Epidemic characterization and molecular genotyping of *Shigella flexneri* isolated from calves with diarrhea in Northwest China

Zhen Zhu†, Mingze Cao†, Xuzheng Zhou, Bing Li and Jiyu Zhang*

**Abstract**

**Background:** The widespread presence of antibiotics resistance genes in pathogenic bacteria can cause enormous problems. Food animals are one of the main reservoirs of intestinal pathogens that pose a potential risk to human. Analyzing the epidemiological characteristics and resistance patterns of *Shigella flexneri* in calves is necessary for animal and human health.

**Methods and results:** A total of 54 *Shigella flexneri* isolates, including six serotypes (1a, 2a, 2b, 4a, 6 and Xv), were collected from 837 fecal samples obtained from 2014 to 2016. We performed pulsed-field gel electrophoresis (PFGE) and applied the restriction enzyme NotI to analyze the genetic relatedness among the 54 isolates and to categorize them into 31 reproducible and unique PFGE patterns. According to the results of antimicrobial susceptibility tests, all 26 *Shigella flexneri* 2a serotypes were resistant to cephalosporin and/or fluoroquinolones. The genes *bla TEM*, *bla OXA* and *bla CTX-M-14* were detected in 19 cephalosporin-resistant *S. flexneri* 2a isolates. Among 14 fluoroquinolone-resistant isolates, the *aac(6')-ib-cr* gene was largely present in each strain, followed by *qnr S* (5). Only one ciprofloxacin-resistant isolate harbored the *qep A* gene. Sequencing the quinolone resistance determining regions (QRDRs) of the fluoroquinolone-resistant isolates revealed two point mutations in *gyr A* (S83 L, D87N/Y) and a single point mutation in *par C* (S80I). Interestingly, two *gyr A* (D87N/Y) strains were resistant to ciprofloxacin.

**Conclusions:** The current study enhances our knowledge of *Shigella* in cattle, although continual surveillance is necessary for the control of shigellosis. The high level of cephalosporin and/or fluoroquinolone resistance in *Shigella* warns us of a potential risk to human and animal health.

**Keywords:** *Shigella flexneri*, Antimicrobial susceptibility, Resistant

**Background**

The majority of Enterobacteriaceae family bacteria, including *Salmonella*, *E. coli* and *Shigella* spp., the major etiological agent of diarrheal disease, are a global public health burden, particularly in low-income countries [1–3]. *Shigella* is phylogenetically distinct from several independent *E. coli* strains and has evolved through convergent evolution [4]. The genus *Shigella* consists of four subgroups differentiated according to their biochemical and serological properties: A (*S. dysenteriae*), B (*S. flexneri*), C (*S. boydii*), and D (*S. sonnei*). All four species of *Shigella* cause shigellosis, but *S. flexneri* is the predominant subgroup found in developing countries, whereas *S. sonnei* is found in industrialized countries [5]. The first *Shigella* species identified was *S. dysenteriae*, followed by *S. flexneri* at the end of the 19th century. Shigellosis became a notorious and widespread epidemic during World War 1 with the transmission of *S. flexneri* strain NCTC1, a 2a lineage [6, 7]. Based on the differing structural characteristics of the antigenic determinants of the O antigen, *S. flexneri* is divided into no fewer than 20 serotypes: 1a, 1b, 1c, 1d, 2a, 2b, 2v, 3a, 3b, 4a, 4v, 4b, 4c, 5a, 5b, X, Xv, Y, Yv, 6, and 7b [8, 9].

Given that shigellosis is a global public health burden, previous studies have focused on the human gastrointestinal pathogens but have ignored animal groups. *Shigella* spp. infect and also cause corresponding clinical...
symptoms in monkeys, cows, pigs, chickens and other animals [10–13]. Indeed, animals that live in environments characterized by poor sanitary hygiene, restricted access to clean drinking water and long-term exposure to contaminated food are prone to dysentery [14, 15]. Many antibiotics are used to control disease and promote growth during the breeding process, leading to the widespread dissemination of antibiotic resistance genes (ARGs). The spread of drug resistance among pathogenic bacteria in humans and animals may be disastrous.

The present study investigated the Shigella epidemic in cows in the northwest region of China. S. flexneri 2a was first isolated from a yak with diarrhea in Tibet in 2014. In this study, we used pulsed-field gel electrophoresis (PFGE) to analyze the relationships among S. flexneri isolates and tested for antimicrobial susceptibility patterns. Our results will help prevent diarrhea in calves and will assist in the selection of effective antibiotics against Shigella.

Methods

Bacterial isolation and identification

Fresh stool samples were isolated from 2014 to 2016 in Northwest China (Gansun, Shanxi, Qinghai, Xinjiang and Tibet) from calves (3 to 20 days) with diarrhea. Samples were stored in transport medium, cultured directly on Salmonella-Shigella (SS) agar and incubated at 37 °C for 24 h to select for Shigella. Resultant colonies (colorless, semitransparent, smooth, and moist circular) [16] were picked and grown on MacConkey (MAC) Agar to verify identity. Colonies were selected and cultured in brain heart infusion broth at 37 °C for 5 h with shaking at 250 rpm. All isolates were confirmed using API20E test strips (bioMérieux Vitek, Marcy-l’Etoile, France) according to the manufacturer’s recommendations. Shigella was serotyped using a commercially available kit (Denka Seiken, Tokyo, Japan) and confirmed by PCR [17].

Antimicrobial susceptibility testing

The antimicrobial susceptibility of S. flexneri isolates was determined via the Kirby–Bauer disc-diffusion method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [18]. The antibiotic discs (OXOID, UK) included penicillin G (P, 10 μg), ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid (AMC, 30 μg), cephalexin (KF, 30 μg), cefazolin (KZ, 30 μg), cefamandole (MA, 30 μg), cefotixin (FOX, 30 μg), ceftriaxone (CRO, 30 μg), cefotaxime (CTX, 30 μg), cefoperazone (CFP, 75 μg), cefepime (FEP, 30 μg), meropenem (MEM, 10 μg), imipenem (IPM, 10 μg), norfloxacin (NOR, 10 μg), enrofloxacin (ENR, 5 μg), levofloxacin (LEV, 5 μg), ciprofloxacin (CIP, 5 μg), erythromycin (E, 15 μg), chloramphenicol (C, 30 μg), tetracycline (TE, 30 μg), streptomycin (S, 10 μg), gentamicin (CN, 10 μg), and amikacin (AK, 30 μg). E. coli strain ATCC25922 was used as a quality control strain in each test batch.

PCR amplification of ARGs

We performed PCR assays that targeted 24 different ARGs using the primers described in Table 1. To determine the underlying resistance mechanism of β-lactam antibiotics, we amplified extended-spectrum β-lactamase (ESBL) genes, specifically blaCTX-M, blasmH, blaTEM, and blaOXA, as well as ampC genes, specifically blaMOX, blaFOX, blaDHA, blaCTI, blaACC, and blaMIR [19–21]. Plasmid-mediated quinolone resistance (PMQR) determinant genes, including qnrA, qnrB, qnrD, qnrS, qepA, and aac(3′)-Ib-cr and four quinolone resistance determining region (QRDR) genes as well as DNA gyrase (gyrA,gyrB) and topoisomerase IV (parC,parE) were amplified to determine the underlying mechanism of quinolone resistance [16, 21–23]. The PCR fragments were sequenced after purification and compared to sequences in GenBank.

PFGE

Genotypes and transmission patterns were determined by performing PFGE according to the method described in a previous study [19]. S. flexneri isolates were digested with the restriction enzyme NotI (TaKaRa, Japan) at 37 °C for 3 h to generate a DNA fingerprinting profile. Salmonella enterica serotype Braenderup strain H9812 was digested with XbaI (TaKaRa, Japan) and used as a molecular size standard. Electrophoresis was performed on the CHEF Mapper XA system (Bio-Rad) with a 1% agarose SeaKem Gold gel (Lonza, USA). Electrophoretic parameters were determined by performing multiple screening runs and included switching times of 2.16 to 54.17 s, a voltage of 6 V/cm, a 120° angle and a run time of 21 h. PFGE images were obtained using a Universal Hood II (Bio-RAD, USA) and analyzed using BioNumerics software version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium). A clustering tree that indicated relative genetic similarity was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and the Dice-predicted similarity value with a 1.0% pattern optimization and 1.5% band position tolerance.

Results

Bacterial isolation and identification

During our epidemiological survey of Shigella, we collected 873 fecal samples from calves with diarrhea and obtained 54 S. flexneri isolates from five provinces in northwest China from 2014 to 2016. Isolate information is shown in detail in Table 2. Among the 54 S. flexneri isolates, there were six serotypes: five (9.26%) isolates were 1a, twenty-six (48.15%) isolates were 2a, four (7.41%) isolates were 2b, six (11.11%) isolates were 4a,
| Target     | Primer sequence (5′ to 3′) | Amplicon size (bp) | Reference                  |
|------------|----------------------------|--------------------|----------------------------|
| **β-lactamase** |                            |                    |                            |
| *blaCTX-M-1* | F: GGTTAAAAATCCTGCGTC      | 873                | Cui et al., 2015 [16]      |
|            | R: TTAAACCCGTCGTTGGTACGGA  |                    |                            |
| *blaCTX-M-2* | F: CGACGCTACCCCCTGCTATT    | 552                | Zong et al., 2008 [17]     |
|            | R: CCAGGTCTAGATTGTTTCCGG   |                    |                            |
| *blaCTX-M-8* | F: TCGGTTAAGCGGATAGTG     | 689                | Zong et al., 2008 [17]     |
|            | R: AACCCACGATGTTGGTACG     |                    |                            |
| *blaCTX-M-9* | F: AGAGTGCAACGGTGATG      | 868                | Cui et al., 2015 [16]      |
|            | R: CCAGTTACAGCCCTTGCG      |                    |                            |
| *blaCTX-M-25* | F: TTGTGAGTACGGGGTTGCA | 497                | Liu et al., 2015 [18]      |
|            | R: GCAGGCACCTCAGGCGAAAAT   |                    |                            |
| **blaSHV**  | F: CGCCGGGTATTCTTATTG       | 1015               | Zong et al., 2008 [17]     |
|            | R: TCTTTCGGATGGCGGACAGTCA  |                    |                            |
| **blaTEM**  | F: ATGATGATTTAACACTTCTCCG  | 876                | This study                 |
|            | R: CCAATGCTTAATCAGTGAG     |                    |                            |
| **blaOXA**  | F: ATTAGGCCCTTACCAAAACCA   | 890                | Cui et al., 2015 [16]      |
|            | R: AAGGTTGCGCGATTGTTGCA    |                    |                            |
| **blaMOX**  | F: GCTGCTCAAGGAGCACAGGAT  | 520                | Cui et al., 2015 [16]      |
|            | R: CACATTGACATAGGTGTGGTGC  |                    |                            |
| **blaFOX**  | F: AACATGGGGTATCAGGGAGATG | 190                | Cui et al., 2015 [16]      |
|            | R: CAAAGCCGGTAACCGGATTTGG |                    |                            |
| **blaDHA**  | F: AACCTTACAGGTTGTGCTGGGT | 405                | Cui et al., 2015 [16]      |
|            | R: CCGTACGCATACTGGCTTTGC  |                    |                            |
| **blaGTT**  | F: TGGCCAGAATCGACAGGCAA    | 462                | Cui et al., 2015 [16]      |
|            | R: TTTCCTTCAGAAGGTGGCTGCG |                    |                            |
| **blaACC**  | F: AACAGCCCTCAAGCCGGATTA  | 346                | Cui et al., 2015 [16]      |
|            | R: TTGGCCGCAATCCCTACTGAGC |                    |                            |
| **blaMVR**  | F: TCAGTAAGCGGTAGTGCAGAG  | 302                | Cui et al., 2015 [16]      |
|            | R: CTTCCACTGCGGCTGCGATTT |                    |                            |
| **PMQRs**   |                            |                    |                            |
| *qnrA*     | F: ATTTTCTACGCCGAGTTTGG    | 516                | Colobatiu et al.,2015 [19] |
|            | R: GATCGGCAAAGTGTGATTTCA  |                    |                            |
| *qnrB*     | F: GATCGTGGAAGCCGAAGAG    | 476                | Colobatiu et al.,2015 [19] |
|            | R: ACGATGCCGGTAGTTGGTCC    |                    |                            |
| *qnrD*     | F: CGAGATCAATTTACCAGGGGATAA | 656            | Cui et al.,2015 [13]       |
|            | R: ACAAGCTGAAGCCGCTG     |                    |                            |
| *qnrS*     | F: ACGACATCCGCAACTGCAAA  | 417                | Colobatiu et al.,2015 [19] |
|            | R: TAAATTGGCACCCCTTACTGAG |                    |                            |
| *aac(6’)-Ib-cr* | F: CCCGCCCTTCTGAGCA     | 544                | Colobatiu et al.,2015 [19] |
|            | R: TTAGGGCCTCATGGTTCCT    |                    |                            |
| *qepA*     | F: CGTTGGTGGGATCATTCC    | 403                | Colobatiu et al.,2015 [19] |
|            | R: CTGGAGCTACTGTGGTACG    |                    |                            |
| **QRDR**   |                            |                    |                            |
| *gyrA*     | F: TACACCCGTCAACATGGAGG   | 648                | Hu et al.,2007 [20]        |
eight (14.81%) isolates were 6, and five (9.26%) isolates were Xv (Fig. 1). Our surveillance of the Gansu isolates identified all of the serotypes, except 4a. All 4a serotypes were isolated from Shanxi, while all Xv and 1a serotypes were from Gansu. Additionally, serotype 2a was widely isolated from each province, with the exception of Xinjiang, and serotype 6 was found only in yaks. Interestingly, Shigella was primarily isolated in the first quarter and fourth quarter, accounting for 54% (29/54) and 30% (16/54), respectively (Fig. 2).

### Antimicrobial susceptibility testing

The 54 S. flexneri isolates were examined for susceptibility to 23 antibiotics. More than 50% of isolates were resistant to 8 antibiotics. Among them, resistance to P (54/54, 100%), AMP (51/54, 94.44%) and TE (49/54, 90.74%) was most common, followed by E (46/54, 85.19%), S (38/54, 70.37%), KZ (34/54, 62.96%), KF (29/54, 53.70%) and CN (29/54, 53.70%). None of the isolates were resistant to IMP, MEM and the fourth-generation cephalosporin FEP. In addition, although a certain number of isolates were resistant to second- and third-generation cephalosporins (MA, FOX, CRO, CTX and CFP) and fluoroquinolones (CIP, NOR, ENR and LEV), these comprised no more than 30% of the total number of isolates, and the resistance rate was lower than those of other antibiotics (Table 3, Fig. 3).

Remarkably, all 26 S. flexneri 2a isolates demonstrated varying degrees of resistance to cephalosporins and/or fluoroquinolones and exhibited multidrug resistance (MDR). The S. flexneri 2a isolates were resistant to 14 diverse cephalosporins/fluoroquinolones. Among them, 73.06% (19/26) of isolates were resistant to cephalosporin, 53.85% (14/26) of isolates were resistant to fluoroquinolones, and 26.92% (7/26) of isolates were resistant to both cephalosporin and fluoroquinolones. Furthermore, isolate GBSF1512433 was resistant to all cephalosporins (with the exception of FEP) and fluoroquinolones (with the exception of CIP). Compared with the S. flexneri 2a isolates collected from beef calves, the 4 yak isolates were sensitive to most cephalosporins and fluoroquinolones but resistant to KF, KZ and MA (Table 4).

### ARGs analysis of cephalosporin- and/or fluoroquinolone-resistant S. flexneri 2a isolates

In this study, only three β-lactamase gene types (bla\text{OXA-1}, bla\text{TEM-1} and bla\text{CTX-M-14}) were identified among the 19 cephalosporin-resistant S. flexneri 2a isolates (Table 5). All isolates harbored bla\text{TEM-1} type ARGs (100%), 15 isolates harbored bla\text{OXA-1} (15/19, 78.95%), and 14 harbored bla\text{CTX-M-14} (14/19, 73.68%). In total, 63.16% (12/19) of isolates harbored three β-lactamase gene types. All S. flexneri 2a isolates from yaks were negative for bla\text{CTX-M} type ARGs.

Both PMQR genes and SNPs in QRDRs were identified for 14 quinolone-resistant isolates (Table 6). According to the PCR results, all quinolone-resistant isolates were positive for aac(6′)-lb-cr but negative for qepA, except strain GBSF1602098. Only five (5/14, 35.71%) strains isolated from Gansu harbored gyrS, and no isolate harbored all three ARGs simultaneously. The point mutations in the QRDR genes play important roles in determining quinolone and/or fluoroquinolone resistance [24]. In the present study, we successfully amplified all four QRDR genes and compared them to reference sequences. We found two point mutations in gyrA and one point mutation each in gyrB and parC (Table 6). All quinolone-resistant strains carried mutations that altered the amino acid sequences of gyrA (S83 L) and parC (S801). In addition, each strain carried the mutation 87 (D → N or Y) in gyrA, with the exception of GBSF1510390. Interestingly, GBSF1505314 and GBSF1602098 harbored the gyrA D87Y mutation, which confers resistance to ciprofloxacin.

### PFGE pattern analysis

PFGE was performed to determine the genetic relatedness among the isolates and to study the molecular epidemiology in specific geographical regions [25]. The PFGE patterns of the 54 NotI-digested S. flexneri isolates were heterogeneous, and multiple PFGE patterns were present among the strains. Thus, diverse factors such as geography and environment may affect PFGE patterns. At an 80% similarity level, S. flexneri isolates generated 31 reproducible and unique PFGE patterns, including 11 common types (CT) and 20 single types (ST) (Fig. 3).
Among all isolates, the majority of *S. flexneri* 2a (26/54, 48.15%) isolates were classified into 11 PFGE patterns (4 CT and 7 ST). These PFGE patterns were closely related to each other, except the Tibet (TYSF1412001) and Qinghai (QYSF1511395) isolates, suggesting the strains isolated from different geographical locations exhibit diverse PFGE patterns and a capricious genetic diversity.

**Discussion**

ARGs are widespread and cause problems when present in pathogens [26]. Over the past decade, MDR *Shigella* has been reported in many countries [27]. However, only a few studies have described the prevalence of *Shigella* in animals worldwide. In the present study, we investigated the epidemiology of *S. flexneri* in cows in northwest China.

| Strain name   | Serotype | Isolation year | Origin | Province |
|---------------|----------|----------------|--------|----------|
| TYSF1412001   | 2a       | 2014           | Yak    | Tibet    |
| GBSF1412056   | 2a       | 2014           | Beef cattle | Gansu    |
| GBSF1501026   | 2a       | 2015           | Beef cattle | Gansu    |
| GBSF1501071   | Xv       | 2015           | Beef cattle | Gansu    |
| GYSF1501076   | 6        | 2015           | Yak    | Gansu    |
| QYSF1501088   | 6        | 2015           | Yak    | Qinghai  |
| XBSF1501093   | 2b       | 2015           | Beef cattle | Xinjiang |
| GBSF1501105   | 2a       | 2015           | Beef cattle | Gansu    |
| SBSF1501123   | 4a       | 2015           | Beef cattle | Shanxi   |
| QYSF1502130   | 6        | 2015           | Yak    | Qinghai  |
| GBSF1502176   | 2a       | 2015           | Beef cattle | Gansu    |
| GYSF1502197   | 6        | 2015           | Yak    | Gansu    |
| SBSF1502219   | 4a       | 2015           | Beef cattle | Shanxi   |
| XBSF1502236   | 2b       | 2015           | Beef cattle | Xinjiang |
| GBSF1503241   | 2a       | 2015           | Beef cattle | Gansu    |
| GYSF1503270   | 1a       | 2015           | Yak    | Gansu    |
| GBSF1503288   | 1a       | 2015           | Beef cattle | Gansu    |
| GBSF1505314   | 2a       | 2015           | Beef cattle | Gansu    |
| SBSF1505331   | 2a       | 2015           | Beef cattle | Shanxi   |
| GBSF1506340   | Xv       | 2015           | Beef cattle | Gansu    |
| GBSF1507358   | 1a       | 2015           | Beef cattle | Gansu    |
| GBSF1509369   | 2a       | 2015           | Beef cattle | Gansu    |
| GBSF1510375   | 2a       | 2015           | Beef cattle | Gansu    |
| GBSF1510390   | 2a       | 2015           | Beef cattle | Gansu    |
| QYSF1511395   | 2a       | 2015           | Yak    | Qinghai  |
| GBSF1511401   | 2a       | 2015           | Beef cattle | Gansu    |
| GYSF1511409   | 2a       | 2015           | Yak    | Gansu    |
| SBSF1512413   | 4a       | 2015           | Beef cattle | Shanxi   |
| GBSF1512419   | 2b       | 2015           | Beef cattle | Gansu    |
| GBSF1512425   | 2a       | 2015           | Beef cattle | Gansu    |
| GBSF1512433   | 2a       | 2015           | Beef cattle | Gansu    |
| GBSF1601015   | Xv       | 2016           | Beef cattle | Gansu    |
| GBSF1601024   | Xv       | 2016           | Beef cattle | Gansu    |
| TYSF1601031   | Xv       | 2016           | Yak    | Tibet    |
| GBSF1601064   | 2a       | 2016           | Beef cattle | Gansu    |
| GYSF1601073   | 6        | 2016           | Yak    | Gansu    |
| GBSF1602082   | 2a       | 2016           | Beef cattle | Gansu    |
| QYSF1602094   | 6        | 2016           | Yak    | Qinghai  |
| GBSF1602098   | 2a       | 2016           | Beef cattle | Gansu    |
| GBSF1602103   | 2a       | 2016           | Beef cattle | Gansu    |
| SBSF1603115   | 4a       | 2016           | Beef cattle | Shanxi   |
| SBSF1603121   | 4a       | 2016           | Beef cattle | Shanxi   |
| GBSF1603138   | 2a       | 2016           | Beef cattle | Gansu    |
| GBSF1603149   | 2a       | 2016           | Beef cattle | Gansu    |
| QYSF1603158   | 6        | 2016           | Yak    | Qinghai  |
| SBSF1604173   | 2a       | 2016           | Beef cattle | Shanxi   |
| GBSF1605203   | Xv       | 2016           | Beef cattle | Gansu    |
| GBSF1608241   | 2a       | 2016           | Beef cattle | Gansu    |
| GYSF1610256   | 2a       | 2016           | Yak    | Gansu    |
| GYSF1610266   | 6        | 2016           | Yak    | Gansu    |
| GBSF1610275   | 1a       | 2016           | Beef cattle | Gansu    |
| GBSF1611283   | 1a       | 2016           | Beef cattle | Gansu    |
| GBSF1611290   | 2a       | 2016           | Beef cattle | Gansu    |

Among all isolates, the majority of *S. flexneri* 2a (26/54, 48.15%) isolates were classified into 11 PFGE patterns (4 CT and 7 ST). These PFGE patterns were closely related to each other, except the Tibet (TYSF1412001) and Qinghai (QYSF1511395) isolates, suggesting the strains isolated from different geographical locations exhibit diverse PFGE patterns and a capricious genetic diversity.

**Fig. 1** *S. flexneri* serotypes collected from 2014 to 2016

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China. During a 2-year survey, 54 *S. flexneri* isolates were obtained. Unfortunately, 16S rRNA gene sequence analysis does not effectively distinguish between closely related strains in a superfamily, such as *Shigella* and *E. coli* [28], and conventional biochemical and serological techniques are also insufficient. Therefore, PFGE was utilized to analyze the molecular characteristics of these isolates, to determine the relatedness among isolates and to study the molecular epidemiology in specific geographical regions. The clustering results allowed us to analyze the epidemiological trends of *S. flexneri*. Characterization of these isolates will be helpful for clinical diagnosis, treatment, prevention and the control of shigellosis [15].

Antimicrobial resistance has emerged as a serious problem [29], particularly for conventional, older-generation antibiotics such as P, AMP, TE, and E. According to the results of our antimicrobial susceptibility tests, cephalosporin and fluoroquinolone resistance rates in our isolates were higher than those in human isolates [19, 26]. Notably, the predominant *S. flexneri* 2a isolates were all resistant to cephalosporins, fluoroquinolones and multiple antibiotics. Two isolates (GBSF1505314 and GBSF1602098) were also resistant to ciprofloxacin, which is the first-line antibiotic.

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**Table 3**: Statistical analysis of the results of antimicrobial susceptibility to 23 antibiotics for 54 *S. flexneri*

| Antibiotic               | Total (n = 54) | Gansu (n = 37) | Shanxi (n = 8) | Xinjiang (n = 2) | Qinghai (n = 5) | Tibet (n = 2) |
|--------------------------|----------------|----------------|---------------|------------------|----------------|--------------|
| Penicillin G (P)         | 54 (100%)      | 37 (100%)      | 8 (100%)      | 2 (100%)         | 5 (100%)       | 2 (100%)     |
| Ampicillin (AMP)         | 51 (94.44%)    | 37 (100%)      | 8 (100%)      | 2 (100%)         | 3 (60%)        | 1 (50%)      |
| Amoxycillin/Clavulanic acid (AMC) | 5 (9.62%)   | 3 (8.11%)      | 1 (12.5%)     | 1 (50%)          |                |              |
| Cephalothin (KF)         | 29 (53.70%)    | 19 (51.35%)    | 5 (62.5%)     | 2 (100%)         | 2 (40%)        | 1 (50%)      |
| Cephazolin (KZ)          | 34 (62.96%)    | 21 (56.76%)    | 7 (87.5%)     | 2 (100%)         | 3 (60%)        | 1 (50%)      |
| Cefarandole (MA)         | 16 (29.63%)    | 12 (32.43%)    | 2 (25%)       | 1 (50%)          | 1 (20%)        | 0            |
| Cefoxitin (FOX)          | 3 (5.56%)      | 2 (5.41%)      | 1 (12.5%)     | 0                | 0              | 0            |
| Ceftriaxone (CRO)        | 12 (22.22%)    | 9 (24.32%)     | 2 (25%)       | 1 (50%)          | 0              | 0            |
| Cefotaxime (CTX)         | 14 (25.93%)    | 10 (27.03%)    | 2 (25%)       | 1 (50%)          | 1 (20%)        | 0            |
| Cefoperazone (CFP)       | 6 (11.11%)     | 6 (16.22%)     | 0             | 0                | 0              | 0            |
| Cefepimide (FEP)         | 0              | 0              | 0             | 0                | 0              | 0            |
| Meropenem (MEM)          | 0              | 0              | 0             | 0                | 0              | 0            |
| Imipenem (IPM)           | 0              | 0              | 0             | 0                | 0              | 0            |
| Norfloxacin (NOR)        | 16 (29.63%)    | 12 (32.43%)    | 3 (37.5%)     | 1 (50%)          | 0              | 0            |
| Enrofloxacin (ENR)       | 13 (24.07%)    | 11 (29.73%)    | 2 (25%)       | 0                | 0              | 0            |
| Levofloxacin (LEV)       | 14 (25.93%)    | 11 (29.73%)    | 2 (25%)       | 1 (50%)          | 0              | 0            |
| Ciprofloxacin (CIP)      | 2 (3.70%)      | 2 (5.41%)      | 0             | 0                | 0              | 0            |
| Erythromycin (E)         | 46 (85.19%)    | 35 (94.59%)    | 6 (75%)       | 2 (100%)         | 3 (60%)        | 0            |
| Tetracycline (TE)        | 49 (90.74%)    | 35 (94.59%)    | 8 (100%)      | 2 (100%)         | 3 (60%)        | 1 (50%)      |
| Chloramphenicol (C)      | 17 (31.48%)    | 10 (27.03%)    | 6 (75%)       | 1 (50%)          | 0              | 0            |
| Streptomycin (S)         | 38 (70.37%)    | 30 (81.08%)    | 4 (50%)       | 2 (100%)         | 2 (40%)        | 0            |
| Gentamicin (CN)          | 29 (53.70%)    | 23 (62.16%)    | 4 (50%)       | 2 (100%)         | 0              | 0            |
| Amikacin (AK)            | 3 (5.56%)      | 3 (8.11%)      | 0             | 0                | 0              | 0            |
treatment for shigellosis. The universal emergence of resistant and MDR strains in animals may be attributable to the unrestricted and excessive use of antibiotics in veterinary clinics. The widespread presence of MDR strains has reduced the selectivity of clinical medications to treat shigellosis [30]. Notably, our PFGE dendrogram showed various genetic patterns for *S. flexneri*, and there were diverse resistance profiles associated with each pattern. Based on these results, *S. flexneri* has the ability to adapt to the selective pressures of different antibiotics.

The high levels of resistance of *S. flexneri* 2a to cephalosporin/fluoroquinolones, which are the most effective treatments for severe gastrointestinal infections caused by pathogenic bacteria, prompted us to study potential molecular resistance mechanisms. The emergence of ESBL-producing *Shigella* spp. has been observed in many countries [31]. In the current study, only 3 ARG genotypes (\textit{blaOXA-1}, \textit{blaTEM-1} and \textit{blaCTX-M-14}) were detected. Among them, the \textit{blaTEM-1} gene was detected in all 19 cephalosporin-resistant isolates. In total, 174 \textit{blaTEM} variants resistant to penicillin and other ß-lactamase antibiotics have been recorded. \textit{TEM-1} confers resistance to ampicillin and cephalothin [32]. \textit{blaOXA-1}-type ARGs are class D ß-lactamases, which were named for their ability to hydrolyze oxacillin [32]. Initially, \textit{blaOXA-1}-beta-lactamases were reported in *P. aeruginosa*,

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**Fig. 3** PFGE dendrogram and antibiotic susceptibility profile of 54 NolI-digested *S. flexneri*. B = Beef cattle; Y = Yak. R = Resistance; N = Sensitive and Intermediary
although now the *bla*<sub>OXA</sub>-type genes, and sequencing results indicated that all the *bla*<sub>OXA</sub> genes were *bla*<sub>OXA-1</sub>. Additionally, *bla<sub>CTX-M</sub>* has become one of the most prevalent extended-spectrum-β-lactamases (ESBLs) [35]. This gene was widely harbored by *S. flexneri* 2a isolated

| Table 4 | Statistical analysis of the cephalosporin and/or fluoroquinolone susceptibility for 26 *S. flexneri* 2a |
|---------|---------------------------------------------|
| Cephalosporin and/or Fluoroquinolones resistance spectrum | Cephalosporin and/or Fluoroquinolones resistance rate No. (%) |
| Total (n = 26) | Gansu (n = 22<sup>a</sup>) | Shanxi (n = 2) | Qinghai (n = 1<sup>b</sup>) | Tibet (n = 1<sup>b</sup>) |
| KF/KZ | 5 (19.23%) | 3 (13.64%) | 0 | 1<sup>a</sup> (100%) | 1<sup>a</sup> (100%) |
| KF/KZ/MA | 3 (11.54%) | 2<sup>a</sup> (9.09%) | 1 (50%) | 0 | 0 |
| KF/KZ/MA/CRO | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |
| KF/KZ/MA/CTX | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |
| KF/KZ/MA/CRO/CFP | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |
| KF/KZ/MA/FOX/CRO/CTX/CFP | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |
| NOR/LEV | 3 (11.54%) | 3 (13.64%) | 0 | 0 | 0 |
| ENR/LEV | 3 (11.54%) | 3 (13.64%) | 0 | 0 | 0 |
| NOR/ENR/LEV/CIP | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |
| KF/KZ/MA/CRO/CTX/NOR/LEV | 2 (7.69%) | 1 (4.55%) | 1 (50%) | 0 | 0 |
| KF/KZ/MA/CRO/CTX/NOR/ENR/LEV | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |
| KF/KZ/MA/CTX/CFP/CIP/NOR/ENR | 2 (7.69%) | 2 (9.09%) | 0 | 0 | 0 |
| KF/KZ/MA/CRO/CTX/CFP/NOR/ENR/CIP | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |
| KF/KZ/MA/FOX/CRO/CTX/CFP/NOR/ENR/LEV | 1 (3.85%) | 1 (4.55%) | 0 | 0 | 0 |

<sup>a</sup>a yak origin *S. flexneri* 2a isolate

| Table 5 | Antimicrobial spectrum and ARGs analysis of *S. flexneri* 2a with resistance to cephalosporin |
|---------|---------------------------------------------------------------|
| Strain name | Antimicrobial spectrum | ARGs in plasmid |
| | | TEM | OXA | CTX-M-9 |
| TYSF1412001 | KF/KZ | TEM-1 | OXA-1 | —— |
| QYSF1511395 | KF/KZ | TEM-1 | —— | —— |
| GBSF1503241 | KF/KZ | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1502176 | KF/KZ | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1602082 | KF/KZ | TEM-1 | —— | CTX-M-14 |
| SBSF1505331 | KF/KZ/MA | TEM-1 | OXA-1 | CTX-M-14 |
| GYSF1511409 | KF/KZ/MA | TEM-1 | —— | —— |
| GYSF1610256 | KF/KZ/MA | TEM-1 | OXA-1 | —— |
| GBSF1510375 | KF/KZ/MA/CRO | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1501105 | KF/KZ/MA/CTX | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1412056 | KF/KZ/MA/CRO/CFP | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1602103 | KF/KZ/MA/FOX/CRO/CTX/CFP | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1601064 | KF/KZ/MA/CRO/CTX/NOR/LEV | TEM-1 | —— | CTX-M-14 |
| SBSF1604173 | KF/KZ/MA/CRO/CTX/NOR/LEV | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1611290 | KF/KZ/MA/CTX/CFP/NOR/ENR | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1501026 | KF/KZ/MA/CTX/CFP/NOR/ENR | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1512425 | KF/KZ/MA/CRO/CTX/NOR/ENR/LEV | TEM-1 | OXA-1 | —— |
| GBSF1602098 | KF/KZ/MA/CRO/CTX/CFP/NOR/ENR/CIP | TEM-1 | OXA-1 | CTX-M-14 |
| GBSF1512433 | KF/KZ/MA/FOX/CRO/CTX/CFP/NOR/ENR/LEV | TEM-1 | OXA-1 | CTX-M-14 |
from beef cattle. Interestingly, all *S. flexneri* 2a isolated from yaks were negative for *blaCTX-M* type ARGs.

Fluoroquinolones are highly effective for the treatment of shigellosis worldwide [36]. The primary mechanism of quinolone resistance involves the accumulation of sequential mutations in QRDRs that encode DNA gyrase and topoisomerase IV [37]. The most prevalent mutations in *Shigella* spp. are the point mutations in *gyrA* codons 83, 87 and 211, and *parC* codon 80 [38, 39]. Novel mutations in QRDRs are also being discovered [39]. In the present study, three mutations in *gyrA* codon 83 (*S* → *L*) and/or 87 (*D* → *N* or *Y*) and *parC* codon 80 (*S* → *I*) were detected in each fluoroquinolone-resistant isolate. All substitutions are responsible for reduced affinity. In addition, the amino acid diversity at the same position may lead to different levels of quinolone resistance [40, 41].

GyrA D87Y mutations were detected in only two ciprofloxacin-resistant isolates. However, the role of this mutation in ciprofloxacin resistance is unclear and requires further investigation.

Over the past few years, PMQR determinants have been deemed the most common ARGs in Enterobacteriaceae worldwide [42]. PMQR determinants mediate only low-level quinolone resistance. However, these resistance genes are usually associated with mobile or transposable elements that allow for dissemination among Enterobacteriaceae. In addition, the presence of PMQR genes may facilitate the selection of QRDR mutations that result in higher levels of quinolone resistance [37, 43, 44]. The aac(6′)-Ib-cr gene encodes an acetyltransferase that is known to reduce quinolone activity. In the present study, all 14 isolates resistant to fluoroquinolones were positive for *aac(6′)-Ib-cr*, indicating the *aac(6′)-Ib-cr* gene is widespread in *S. flexneri* 2a. Compared with the *aac(6′)-Ib-cr* gene, the transmembrane segment efflux pump *qepA* gene was scarcely detected in *Shigella*, and we found only one ciprofloxacin-resistant isolate that was *qepA*-positive. The qnr family (which includes the first PMQR genes) contains a variety of subtypes, including *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* and several qnr family genes that have been reported in *Shigella* [39, 45]. The Qnr proteins protect DNA gyrase against quinolones and facilitate the selection of QRDR mutations that improve resistance to these antimicrobials.

### Conclusion

In conclusion, cephalosporin and/or fluoroquinolone resistance in *Shigella* has been widely reported. To increase our understanding of *Shigella* in cattle, we investigated *Shigella* in calves with diarrhea and analyzed the genetic relatedness, antimicrobial susceptibility, QRDR mutations, and prevalence of PMQR and β-lactamase in *S. flexneri* 2a isolates from five provinces in northwest China. However, this study also had limitations, including the lack of a systematic surveillance system to prospectively or retrospectively detect and analyze shigellosis in veterinary clinics. Furthermore, we are unable to effectively monitor and control antibiotic abuse and the resulting spread of ARGs. Therefore, it is essential to continually monitor rates of shigellosis and the development of resistance patterns.

### Table 6 Antimicrobial spectrum and amino acid types in QRDR and PMQRs genes analysis of *S. flexneri* 2a with resistance to fluoroquinolones

| Strain name | Antimicrobial spectrum | QRDR | ARGs in plasmid |
|-------------|------------------------|------|-----------------|
|             |                        | *gyrA* | *parC* | aac(6′)-Ib-cr | qnrS | qepA |
|             |                        | 83    | 87    | 80            |      |      |
| GBSF1509369 | NOR/LEV                | S83 L | D87N  | S800          | +    | −    |
| GBSF1511401 | NOR/LEV                | S83 L | D87N  | S800          | +    | −    |
| GBSF1608241 | NOR/LEV                | S83 L | D87N  | S800          | +    | −    |
| GBSF1510390 | ENR/LEV                | S83 L | D87D  | S800          | +    | +    |
| GBSF1603138 | ENR/LEV                | S83 L | D87N  | S800          | +    | −    |
| GBSF1603149 | ENR/LEV                | S83 L | D87N  | S800          | +    | −    |
| GBSF1505314 | NOR/ENR/LEV/CIP        | S83 L | D87Y  | S800          | +    | +    |
| GBSF1601064 | KF/KZ/MA/CTX/NOV/LEV   | S83 L | D87N  | S800          | +    | −    |
| SBSF1604173 | KF/KZ/MA/CTX/NOV/LEV   | S83 L | D87N  | S800          | +    | −    |
| GBSF1611290 | KF/KZ/MA/CTX/CFP/NOR/ENR | S83 L | D87N  | S800          | +    | −    |
| GBSF1501026 | KF/KZ/MA/CTX/CFP/NOR/ENR | S83 L | D87N  | S800          | +    | +    |
| GBSF1512425 | KF/KZ/MA/CTX/NOV/ENR/LEV | S83 L | D87N  | S800          | +    | +    |
| GBSF1602098 | KF/KZ/MA/CTX/CFP/NOR/ENR/CTP | S83 L | D87Y  | S800          | +    | −    |
| GBSF1512433 | KF/KZ/MA/FOX/CTP/NOV/ENR/LEV | S83 L | D87Y  | S800          | +    | −    |

*: Presence corresponding genes
−: Absence corresponding genes.
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