%) | |
| *icsA* | 448 (99.8%) | 67 (98.5%) | |
| *icsB* | 439 (97.8%) | 66 (97.1%) | |
| *sigA* | 415 (92.4%) | 64 (94.1%) | |
| *sat* | 421 (93.8%) | 0 | |
| *pic* | 396 (88.2%) | 0 | |
| *sepA* | 394 (87.8%) | 0 | |

**Note:** [#]Fisher's exact test.

among VRG-negative (*ial, virF, set, sat, pic*, and *sepA*) *S. flexneri* isolates than among VRG-positive *S. flexneri* isolates ($P<0.05$) (Figure 3). With fewer VRGs-negative strains among *S. flexneri* (*ipaH, ipaB*, and *icsA*) and among *S. sonnei*, no correlation tests were done between these VRGs and antimicrobial resistance. The resistance rates were similar between VRG-positive *S. flexneri* and VRG-positive *S. sonnei* isolates, except for the resistance to CRO and CAZ, which was more commonly found among VRG-positive *S. flexneri* than among VRG-positive *S. sonnei* ($P<0.05$) (Supplementary Figure 1 and 2).

## Discussion

Shigellosis is an invasive bacterial infection of the human colon that manifests a spectrum of clinical presentations, ranging from short-lasting watery diarrhea to inflammatory bowel disease.[3] In this study, we explored the incidence rate of shigellosis during 2011–2015 in Anhui, China, and showed that there was an obvious decline. *Shigella flexneri* was the most commonly isolated species, similar to that previously reported for several regions in China.[16–18]

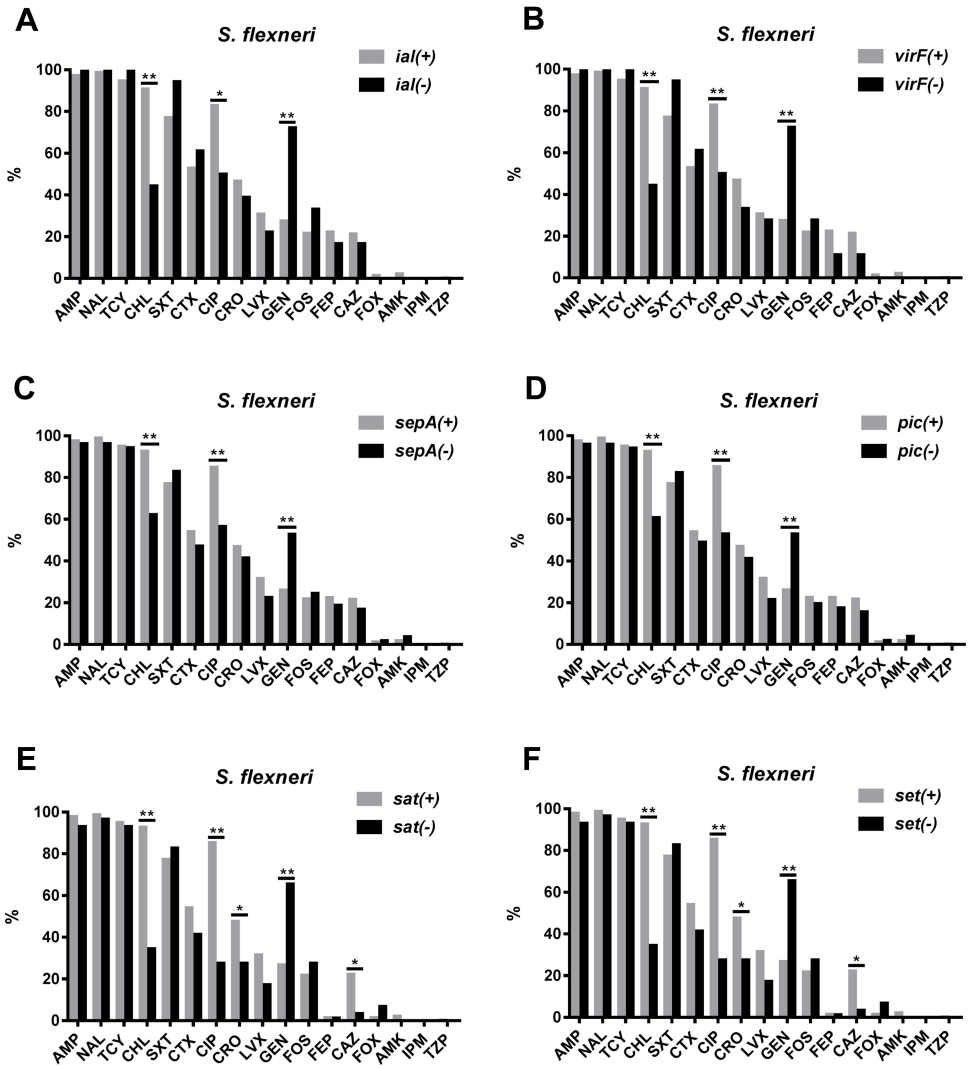


**Figure 3** Comparison of the antimicrobial resistance rates between virulence-related gene (VRG)-positive (*ial, virF, sepA, pic, sat*, and *set*) and VRG-negative *S. flexneri*. (**A**) Comparison of the antimicrobial resistance rates between *ial*-positive and *ial*-negative *S. flexneri*. (**B**) Comparison of the antimicrobial resistance rates between *virF*-positive and *virF*-negative *S. flexneri*. (**C**) Comparison of the antimicrobial resistance rates between *sepA*-positive and *sepA*-negative *S. flexneri*. (**D**) Comparison of the antimicrobial resistance rates between *pic*-positive and *pic*-negative *S. flexneri*. (**E**) Comparison of the antimicrobial resistance rates between *sat*-positive and *sat*-negative *S. flexneri*. (**F**) Comparison of the antimicrobial resistance rates between *set*-positive and *set*-negative *S. flexneri*. *, $P<0.05$; **, $P<0.001$.

However, we found that the detection rate of *S. sonnei*, which is the predominant species in developed countries, showed a clear yearly increase. This was likely due to improvements in socioeconomic levels and environmental conditions.[18,19]

In this study, shigellosis was detected in all age groups; however, children under 5 years of age had higher incidence rates, which was consistent with previous reports.[11,17,20] Children under 5 years of age are likely to be more susceptible to shigellosis because of low immune function and the lack of previous exposure.[21,22] In this study, we observed that the incidence of *S. flexneri* was markedly higher among males than among females, similar to that reported in previous studies.[16,17] One explanation may be that males have a higher risk of exposure to *Shigella*-contaminated environments, such as men having sex with men.[23] However, there was no significant difference in the incidence of *S. sonnei* between males and females, which may have been due to the small number of isolated samples.

Effective antimicrobial therapy for shigellosis can significantly relieve symptoms and interrupt bacterial shedding.[3] The emergence and dissemination of antimicrobial resistance among *Shigella* strains in recent years have complicated the therapeutic management of severe shigellosis.[6,7,24] Our data showed that *Shigella* isolates in

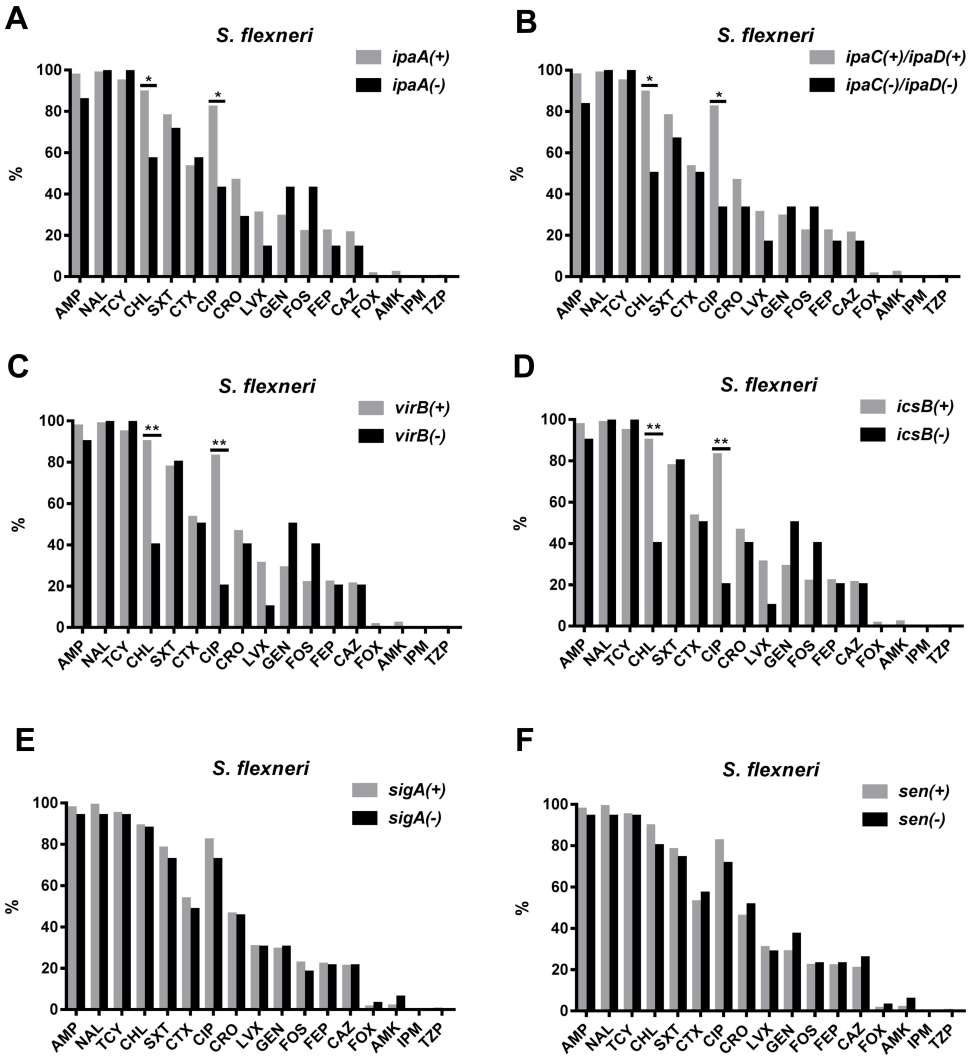


**Figure 4** Comparison of the antimicrobial resistance rates between virulence-related gene (VRG)-positive (*ipaA, ipaC, ipaD, virB, icsB, sigA*, and *sen*) and VRG-negative *S. flexneri*. (**A**) Comparison of the antimicrobial resistance rates between *ipaA*-positive and *ipaA*-negative *S. flexneri*. (**B**) Comparison of the antimicrobial resistance rates between *ipaC*/*ipaD*-positive and *ipaC*/*ipaD*-negative *S. flexneri*. (**C**) Comparison of the antimicrobial resistance rates between *virB*-positive and *virB*-negative *S. flexneri*. (**D**) Comparison of the antimicrobial resistance rates between *icsB*-positive and *icsB*-negative *S. flexneri*. (**E**) Comparison of the antimicrobial resistance rates between *sigA*-positive and *sigA*-negative *S. flexneri*. (**F**) Comparison of the antimicrobial resistance rates between for *sen*-positive and *sen*-negative *S. flexneri*. *, $P<0.05$; **, $P<0.001$.

China had a high resistance to AMP, NAL, TCY, and SXT, suggesting that these drugs can no longer be recommended as empirical therapy for shigellosis. In this study, the *Shigella* isolates were observed to be susceptible to TZP, IMP, AMK, and FOX, indicating that these antibiotics may be the preferred treatment options in Anhui, China.

In our study, the most frequently detected antimicrobial resistance pattern was AMP–NAL–TCY (90.2% in *S. flexneri*, 92.6% in *S. sonnei*), which indicated that MDR among *Shigella* isolates was widespread in Anhui, China. According to the WHO guidelines for the control of shigellosis, fluoroquinolones and third-generation cephalosporins are effective treatments for this disease.[2] However, several studies have reported that the rate of resistance to CIP and CTX in *Shigella* isolates has gradually increased in recent years,[11,25] which was also observed in our study. Of note, our data showed that the susceptibility to CIP and LVX was higher among *S. sonnei* isolates than among *S. flexneri* isolates (80.9% *vs* 50.3%, $P<0.0001$; 89.7% *vs* 69.5%, $P=0.001$, respectively), suggesting that resistance to fluoroquinolones in Anhui, China, was more pronounced for *S. flexneri* than for *S. flexneri*. Moreover, the susceptibility to GEN was higher among *S. flexneri* isolates than among *S. sonnei* isolates (71.3% *vs* 7.4%, $P<0.0001$), suggesting that GEN may be effective as a second-line treatment for *S. flexneri* infection in Anhui, China.

All *Shigella* species possess a large virulence plasmid and a single circular chromosome. The T3SS-associated

*ipa* genes are both necessary for the invasion of epithelial cells and the development of shigellosis.[26] The *ipaH* gene, which encodes invasion plasmid antigen H, is commonly used for the molecular identification of *Shigella spp*. using PCR assays. In our study, all the isolates harbored *ipaH*, which was consistent with previous reports.[27] Most of the *Shigella* isolates in this study harbored *ipaA, ipaB, ipaC*, and *ipaD*, genes that regulate the secretion and translocation of several effector proteins and play a key role in intracellular actin polymerization and depolymerization.[28] The *ial* gene was reported to be responsible for *Shigella* epithelial cell penetration and cell-to-cell dissemination.[29] In our study, the *ial* gene was present in 96.0% of the *S. flexneri* isolates and 75.0% of the *S. sonnei* isolates. Notably, the prevalence of the *ial* gene in *S. sonnei* was significantly lower than that in *S. flexneri*, suggesting that *S. sonnei* may be less aggressive.

The expression of *Shigella* virulence genes is regulated by a heat-stable, nucleoid-structuring protein. When the conditions are favorable for invasion, a transcriptional cascade is initiated through the activation of the *virF* gene, which subsequently turns on the transcription of the regulatory *virB* gene.[30] In this study, 92.0% of the *S. flexneri* isolates and 73.5% of the *S. sonnei* isolates were found to harbor both *virF* and *virB*, indicating that there might be other pathways for regulating gene expression. Five of the 449 *S. flexneri* isolates tested possessed *virF*, but not *virB*, suggesting that *virF* may regulate the expression of virulence genes through additional pathways. Interestingly, *virB*, but not *virF*, was detected in 3.0% of the *S. flexneri* isolates and in 21.1% of the *S. sonnei* isolates, which may have been due to the loss of the *virF* gene.

IcsB was previously shown to be required for *S. flexneri* evasion of autophagy at late stages infection (4–6 h) through inhibition of the binding of the autophagy protein Atg5 to the *Shigella* surface protein IcsA.[31] In this study, 97.3% of *S. flexneri* and 91.3% of *S. sonnei* harbored both *icsA* and *icsB*, indicating that there might be other pathways involved in the prevention of the autophagic recognition of *icsA*.

The SPATE genes comprise an extended family of secreted autotransporters in Gram-negative bacteria. They exert various functions, including as proteases or mucinases, that can directly or indirectly be toxic to intestine cells.[32] In this study, most of the *S. flexneri* isolates harbored two or more SPATE genes, which is consistent with the results of previous studies.[33,34] We also found that the *sat* gene was the most commonly identified SPATE gene among the *S. flexneri* isolates, but was absent among the *S. sonnei* isolates. This indicated that *sat*-encoded toxin might be a major contributor to the virulence of *S. flexneri* isolates. Similar to *sat*, the class II SPATE genes (*pic* and *sepA*) were also absent among the *S. sonnei* isolates in the present study, which indicated that *pic* and *sepA* toxins played minor roles in the pathogenicity of *S. sonnei.*

Two novel enterotoxins (ShET-1 and ShET-2) have recently been described in *Shigella* isolates. ShET-1 (encoded by *set*) and ShET-2 (encoded by *sen*) can alter electrolyte and water transport in the small intestine, which is closely related to the symptoms of dehydration in shigellosis.[10,35] In our study, no significant difference was found in the distribution of the *sen* gene between *S. flexneri* and *S. sonnei*. Interestingly, the *set* gene was not detected among the *S. sonnei* isolates in this study, suggesting that *S. sonnei* may be less aggressive than *S. flexneri.*

This is the first study to demonstrate the correlation between VRGs and antimicrobial resistance among *S. flexneri* in China. With fewer VRG-negative strains among *S. flexneri* (*ipaH, ipaB*, and *icsA*) and among *S. sonnei*, no correlation tests were done between these VRGs and antimicrobial resistance. In this study, we observed that resistance to CHL and CIP was more common among VRG-positive (except for *sigA* and *sen*) *S. flexneri* than among VRG-negative *S. flexneri*; this indicated that, except for *sigA* and *sen*, all the *S. flexneri* VRGs were positively correlated with resistance to CHL and CIP. Furthermore, resistance to CRO and CAZ was more commonly detected among *sat*- and *set*-positive *S. flexneri* isolates than among *sat*- and *set*-negative *S. flexneri*, indicating that the *sat* and *set* genes were also positively correlated with resistance to third-generation cephalosporins. Interesting, CEN resistance was more frequent among VRG-negative (*ial, virF, set, sat, pic*, and *sepA*) *S. flexneri* than among VRG-positive *S. flexneri*, implying that the *ial, virF, set, sat, pic*, and *sepA* VRGs in *S. flexneri* were negatively correlated with CEN resistance.

## Conclusion

Our study demonstrated that *S. flexneri* remains the predominant species in Anhui, China and that the most frequent MDR pattern was AMP–NAL–TCY. This study also highlighted that *S. flexneri* strains harboring several VRGs were associated with antimicrobial resistance. In summary, we identified the correlation between the VRGs and

antimicrobial resistance, thereby improving our understanding of *Shigella* virulence. Further investigation of VRG expression and the genetic mechanisms underlying the antimicrobial resistance of *Shigella* isolates is required to help guide empirical antimicrobial therapy for the treatment of shigellosis.

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## Disclosure

The authors report no conflicts of interest in this work.

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