

Epidemic and molecular characterization of fluoroquinolone-resistant *Shigella dysenteriae* 1 isolates from calves with diarrhea

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Abstract

Background: The widespread distribution of antimicrobial-resistant *Shigella* has become a recurrent challenge in many parts of the developing world. Previous studies indicate that the host of *Shigella* has expanded from humans to animals. This study aimed to investigate the prevalence of fluoroquinolone resistance and associated molecular characterization of *S. dysenteriae* 1 isolated from calves.

Methods and Results: All 38 unduplicated *S. dysenteriae* 1 isolates were collected from calves in Gansu Province from October 2014 to December 2016. According to MLST and PFGE analysis, these isolates were separated into 4 and 28 genotypes, respectively. The most common STs identified were ST228 (34.21%, 13/38) and ST229 (39.47%, 15/38), which were first found in the present study. All isolates harbored virulence genes, and the incidence of the five virulence genes were *ipaH* (100%), *ipaBCD* (92.11%), *stx* (73.68%), *ial* (57.89%), and *sen* (28.95%). According to the results of antimicrobial susceptibilities, 76.32% (29/38) of *S. dysenteriae* isolates were resistant to fluoroquinolone and showed multidrug resistance. In a study on the polymorphism of QRDR of *gyrA/B* and *parC/E* genes, we identified two mutations in *gyrA* (Ser83→Leu and Asp87→Asn) and *parC* (Ser80→Ile and Ser83→Leu), respectively. Among them, 55.17% (16/29) of resistant strains had the *gyrA* point mutations (Ser83→Leu) and *parC* point mutation (Ser83→Leu). Moreover, 41.38% (12/29) of isolates had all five point mutations of *gyrA* and *parC*. In addition, the prevalence of the PMQR determinant genes was also investigated. All 29 fluoroquinolone-resistant isolates were positive for the *aac(6')-Ib-cr* gene but negative for *qepA*, except SD001. In addition, only 6 (20.69%, 6/29) isolates harbored the *qnr* gene, including two with *qnrB* (6.90%, 2/29) and four with *qnrS* (13.79%, 4/29).

Conclusion: Given the increased common emergence of multidrug resistant isolates, uninterrupted surveillance will be necessary to understand the actual epidemic burden and control this infection.

Background

Shigella is one of the major pathogens that causes diarrheal diseases in humans and animals.^{1,2} Since the first highly toxigenic species, *Shigella dysenteriae*, was isolated in 1898,³ four species has been described in *Shigella* genus, and each subgroup can be divided into multiple subtypes according to biochemical and serological properties.^{4,5} Worldwide, there are approximately 164.7 million shigellosis infection cases in humans annually,⁵ while there are few data showing the prevalence in animals.

Antimicrobial therapy is the most effective way to fight shigellosis, so various kinds of drugs are widely used.⁶ Fluoroquinolone, especially ciprofloxacin, was the first-line antibiotic for the treatment of shigellosis as recommended by the WHO.⁷ However, antibiotic resistance usually occurs in the environment where antibiotics are frequently used.^{8,9} Furthermore, the accelerating accumulation and transmission of antimicrobial resistance genes (ARGs) among multidrug resistant (MDR) pathogens pose major difficulties in treating infections.¹⁰

ARGs are widespread but cause problems only when present in pathogens.¹⁰ The virulence gene has become a significant marker of pathogenic bacteria. *S. dysenteriae* possesses diverse virulence genes, located on both the chromosome and/or the plasmid.¹¹ The common genetic loci are invasion plasmid antigen H (*ipaH*), invasion plasmid antigen genes (*ipaBCD*) and invasion associated locus (*ial*). In addition, the *Shigella* enterotoxin genes *set1A*, *set1B* (*ShET-1*), *sen* (*ShET-2*) and Shiga toxin gene *stx* were also reported to be responsible for initial watery diarrhea.^{12–14}

In this study, we aimed to investigate the epidemiology and molecular characteristics of *S. dysenteriae* isolates from cattle farms in Gansu, China. Our analysis includes the antimicrobial resistance profiles, the molecular characterization of the mechanisms of resistance to fluoroquinolones, virulence gene profiles and the molecular characterization of *S. dysenteriae* isolates by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Materials And Methods

Bacterial strains and serotyping

From October 2014 to December 2016, we tracked the calf diarrhea caused by *Shigella* from 26 farms in northwestern regions of China. A total of 3321 fresh stool samples from calves with diarrhea (3 to 20 days) were collected for detecting *Shigella*. All of the isolates were collected directly from fresh stool samples following plating on *Salmonella-Shigella* (SS) selective agar and confirming on MacConkey (MAC) agar with 37 °C for 24 h. The colorless, semitransparent, smooth, and moist circular plaques were considered presumptive *Shigella* for further 16S rDNA (TaKaRa, Japan) and biochemical confirmation.⁶

The biochemical reactions of the presumptive positive *Shigella* isolates were tested by API20E kits (bioMérieux, France), and the serotype was tested by a commercially available agglutinating antibody kit (Denka Seiken, Tokyo, Japan) according to the manufacturers' recommendations.

Multilocus sequence typing (MLST)

All isolates were subjected to MLST according to the protocols described in the EcMLST database (<http://www.shigatox.net/ecmlst>). PCR amplification conditions of 15 housekeeping genes were as follows: 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 90 s, and 72 °C for 1 min; and 72 °C for 5 min with ExTaq DNA polymerase (Takara, Dalian, China). The PCR products were sequenced bidirectionally, and sequences of the 15 housekeeping genes were edited by using SeqMan 7.0. Then, these sequences were uploaded to the EcMLST website for comparison, which allowed us to determine the gene and ST type.¹⁵ Clustering and minimum spanning tree (MST) analysis were used to infer relationships among the isolates using the fingerprint analysis software BioNumerics (version 7.1).

Pulsed-field gel electrophoresis (PFGE)

DNA fingerprinting was performed by PFGE according to the method from the CDC of China. Intact agarose-embedded chromosomal DNA of *S. dysenteriae* isolates was digested with restriction enzyme *Xba*I (TaKaRa, Japan), and restriction fragments were separated on the CHEF Mapper XA system (Bio-Rad) in 1% SeaKem Gold agarose gel (Lonza, USA) in 0.5x TBE buffer with a size range of 30–700 kb. Electrophoretic parameters were determined by multiple screening for the most suitable value, which included switching times from 2.16 to 54.17 s, voltage 6 v/cm, angle 120° and run time 21 h. *Salmonella enterica* serotype *Braenderup* strain H9812 was used as a molecular size marker for this analysis.

The gel image was captured by Universal Hood II (Bio-RAD, USA), and the PFGE profiles were analyzed by BioNumerics (version 7.1). Degrees of homology were determined by comparing the bands, and the clustering tree indicating relative genetic similarity was constructed by the unweighted pair group method with arithmetic averages (UPGMA).

Detection of virulence genes

All *S. dysenteriae* isolates were examined for the presence of seven virulence genes, invasion plasmid antigen H (*ipaH*), invasion plasmid antigen genes (*ipaBCD*), invasion associated locus (*ial*), Shiga toxin gene (*stx*), and *Shigella* enterotoxin genes (*set1A*, *set1B*, and *sen*) by PCR according to published procedures.^{13,16} The primers for these virulence genes are listed in Table S1. Amplification products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide.

Determination of antimicrobial susceptibility

All of the isolated strains were tested for their antimicrobial susceptibility against a variety of 20 antibiotic discs (Oxoid, UK) on Mueller–Hinton agar following the guidelines of the Clinical and Laboratory Standards Institute (CLSI).¹⁷ The antimicrobial discs used in this study were norfloxacin (NOR, 10 µg), enrofloxacin (ENR, 5 µg), levofloxacin (LEV, 5 µg), ciprofloxacin (CIP, 5 µg), ofloxacin (OFX, 5 µg), ampicillin (AMP, 10 µg), amoxycillin/clavulanic acid (AMC, 30 µg), cephazolin

(KZ, 30 µg), cefoxitin (FOX, 30 µg), ceftriaxone (CRO, 30 µg), cefotaxime (CTX, 30 µg), cefepime (FEP, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 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* ATCC 25922 was used for quality control organisms according to the CLSI standard.

Detection of fluoroquinolone resistance genes

To understand the underlying mechanism of fluoroquinolone resistance, four quinolone resistance–determining region (QRDR) genes, DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), and six plasmid-mediated quinolone resistance (PMQR) determinant genes, *qnrA*, *qnrB*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr* and *qepA*, were amplified for fluoroquinolone-resistant isolates in our study.^{4,18,19} The obtained PCR fragments were sequenced after purification and analyzed through comparison with sequences in GenBank. The primer information is listed in Table S2.

Results

Bacterial isolation and identification

During our routine surveillance of bacillary dysentery, a total of 136 *Shigella* isolates were collected in this study, including 54 *S. flexneri* (1.63%, 54/3321), 44 *S. sonnei* (1.32%, 44/3321), and 38 *S. dysenteriae* (1.14%, 38/3321), and no *S. boydii* was found. All 38 *S. dysenteriae* were only found on six farms in Gansu Province.

Detailed information on the study isolates is listed in Table S3. Among them, 20 isolates were isolated from 3 beef cattle farms, and 18 isolates were isolated from 3 dairy farms. There are 14 and 10 *S. dysenteriae* isolates from the same farms of Zhangye and Jinchang, respectively.

All 38 *S. dysenteriae* isolates were type 1 according to the results of the serotype reactions. In addition, based on the typical biochemical characteristics of *Shigella* spp., analysis of biochemical reactions indicated the presence of 3 biotypes among these isolates (Table 1, Table S3). Among these BTs, BT2 (ability to ferment glucose, arabinose, and melibiose) was the predominant biotype, accounting for 86.84% (33/38). Furthermore, BT2 was widely isolated from each locus, with the exception of Baiyin.

Table 1
Biochemical characteristics of *S.dysentery* 1 isolates.

Biotype		Total (n = 38)	ZY (n = 14)	BY (n = 3)	LX (n = 3)	LZ (n = 5)	WW (n = 3)	JC (n = 10)
BT1	glucose+, mannose+, arabinose+, melibiose-	3 (7.89%)	0	3 (100%)	0	0	0	0
BT2	glucose+, mannose-, arabinose+, melibiose+	33 (86.84%)	14 (100%)	0	3 (100%)	5 (100%)	1 (33.33%)	10 (100%)
BT3	glucose+, mannose+, arabinose+, melibiose+	2 (5.26%)	0	0	0	0	2 (66.67%)	0
ZY: Zhangye; BY: Baiyin; LX: Linxia; LZ: Lanzhou; WW: Wuwei; JC: Jinchang.								

MLST-based genotype analysis

Thirty-eight *S. dysenteriae* isolates belonged to 4 STs, ST57, ST191, ST228 and ST229. Among them, ST57 and ST191 were previously reported, while ST228 and ST229 were unique. The allele number for each locus and the designation of the ST are listed in Fig. 1 and shared on the EcMLST website. The most common STs identified were ST228 (n = 13) and ST229 (n = 15), accounting for 73.68% (28/38). ST228 and ST229 were major ST types for dairy cows and beef cattle, respectively. In

addition, these two ST types were different by 3 loci: *arcA*, *clpX*, *mutS*. The other ST type, ST57 includes 2 isolates from beef cattle and 5 isolates from dairy cows. ST191 was only isolated from three steers in Linxia (Fig. 1, Fig. 2).

PFGE-based genotype analysis

The genotypes and genetic relatedness of the 38 isolates were further determined by using PFGE. PFGE patterns of these *S. dysenteriae* isolates were heterogeneous; however, multiple PFGE patterns were present among these strains (Fig. 3). With approximately 80% similarity, *Xba*I-digested *S. dysenteriae* type 1 could be divided into 28 distinctive PFGE patterns (PT) and belonged to two major groups: A (A1-A4) and B (B1-B3), about 66% similarity. Interestingly, most strains in different geographical locations can be clustered individually, suggesting high genetic diversity among *S. dysenteriae* 1.

Prevalence of virulence genes

The frequencies of some major *Shigella* virulence genes in this study. A total of five virulence genes were detected in those isolates involving *ipaH*, *ipaBCD*, *ial*, *sen*, and *stx*. The most frequently observed virulence genes are *ipaH* (100%), *ipaBCD* (92.11%), *stx* (73.68%), and *ial* (57.89%). The *Shigella* enterotoxin genes *sen* (28.95%) are occasionally present in Wuwei and Jinchang isolates. None of the study strains possessed the *set1A* or *set1B* gene.

Regarding the differences in virulence gene distributions, the 38 *S. dysenteriae* isolates fell into 5 gene profile types (VT) (Table 2). Among these VTs, VT IV (n = 17) and VT V (n = 11) were the most common, accounting for 44.34% and 28.95%, respectively. One interesting finding was the presence of the same and/or similar VTs in the same locus. In addition, 92.11% of isolates carried two or more virulence genes. Three Linxia isolates (7.89%) belonged to VT I, which was only positive for *ipaH*.

Table 2
Statistical the rate of each Virulence genes profile in S.dysentery serotype 1 isolates.

VT	Virulence genes profiles	No. (%) isolates						
		Total (n = 38)	ZY (n = 14)	BY (n = 3)	LX (n = 3)	LZ (n = 5)	WW (n = 3)	JC (n = 10)
I	<i>ipaH</i> + <i>ipaBCD</i> - <i>ial</i> - <i>sen</i> - <i>stx</i> -	3 (7.89%)	0	0	3 (100%)	0	0	0
II	<i>ipaH</i> + <i>ipaBCD</i> + <i>ial</i> - <i>sen</i> - <i>stx</i> -	2 (5.26%)	0	2 (66.67%)	0	0	0	0
III	<i>ipaH</i> + <i>ipaBCD</i> + <i>ial</i> + <i>sen</i> - <i>stx</i> -	5 (13.16%)	2 (14.29%)	0	0	2 (40%)	1 (33.33%)	0
IV	<i>ipaH</i> + <i>ipaBCD</i> + <i>ial</i> + <i>sen</i> - <i>stx</i> +	17 (44.34%)	12 (85.71%)	1 (33.33%)	0	3 (60%)	0	1 (10%)
V	<i>ipaH</i> + <i>ipaBCD</i> + <i>ial</i> - <i>sen</i> + <i>stx</i> +	11 (28.95%)	0	0	0	0	2 (66.67%)	9 (90%)
VT: Virulence genes profiles type.								
ZY: Zhangye; BY: Baiyin; LX: Linxia; LZ: Lanzhou; WW: Wuwei; JC: Jinchang.								

Antimicrobial resistance profiles

The antimicrobial resistance profiles of the 20 antimicrobials for 38 *S. dysenteriae* are shown in Table 3. All *S. dysenteriae* isolates were uniformly multidrug resistant to at least 3 types of antimicrobial agents. Among them, resistance to E was the most common (36,94.74%), followed by AMP (35, 92.11%), KZ (33, 86.84%), CRO (32, 84.21%), CTX (32, 84.21%), TE (31, 81.58%), CN (31, 81.58%), ENR (26, 68.42%), LEV (25, 65.79%), CIP (25, 65.79%), NOR (15, 39.47%), OFX (15, 39.47%), C (15,

39.47%), and S (15, 39.47%). Fortunately, all 38 isolates were sensitive to AMC, FOX, FEP, MEM, IPM and AK. However, the resistance rate of fluoroquinolone antibiotics has reached 39.47–68.42%, which is not optimistic.

Table 3
Statistical and analysis the result of antimicrobial susceptibility to 20 antibiotics for 38 *S.dysentery*.

Antimicrobial	Antimicrobial resistance rate No. (%)						
	Total (n = 38)	Beef farm(n = 20)			Dairy farm(n = 18)		
Zhangye(n = 14)	Baiyin(n = 3)	Linxia(n = 3)	Jinchang(n = 10)	Lanzhou(n = 5)	Wuwei(n = 3)		
Norfloxacin	15(39.47%)	12(85.71%)	3(100%)	0	0	0	0
Enrofloxacin	26(68.42%)	13(92.86%)	0	0	10(100%)	0	3(100%)
Levofloxacin	25(65.79%)	12(85.71%)	0	0	10(100%)	0	3(100%)
Ciprofloxacin	25(65.79%)	12(85.71%)	0	0	10(100%)	0	3(100%)
Ofloxacin	15(39.47%)	12(85.71%)	3(100%)	0	0	0	0
Ampicillin	35(92.11%)	14(100%)	3(100%)	0	10(100%)	5(100%)	3(100%)
Amoxycillin/Clavulanic acid	0	0	0	0	0	0	0
Cephazolin	33(86.84%)	14(100%)	3(100%)	0	9(90%)	5(100%)	2(66.67%)
Cefoxitin	0	0	0	0	0	0	0
Ceftriaxone	32(84.21%)	14(100%)	0	0	10(100%)	5(100%)	3(100%)
Cefotaxime	32(84.21%)	14(100%)	0	0	10(100%)	5(100%)	3(100%)
Cefepime	0	0	0	0	0	0	0
Meropenem	0	0	0	0	0	0	0
Imipenem	0	0	0	0	0	0	0
Erythromycin	36(94.74%)	12(85.71%)	3(100%)	3(100%)	10(100%)	5(100%)	3(100%)
Chloramphenicol	15(39.47%)	12(85.71%)	3(100%)	0	0	0	0
Tetracycline	31(81.58%)	12(85.71%)	3(100%)	3(100%)	10(100%)	0	3(100%)
Streptomycin	15(39.47%)	12(85.71%)	3(100%)	0	0	0	0
Gentamicin	31(81.58%)	12(85.71%)	3(100%)	3(100%)	10(100%)	0	3(100%)
Amikacin	0	0	0	0	0	0	0

Moreover, most of the isolates (29/38, 76.32%) were resistant to fluoroquinolone antibiotics, there were 4 isolates resistant to other fluoroquinolones that is not CIP. These resistant isolates were divided into 5 antibiotic-resistance profile patterns (Table 4). All of the fluoroquinolone-resistant isolates were multidrug resistant (MDR), and 41.8% (12/29) of fluoroquinolone-resistant isolates were resistant to each class of antibiotics. In addition, the resistance profiles of strains isolated from the same farms were very similar.

Table 4

Antimicrobial profile and amino acid types in QRDRs and PMQRs analysis of *S.dysentery* with resistance to fluoroquinolones.

Strain name	Antimicrobial profile	QRDRs			PMQR			
		<i>parC</i>	<i>aac(6)-Ib-cr</i>	<i>qnr</i>	<i>qepA</i>			
<i>gyrA</i>		80 (S)	83 (S)					
83 (S)	87 (D)							
SD020	ENR/KZ/CRO/CTX	L	D	I	L	+	-	-
SD002	NOR/OFX/KZ/E/C/TE/S/CN	L	D	S	L	+	-	-
SD026	NOR/OFX/KZ/E/C/TE/S/CN	L	D	S	L	+	-	-
SD036	NOR/OFX/KZ/E/C/TE/S/CN	L	D	S	L	+	-	-
SD015	ENR/LEV/CIP/ CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD016	ENR/LEV/CIP/ CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD004	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD007	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD008	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	<i>qnrB</i>	-
SD012	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD014	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD019	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD023	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	<i>qnrB</i>	-
SD024	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD029	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD030	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD035	ENR/LEV/CIP/ KZ/CRO/CTX/E/TE/CN	L	D	S	L	+	-	-
SD001	NOR/ENR/LEV/CIP/OFX/ KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	-	+
SD006	NOR/ENR/LEV/CIP/OFX/ KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	-	-
SD009	NOR/ENR/LEV/CIP/OFX/ KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	-	-
SD011	NOR/ENR/LEV/CIP/OFX/ KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	-	-
SD013	NOR/ENR/LEV/CIP/OFX/ KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	-	-

Table 4
contain

Strain name	Antimicrobial profile	QRDRs			PMQR			
<i>gyrA</i>		<i>parC</i>		<i>aac(6')-lb-cr</i>	<i>qnr</i>	<i>qepA</i>		
83 (S)	87 (D)	80 (S)	83 (S)					
SD017	NOR/ENR/LEV/CIP/OFX/KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	-	-
SD018	NOR/ENR/LEV/CIP/OFX/KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	-	-
SD022	NOR/ENR/LEV/CIP/OFX/KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	<i>qnrS</i>	-
SD028	NOR/ENR/LEV/CIP/OFX/KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	<i>qnrS</i>	-
SD031	NOR/ENR/LEV/CIP/OFX/KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	<i>qnrS</i>	-
SD032	NOR/ENR/LEV/CIP/OFX/KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	<i>qnrS</i>	-
SD034	NOR/ENR/LEV/CIP/OFX/KZ/CRO/CTX/E/C/TE/S/CN	L	N	I	L	+	<i>qnrS</i>	-

ARG analysis for fluoroquinolone-resistant *S. dysenteriae* isolates

To determine the molecular characterization in fluoroquinolone-resistant *S. dysenteriae*, both SNPs in QRDR of *gyrA/B* and *parC/E* genes and PMQR genes were analyzed. In 29 fluoroquinolone-resistant isolates, there was no strain that displayed mutations in the *gyrB* and *parE* genes, although some mutations in *gyrA* and *parC* were identified in each resistant isolate (Table 4). All resistant isolates in the present study carried common mutations in *gyrA* codon 83 (S→L) and *parC* codon 83 (S→L). Furthermore, 12 Zhangye isolates showed an additional 87 (D→N) and 80 (S→I) mutations in *gyrA* and *parC*, respectively.

The PMQR genes *qnr*, *aac(6')-lb-cr*, and *qepA* occur worldwide and are increasingly detected in clinical isolates of Enterobacteriaceae.^{20,21} In our study, *aac(6')-lb-cr* was the most common PMQR gene and harbored by each isolate; however, all isolates were negative for *qepA*, except SD001. Only seven (7/29, 24.14%) isolates harbored the *qnr* gene, and no isolate harbored *qnr*, *aac(6')-lb-cr*, and *qepA* simultaneously. Interestingly, the *qnrB* (n = 2) gene and *qnrS* (n = 5) were detected in Jinchang and Zhangye, respectively.

Discussion

Invasive diarrhea due to *Shigella* species remains an important public health problem in developing countries.²² All four species of *Shigella* can cause shigellosis, but *S. flexneri* and *S. sonnei* are the most prevalent²³. Present study confirmed the existence of diverse *S. dysenteriae* isolates in cows in local epidemiological studies. The rate of *S. dysenteriae* isolation from the diarrhea of cows was 1.14% (38/3321), and all the *S. dysenteriae* isolates were type 1.

Strain molecular characterization is important for epidemiological studies. In the present study, 38 *S. dysenteriae* isolates from cows were separated into 4 STs. Comparing the 15 housekeeping genes, only 4 allele genes (*aspC*, *cyaA*, *icdA*, and *uidA*) were identical. In our study, the majority of isolates (n = 13 and n = 15) belonged to new ST types ST228 and ST229.

However, ST148, ST252, and ST1739 were previously reported in human isolates.²⁴ PFGE is the most common typing procedure currently used with *Shigella* spp. because of its high discriminatory power.²⁵ Taking PFGE as a reference epidemiological tool, the *S. dysenteriae* isolates in this study were heterogeneous and distributed into 28 PTs. The clustering of these diverse PTs allows us to learn more about the epidemiological characteristics of *S. dysenteriae* in specific geographical regions.

The ability of *Shigella* spp. to cause shigellosis is attributed to the expression of arrays of virulence genes associated with colonization, invasion/penetration and toxin-mediated disease.²⁶ Virulence genes responsible for the pathogenesis of shigellosis are often multifactorial and coordinately regulated.²⁷ Estimating the existence of virulence determinants in *Shigella* would help us better understand its pathogenicity.²⁶ *lpaH* takes responsibility for the strain spread from cell to cell and the modification of host response to infection.²⁸ All *Shigella* spp. were positive for *ipaH* as a typical marker because this gene exists in multiple copies on both the chromosome and the invasion plasmid. Additionally, in this study, most isolates simultaneously harbored the *stx*, *ial* and *sen* genes. The diversity of the observed virulence genes results in dysentery in hosts. Notably, these virulence genes are indistinguishable from human *Shigella*.

For shigellosis, antibiotic therapy can reduce the duration and severity of the illness.²⁹ Based on previous reports, fluoroquinolones and third-generation cephalosporins are the best choice for empiric treatment for severe bacterial diarrheas caused by *S. dysenteriae*.³⁰ However, resistance will emerge to any antimicrobial agent used intensively, and the differential selection pressures of antimicrobials lead to differences in drug resistance.³¹ These factors have contributed to *Shigella* strains acquiring resistance to the latest drug of choice, together with other effective drugs after therapy shigellosis. In addition, the resistance rate of *S. dysenteriae* 1 isolates to fluoroquinolones has achieved at an alarming rate, and the isolates have demonstrated multidrug resistance. Our current study indicated that continuous surveillance of the resistance pattern in pathogens would be essential for the choice of appropriate antimicrobial therapy and control the spread of *Shigella*.

The most common mechanism of the high level resistance to quinolone has been mostly attributed to accumulation of sequential mutations in DNA gyrase and DNA topoisomerase IV, typically of *gyrA* at codon 83 and/or 87, and of *parC* at codon 80.^{16,32} In addition, different mutation loci and different mutations at the same locus may result in different quinolone susceptibility levels.^{33,34} To date, limited studies have examined quinolone resistance mechanisms among *Shigella* isolates in animals. In the current study, we successfully investigated fluoroquinolone resistance-conferring mutations by sequencing QRDR of *S. dysenteriae* 1 isolated in different geographical locations. According to our results, only two main combinations of mutations were observed: *gyrA* codons 83 (Ser→Leu), and *parC* codons 83 (Ser→Leu) (55.17%, 16/29); *gyrA* codons 83 (Ser→Leu), 87 (Asp→Asn) and *parC* codons 80 (Ser→Ile), 83 (Ser→Leu) (41.38%, 12/29). This phenomenon of high resistance rate and single mutation pattern is likely related to the large-scale use of single quinolone antibiotics by veterinarians.

Generally, the PMQR determinants are located on mobile genetic elements, which may be associated with mobile or transposable elements among members of the Enterobacteriaceae family.³⁵ Over the past few years, the dissemination of PMQR genes among quinolone-resistant *Shigella* isolates has been surveyed in some studies and has emerged as an important issue across the world.^{36–39} It has been reported that *aac(6′)-Ib-cr* significantly increases the frequency of selection of chromosomal mutants to reduce ciprofloxacin activity by N-acetylation at the amino nitrogen on its piperazinyl substituent.⁴⁰ The *aac(6′)-Ib-cr*-positive *Shigella* was first isolated in 1998,⁴¹ however, the most striking finding of our previous and present study was the *aac(6′)-Ib-cr* allele wide penetration in all quinolone-resistant isolates.^{42,43} The *qnr* family could protect DNA gyrase against quinolones and confer low-level resistance to nalidixic acid and reduced susceptibility to fluoroquinolone, especially to ciprofloxacin.⁴⁴ The *qnr* gene has occurred worldwide and contains a variety of subtypes, but it was not a common epidemic genotype according to our study. Otherwise, the plasmid-mediated *qepA* gene can encode a 14-transmembrane segment efflux pump and extrude several quinolones from bacteria, thereby conferring low levels of

resistance to these antimicrobial agents and favoring the selection of new alterations that are able to induce full quinolone resistance.^{45,46} Compared with other PMQR determinants, *qepA* is a new resistance gene, first described in 2007,⁴⁷ so it is not difficult to understand the lowest frequencies.

The QRDR mutation and PMQR determinants are both important to quinolone resistance. Compared with mutations in the QRDR, PMQR could not be the direct reason for quinolone and fluoroquinolone resistance.⁴⁸ However, the presence of PMQR genes may facilitate the selection of QRDR mutations that result in higher levels of quinolone resistance.

In conclusion, this study described fluoroquinolone resistance and virulence genes among *S. dysenteriae* strains isolated from cows and the molecular characterization involved. To systematically understand *S. dysenteriae*, PFGE and MLST methods were applied to genetically characterize the 38 isolates. PFGE based on *Xba*I digestion divided the 38 isolates into 28 PTs, while MLST based on 15 housekeeping genes differentiated the 38 isolates into 4 STs. Although MLST provided suitable discrimination in *S. dysenteriae* subtyping, PFGE might exhibit a higher discriminatory ability. Overall, the data from this study will provide a useful typing resource, which will provide a scientific basis for addressing clinical and epidemiological issues regarding *S. dysenteriae*. Given this knowledge, continuous and extensive surveillance will be essential to explore and prevent the spread of the epidemic.

Declarations

Ethics approval and consent to participate

This study has gained ethical approval for this study. Our study was conducted according to the Ethics Committee of Animal Experiments at the Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agricultural Sciences in Lanzhou, China. And we gained consent from the owners of the animals for them to be used in the study.

Consent for publication

All the authors agreed to the publication of the paper.

Availability of data and material

The data supporting the findings of this study are contained within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Z.Z. and J.Y.Z. designed the study; Z.Z., M.Z.C., Y.X.S., X.Z.Z., G.H. L and B.L. generated and provided the dataset; Z.Z., and W.W.W. performed the experiments, analyzed the data, and wrote the manuscript. All authors have read and approved the final manuscript

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References

1. Mather AE, Baker KS, McGregor H, Coupland P, Mather PL, Deheer-Graham A, Parkhill J, Bracegirdle P, Russell JE, Thomson NR. Bacillary dysentery from World War 1 and NCTC1, the first bacterial isolate in the National Collection. *The Lancet*. 2014;384:1720.
2. Priamukhina NS, Kileso VA, Tikhomirov ED. Animal carriers of *Shigella* and their possible epidemiological importance. *Zh Mikrobiol Epidemiol Immunobiol*. 1984;11:20–4.
3. Baker KS, Mather AE, McGregor H, Coupland P, Langridge GC, Day M, Deheer-Graham A, Parkhill J, Russell JE, Thomson NR. The extant World War 1 dysentery bacillus NCTC1: a genomic analysis. *The Lancet*. 2014;384:1691–7.
4. Cui X, Wang J, Yang C, Liang B, Ma Q, Yi S, Li H, Liu H, Li P, Wu Z, Xie J, Jia L, Hao R, Wang L, Hua Y, Qiu S, Song H. 2015, Prevalence and antimicrobial resistance of *Shigella flexneri* serotype 2 variant in China. *Front Microbiol*. 6,435.
5. Perepelov AV, Shekht ME, Liu B, Shevelev SD, Ledov VA, Senchenkova SN, L'Vov VL, Shashkov AS, Feng L, Aparin PG, Wang L, Knirel YA. *Shigella flexneri* O-antigens revisited: final elucidation of the O-acetylation profiles and a survey of the O-antigen structure diversity. *FEMS Immunology Medical Microbiology*. 2012;66:201–10.
6. Tajbakhsh M, Garcia Migura L, Rahbar M, Svendsen CA, Mohammadzadeh M, Zali MR, Aarestrup FM, Hendriksen RS. Antimicrobial-resistant *Shigella* infections from Iran: an overlooked problem? *J Antimicrob Chemoth*. 2012;67:1128–33.
7. WHO. International note on antibiotics in the management of shigelliosis. *Wkly Epidemiol Rec*. 2004;79:202–3.
8. Poole K, Srikumar R. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Curr Top Med Chem*. 2001;1:59.
9. McEwen SA, Fedorka-Cray PJ. Antimicrobial use and resistance in animals. *Clin Infect Dis*. 2002;34:93–106.
10. Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DGJ. The structure and diversity of human, animal and environmental resistomes. *Microbiome*. 2016;4:54.
11. Dutta S, Nandy S, Jain P, Yoshida S, Matsushita S. Molecular characterization of serologically atypical provisional serovars of *Shigella* isolates from Kolkata, India. *J Med Microbiol*. 2014;63:1696–703.
12. Vargas M, Gascon J, Maria TJDA, Vila J. Prevalence of *Shigella* Enterotoxins 1 and 2 among *Shigella* Strains Isolated from Patients with Traveler's Diarrhea. *J Clin Microbiol*. 1999;37:3608–11.
13. Das A, Natarajan M, Mandal J. The Emergence of Quinolone Resistant *Shigella sonnei*, Pondicherry, India. *Plos One*. 2016;11:e160290.
14. Faruque SM, Khan R, Kamruzzaman M, Yamasaki S, Ahmad QS, Azim T, Nair GB, Takeda Y, Sack DA. Isolation of *Shigella dysenteriae* type 1 and *S. flexneri* strains from surface waters in Bangladesh: comparative molecular analysis of environmental *Shigella* isolates versus clinical strains. *Appl Environ Microbiol*. 2002;68:3908–13.
15. Schaumburg F, Alabi AS, Kaba H, Lell B, Becker K, Grobusch MP, Kremsner PG, Mellmann A. Molecular characterization of *Shigella* spp. from patients in Gabon 2011–2013. *T Roy Soc Trop Med H*. 2015b;109:275–9.
16. Talukder KA, Khajanchi BK, Islam MA, Islam Z, Dutta DK, Rahman M, Watanabe H, Nair GB, Sack DA. Fluoroquinolone Resistance Linked to Both *gyrA* and *parC* Mutations in the Quinolone Resistance–Determining Region of *Shigella*

dysenteriae Type 1. Curr Microbiol. 2006;52:108–11.

17. Clinical Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility testing; Twenty-second informational supplement. m100 –s23. Wayne: Clinical and Laboratory Standards Institute; 2013.
18. Colobatiu L, Tabaran A, Flonta M, Oniga O, Mirel S, Mihaiu M. First description of plasmid-mediated quinolone resistance determinants and β -lactamase encoding genes in non-typhoidal *Salmonella* isolated from humans, one companion animal and food in Romania. Gut Pathog. 2015;7:16.
19. Hu LF, Li JB, Ye Y, Li X. 2007, Mutations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in clinical strains of fluoroquinolone-resistant *Shigella* in Anhui, China. J. Microbiol. 45, 168 – 70.
20. Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. The Lancet Infectious Diseases. 2006;6:629–40.
21. Pu X, Pan J, Gu Y, Zheng W, Li J, Yu H. Complete Sequences and Characterization of Two Novel Plasmids Carrying *aac(6')-Ib-cr* and *qnrS* Gene in *Shigella flexneri*. Microb Drug Resist. 2016;22:115–22.
22. Ansaruzzaman M. Isolation and characterization of provisional serovar *Shigella boydii* E16553 from diarrhoeal patients in Bangladesh. J Med Microbiol. 2005;54:477–80.
23. Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, Marohn ME, Antonio M, Hossain A, Mandomando I, Ochieng JB, Oundo JO, Qureshi S, Ramamurthy T, Tamboura B, Adegbola RA, Hossain MJ, Saha D, Sen S, Faruque ASG, Alonso PL, Breiman RF, Zaidi AKM, Sur D, Sow SO, Berkeley LY, O'Reilly CE, Mintz ED, Biswas K, Cohen D, Farag TH, Nasrin D, Wu Y, Blackwelder WC, Kotloff KL, Nataro JP, Levine MM. *Shigella* Isolates From the Global Enteric Multicenter Study Inform Vaccine Development. Clin Infect Dis. 2014;59:933–41.
24. Chattaway MA, Schaefer U, Tewolde R, Dallman TJ, Jenkins C. Identification of *Escherichia coli* and *Shigella* Species from Whole-Genome Sequences. J Clin Microbiol. 2017;55(2):616–23.
25. Navia MM, Capitano L, Ruiz J, Vargas M, Urassa H, Schelleberg D, Gascon J, Vila J. Typing and characterization of mechanisms of resistance of *Shigella* spp. isolated from feces of children under 5 years of age from Ifakara. Tanzania J Clin Microbiol. 1999;37:3113–7.
26. Zhang CL, Liu QZ, Wang J, Chu X, Shen LM, Guo YY. Epidemic and virulence characteristic of *Shigella* spp. with extended-spectrum cephalosporin resistance in Xiaoshan District, Hangzhou, China. BMC Infect Dis. 2014;14:260.
27. Qu M, Zhang X, Liu G, Huang Y, Jia L, Liang W, Li X, Wu X, Li J, Yan H, Kan B, Wang Q. An eight-year study of *Shigella* species in Beijing, China: serodiversity, virulence genes, and antimicrobial resistance. J Infect Dev Ctries. 2014;8:904–8.
28. Parsot C. *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. Fems Microbiol Lett. 2005;252:11–8.
29. Pazhani GP, Niyogi SK, Singh AK, Sen B, Yamasaki S, Taneja N, Ramamurthy T, Kundu M. Molecular characterization of multidrug-resistant *Shigella* species isolated from epidemic and endemic cases of shigellosis in India. J Med Microbiol. 2008;57:856–63.
30. Gendrel D, Cohen R. Diarrhées bactériennes et antibiotiques: les recommandations européennes. Archives de Pédiatrie. 2008;15:93–6.
31. Heddini A, Cars O, Qiang S, Tomson G. Antibiotic resistance in China—a major future challenge. The Lancet. 2009;373:30.
32. Dutta S, Kawamura Y, Ezaki T, Nair GB, Iida K, Yoshida S. Alteration in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV in Quinolone-resistant *Shigella dysenteriae* serotype 1 clinical isolates from Kolkata, India. Antimicrob Agents Chemother. 2005;49:1660–1.
33. Kim JY, Kim SH, Jeon SM, Park MS, Rhie HG, Lee BK. Resistance to fluoroquinolones by the combination of target site mutations and enhanced expression of genes for efflux pumps in *Shigella flexneri* and *Shigella sonnei* strains isolated in Korea. Clin Microbiol Infect. 2008;14:760–5.
34. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother. 2003;51:1109–17.

35. Folster JP, Pecic G, Bowen A, Rickert R, Carattoli A, Whichard JM. Decreased Susceptibility to Ciprofloxacin among *Shigella* Isolates in the United States, 2006 to 2009. *Antimicrob Agents Ch.* 2011;55:1758–60.
36. Pu XY, Pan JC, Zhang W, Zheng W, Wang HQ, Gu YM. Quinolone resistance-determining region mutations and the plasmid-mediated quinolone resistance gene *qnrS* played important roles in decreased susceptibility to fluoroquinolones among *Shigella* isolates in southeast China between 1998 and 2013. *Int J Antimicrob Agents.* 2015;45:438–9.
37. Xiong W, Sun Y, Ding X, Wang M, Zeng Z. Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. *Front Microbiol.* 2015;6:194.
38. Tian L, Zhu X, Chen Z, Liu W, Li S, Yu W, Zhang W, Xiang X, Sun Z. Characteristics of bacterial pathogens associated with acute diarrhea in children under 5 years of age: a hospital-based cross-sectional study. *BMC Infect Dis.* 2016;16:253.
39. Gu B, Qin T, Fan W, Bi R, Chen Y, Li Y, Ma P. Novel mutations in *gyrA* and *parC* among *Shigella sonnei* strains from Jiangsu Province of China, 2002–2011. *Int J Infect Dis.* 2017;59:44–9.
40. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park H, Bush C, Hooper K, D.C. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med.* 2006;12:83–8.
41. Pu XY, Pan JC, Wang HQ, Zhang W, Huang ZC, Gu YM. Characterization of fluoroquinolone-resistant *Shigella flexneri* in Hangzhou area of China. *J Antimicrob Chemoth.* 2009;63:917–20.
42. Zhu Z, Cao M, Zhou X, Li B, Zhang J. Epidemic characterization and molecular genotyping of *Shigella flexneri* isolated from calves with diarrhea in Northwest China. *Antimicrob Resist Infect Control.* 2017;6:92.
43. Zhu Z, Shi Y, Zhou X, Li B, Zhang J. Molecular characterization of fluoroquinolone and/or cephalosporin resistance in *Shigella sonnei* isolates from yaks. *BMC Vet Res.* 2018;14:177.
44. Taneja N, Kumar A, Appannanavar S, Verma G, Sharma M. Plasmid-mediated quinolone resistance in *Shigella* isolates over a decade in India. *J Glob Antimicrob Res.* 2014;2:59–60.
45. Yamane K, Wachino JI, Suzuki S, Kimura K, Shibata N, Kato H, Shibayama K, Konda T, Arakawa Y. New Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, Found in an *Escherichia coli* Clinical Isolate. *Antimicrob Agents Ch.* 2007;51:3354–60.
46. Ruiz J. 2019, Transferable Mechanisms of Quinolone Resistance from 1998 Onward. *Clin. Microbiol. Rev.* 32.
47. Perichon B, Courvalin P, Galimand M. Transferable Resistance to Aminoglycosides by Methylation of G1405 in 16S rRNA and to Hydrophilic Fluoroquinolones by QepA-Mediated Efflux in *Escherichia coli*. *Antimicrob Agents Ch.* 2007;51:2464–9.
48. Yang H, Duan G, Zhu J, Zhang W, Xi Y, Fan Q. Prevalence and characterisation of plasmid-mediated quinolone resistance and mutations in the gyrase and topoisomerase IV genes among *Shigella* isolates from Henan, China, between 2001 and 2008. *Int J Antimicrob Ag.* 2013;42:173–7.

Figures

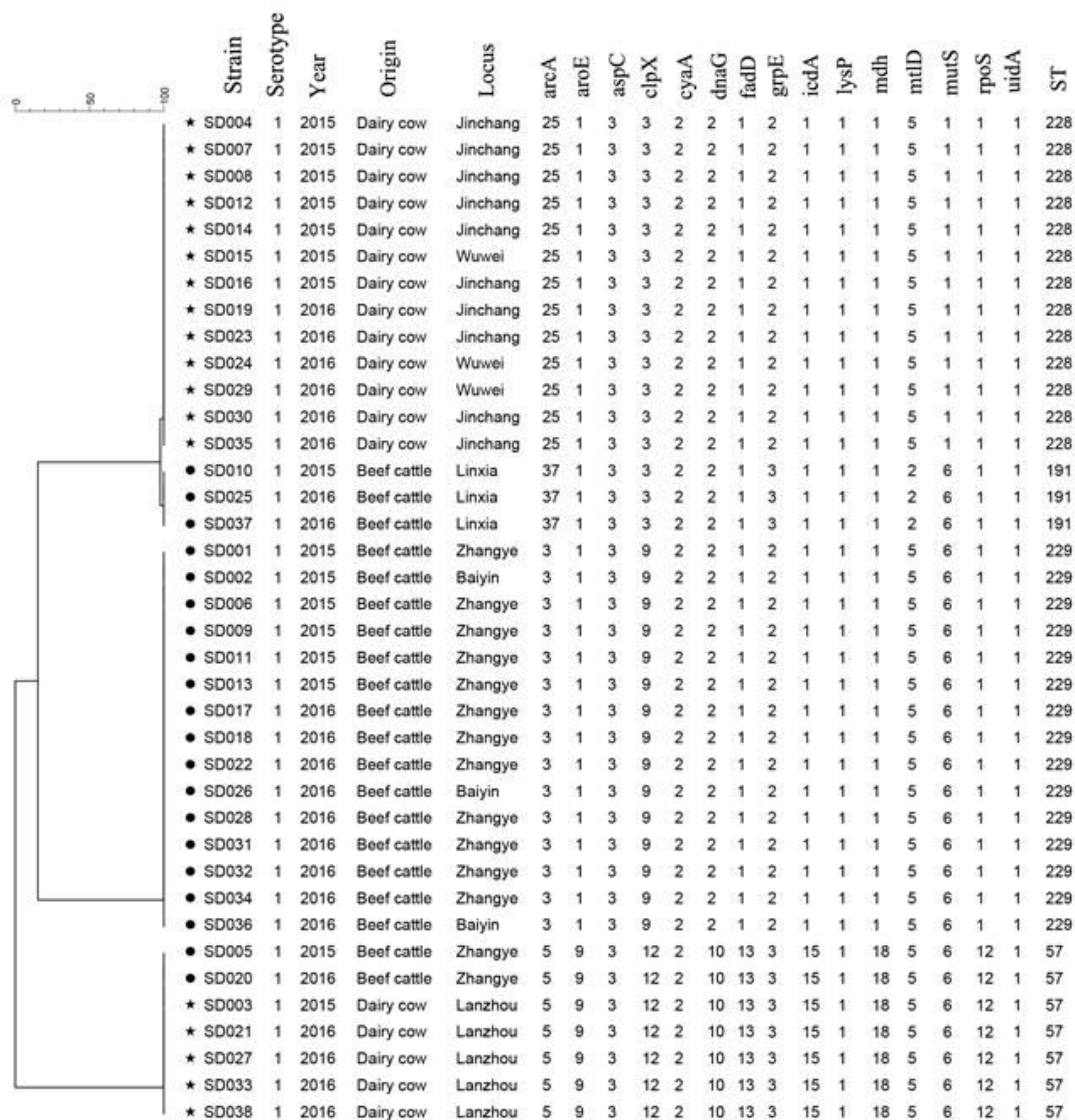


Figure 1

MLST clustering tree of *S. dysenteriae* isolates from 2014 to 2016 in diarrhea calves. The 38 isolates were analyzed using a 15 allele MLST as described in the Materials and Methods.

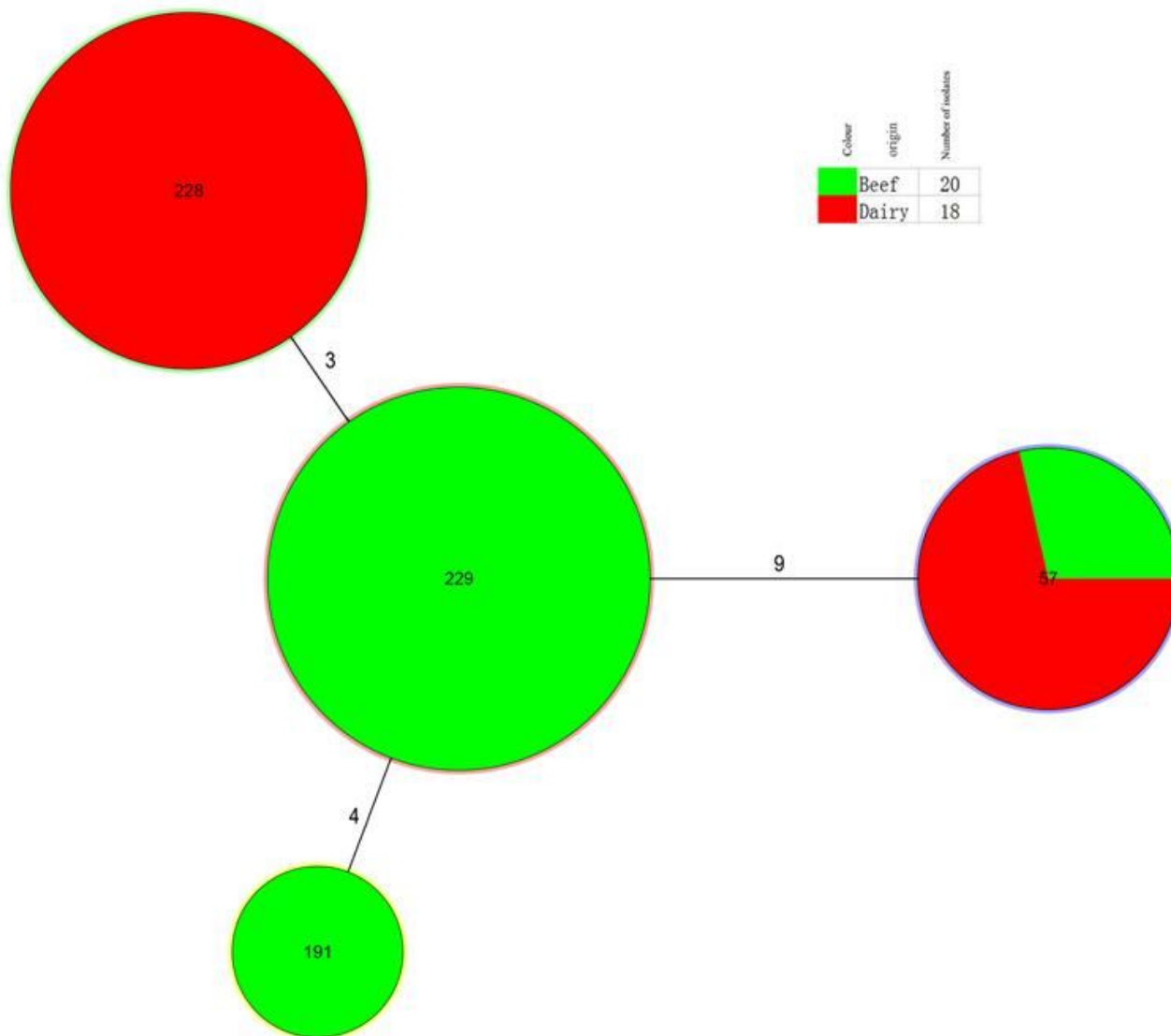


Figure 2

Minimum spanning tree of the isolates of 38 *S. dysenteriae* isolates from diarrhea calves based on multilocus sequence typing (MLST). The minimum spanning tree was constructed using the 4 identified STs obtained from the 38 isolates using BioNumerics Software. Each circle corresponds to a single ST. The shadow zones of circles' filling in green correspond to beef, and red to dairy cows. The size of the circle is proportional to the number of isolates and the numbers over the branches means the difference between the two ST types. The corresponding color, number of isolates and background information are shown to the right of the minimum spanning tree

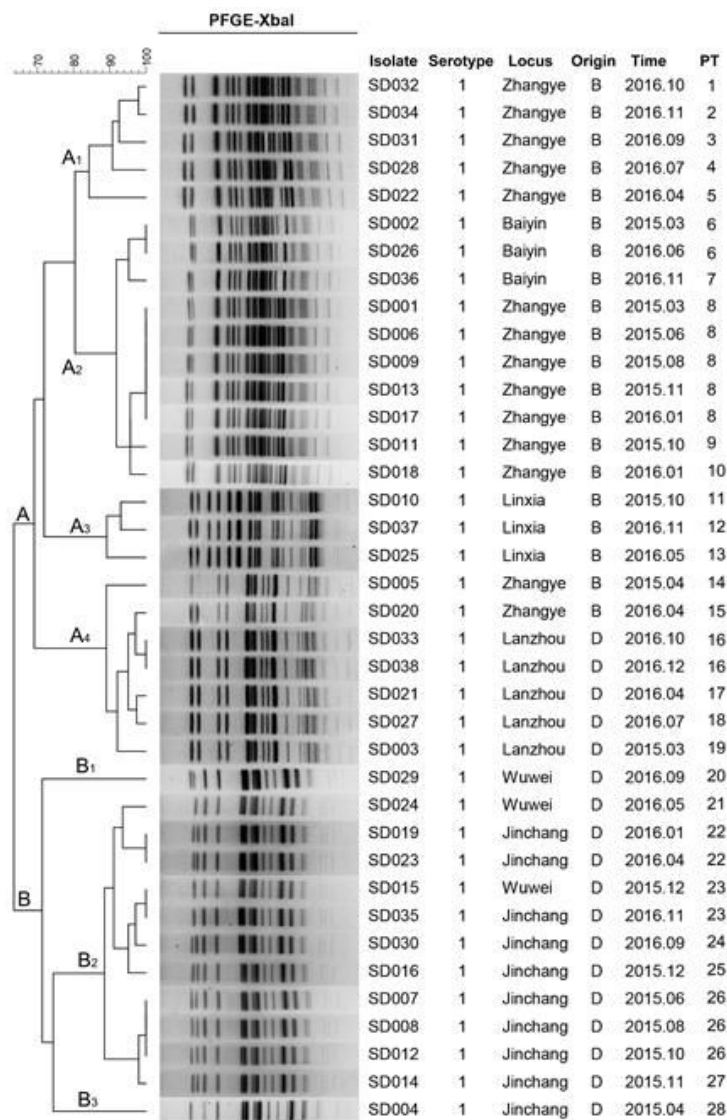


Figure 3

Dendrogram of 38 XbaI-digested *S. dysenteriae* isolates based on the cluster analysis of PFGE patterns. The dendrogram was constructed using the UPGMA clustering method. The PFGE pattern and background information for each strain are listed on the right side of the dendrogram

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