Virulence factors and molecular characteristics of *Shigella flexneri* isolated from calves with diarrhea

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Abstract

Background

The natural hosts of *Shigella* are conventionally humans and other primates; however, the host range of *Shigella* has been shown to expand to many animals. Although *Shigella* is becoming a huge threat to animals, there is limited information on the genetic background of local strains. The purpose of this study was to assess the presence of virulence factors and the molecular characteristics of *S. flexneri* isolated from calves with diarrhea.

Results

Fifty-four *S. flexneri* isolates possessed four typical biochemical characteristics of *Shigella*. The prevalences of *ipaH*, *virA*, *ipaBCD*, *ial*, *sen*, *set1A*, *set1B* and *stx* were 100%, 100%, 77.78%, 79.63%, 48.15%, 48.15% and 0, respectively. MLVA based on 8 VNTR loci discriminated the isolates into 39 different MTs, PFGE based on *Not*I digestion divided the 54 isolates into 31 PTs, and MLST based on 15 housekeeping genes differentiated the isolates into 7 STs.

Conclusion

Our findings of this study have enriched our knowledge of the molecular characteristics of *S. flexneri* collected from diarrheal calves, which will be important for addressing clinical and epidemiological issues regarding Shigellosis.

Background

Shigellosis or blood dysentery is widespread in underdeveloped or developing regions with poor hygiene and limited access to clean drinking water, and has become a serious threaten to public health [1, 2]. Shigellosis is caused by nonmotile, facultative anaerobic Gram-negative bacilli of the Enterobacteriaceae family, includeing *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* [3–5]. *Shigella* species have highly effective on invasive systems that enable the bacteria to invade and multiply within the human intestinal epithelia, ultimately leading to severe inflammatory colitis, which is -named bacillary dysentery or shigellosis [4].

The various virulence factors located in the chromosome or large virulent *inv* plasmids is recognized as a crucial part that is related to the pathogenesis of shigellosis [6]. Moreover, these different virulence factors are associated with the colonization of intestinal cells and intracellular invasion, which may partly explain why there are various manifestations are detected in clinic, such as intestinal inflammatory responses and watery diarrhea [1].Bacterial cell-to-cell movement and dissemination within epithelial cells of intestine are allowed by *iphH* gene which encoded by chromosomal DNA and/or recombinant plasmid, while *ial* which are encoded by plasmid (invasion-associated locus) enables *Shigella* bacteria to penetrate into intestinal epithelial tissues [7, 8]. The chromosomal genes *set1A* and *set1B* encode *Shigella* enterotoxin 1 (*ShET-1*) [9, 10], which is easily detected in all *S. flexneri* 2a. *Shigella* enterotoxin 2 which encoded by the gene *sen*, is located on a large plasmid associated with the virulence of *Shigella* and is found in most of *Shigella* of different serotypes and in enteroinvasive *Escherichia coli* (EIEC) [11, 12].It's recognized that *ShET-1* and *ShET-2*, except for their enterotoxic activity, play significant roles in the transport of electrolytes and water in the intestine [12]. *VirA* located on large virulent plasmids has a great impact in intercellular spreading and invasion [13]. On the other hand, the type III secretion system (T3SS) is regarded as an important component for bacterial entry and is also composed of several proteins, including a needle-shaped oligomer anchored in the protein complex that connects the inner and outer bacterial membranes. The tip of the needle is an oligomer composed of the invasion plasmid antigens *ipaB*, *ipaC*, and *ipaD* [14–16]. Futhermore, the upstream *ipaB* region is often used as a marker to detect the *ipaBCD* gene.

The natural hosts of *Shigella* are conventionally humans and other primates [4]. However, it's reported that monkeys, rabbits, calves, fish, chickens and piglets are infected with *Shigella* as new hosts [4, 17–21]. In recent years, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* are isolated from cows. Although *Shigella* is becoming a huge threat to animals, there is limited information on the

genetic background of isolated strains. Therefore, aim to indentify molecular genotype and determine the genetic relatedness diversity of local *S. flexneri* strains, we performed the test via the methods of MLST, MLVA and PFGE.

Results

Biochemical characterization

A total of 54 *S. flexneri* of six serotypes, including 1a (n=5), 2a (n=26), 2b (n=4), 4a (n=6), 6 (n=8), and Xv (n=5), were analyzed in this study. Based on the results of the biochemical reaction assays, we observed that all 54 *S. flexneri* isolates possessed 4 typical *Shigella* biochemical characteristics (Table 1). Among these BTs, BT4 (the ability to ferment glucose, mannitol, arabinose, and melibiose) was the predominant biotype, accounting for 70.37% (38/54) of all BTs. Furthermore, BT4 was widely found in each serotype, except serotype 6. *S. flexneri* 2a was distributed among all four biochemical phenotypes and mainly in BT4 (22/26, 84.62%). However, the other five serotype strains only had one or two biochemical phenotypes.

Virulence factors

The frequencies of the virulence factor profiles in the *S. flexneri* isolates are listed in Fig 1. A total of seven virulence factors were detected in those isolates, including *ipaH* (100%), *virA* (100%), *ipaBCD* (92.59%), *ial* (77.78%), *sen* (79.63), *set1A* (48.15%) and *set1B* (48.15%). None of the studied strains possessed the *stx* gene. The *Shigella* enterotoxin genes *set1A* and *set1B* were only present in *S. flexneri* 2a, and all of these serotype isolates were positive for these two genes.

Regarding the differences in the distributions of the virulence factors, the 54 *S. flexneri* isolates fell into seven gene profile types (VT) (Table 2). Among these VTs, VT4 (positive for *ipaH*, *virA*, *ipaBCD*, *ial*, *sen*) and VT6 (positive for *ipaH*, *virA*, *ipaBCD*, *ial*, *sen*, *set1A*, *set1B*) were the most common, accounting for 29.63% and 44.44% of all VTs, respectively. Furthermore, 92.59% of the isolates carried two or more virulence factors. In addition, the virulence factor types were associated with the *S. flexneri* serotype. VT1 was only found in 4a, and VT4 was present in isolates from each serotype, except 2a. *S. flexneri* 2a major belonged to VT6 (24/26, 92.31%).

MLST-based genotype analysis

MLST was performed to analyze the genotypic diversity of *S. flexneri* isolates based on 15 housekeeping genes. The 54 isolates were divided into seven STs, including ST68, ST100, ST103, ST120, ST124, ST135 and ST227. Among them, ST227 was novel, while the six other STs have previously been reported. These seven STs belonged to several clonal complexes (CCs): CC10 (ST100 and ST103), CC26 (ST68), and others (ST120, ST124, ST135 and ST227). The clustering tree (Fig 2) based on the MLST data showed that ST68 was a singleton type and that the other six STs contained two or more isolates. The most common ST was ST100 (n=33, 61.11%), including isolates of serotypes 1a, 2a, and Xv. All the isolates of ST124 and ST227 belonged to *S. flexneri* 6 and 4a, respectively. The cluster tree indicated that isolates belonging to the same serotype closely clustered based on the province of isolation. In addition, according to the minimum spanning tree (MST) based on the allele, it was found that ST100, ST120 and ST135 had closer relationships and only differed in *aspC*, while ST68, ST124 and ST227 were very different from the other STs (Fig 3).

MLVA-based genotype analysis

MLVA based on eight VNTR loci was performed to further characterize the isolated *S. flexneri* strains. The copy numbers of the eight VNTR loci are listed in Fig 4. Overall, the 54 isolates based on the unique MLVA profiles were discriminated into 39 different MLVA types (MTs). Among them, twenty-eight MTs belonged to the singleton type, and the other ten MTs contained no more than three isolates. The MLVA cluster tree of the isolates showed that they were divided into five clusters, designated A to E, with a low coefficient of similarity from 20%-60% (Fig 4). Each cluster was further divided into many subclusters. MLVA can cluster different serotype strains separately and distinguish between the same serotype strains. The main cluster, cluster C, was observed clustering *S. flexneri* 2a isolates and further divided into 15 MTs. Additionally, clusters A (except GBSF1502176), D and E only clustered the Xv, 2b, and 6 serotype strains, respectively. The results showed differences based on the geographical origin and time span in the same serotype.

PFGE-based genotype analysis

The genotypes and genetic relatedness diversity of the 54 *S. flexneri* isolates were assessed by PFGE. *Not*I-digested *S. flexneri* chromosomal DNA generated 31 reproducible unique PFGE patterns (PTs), each with 11-16 bands (Fig 5). Eleven patterns were represented by more than one isolate, with PT20 (n = 8) containing the most isolates, followed by PT18 (n =5). The dendrogram of *S. flexneri* isolates showed low similarity (40%-60%) and could be classed into three gross clusters on the basis of their serotypes: clusters A, B and C. Isolates belonging to the same serotype but recovered in different years showed clear relatedness, as indicated by their grouping in the same clusters. The majority of serotype 2a isolates, with the exception of isolate QYSF1511395, grouped together in cluster B. The QYSF1511395 strain isolated from Qinghai Province clustered independently in cluster C. Isolates 1a, 2b and Xv clustered into cluster B and were closely related to the serotype 2a isolates. However, the isolates of serotypes 4a and 6 were assigned to cluster A with a relatively close relationship, but different serotype strains clustered separately.

Discussion

Shigella is an important invasive enteric infectious pathogen known for its sporadic, epidemic and pandemic spread [3], which is still a landmark cause of inflammatory diarrhea and dysentery, posing a serious challenge to public health, especially tracked in most middle-income countries and regions with substandard hygiene and poor qualitily water supplies [22]. All four kinds of *Shigella* can cause shigellosis, but *S. flexneri* is the most common bacterial preparation in shigellosis [23]. The traditional hosts of this pathogen are limited in primates; however, the range of host has been extended to many animals in recent decades [4]. Better understanding of the hosts of *Shigella* is needed to assess its potential effects on animal health, otherwise, it's a challenge for preventing the disease caused by *Shigella.*

Pathogenesis of *Shigella* is contributed to the organism's ability to invade, replicate and spread intercellularly within the colonic epithelium. Pathogenic factors are the reasons that pathogenic Shigella invades intestinal epithelial cells, leading to dysentery and other intestinal clinical symptoms in the host [24], and its pathogenesis is often multifactorial and coordinated [25]. Virulence factor has become an important indicator of pathogenic bacteria.

Based on the detection of virulence factors, the *Shigella* isolates used in the present study had vast genetic diversity. Our results showed that *ipaH* and *virA* were found in each strain, in agreement with the previous study which demonstrating that *ipaH* is carried by all four *Shigella* species as well as by enteroinvasive *E. coli* (EIEC). Multiple copies (*ipaH*1.4, *ipaH*2.5, *ipaH*4.5, *ipaH*7.8 and *ipaH*9.8) on large plasmids and chromosomes may explain why the *ipaH* gene tested positive in all isolates. Therefore, as a diagnostic tool for detecting *Shigella*, the *ipaH* gene is often an appealing target, even in the absence of the plasmid [26]. *VirA* was initially thought to be an invasion in *Shigella*; however, structural analysis showed that *VirA* lacks lacked papain like protease activity to promote tubulin division. *VirA* belongs to the GTPase activating protein family, which is involved in the cleavage of single membrane into vacuole. Previous studies have shown that *VirA* is often present in *Shigella*, and it is an important terminal point for bacteria to invade host cells and nucleate actin at one end of bacteria [9, 27].

Expert opinion suggested that T3SS is essential for host cell invasion and intracellular survival among those virulence factors, whereas *IpaB*, *IpaC*, and *IpaD* are key factors of virulent *Shigella* [9, 28, 29]. Unlike the *ipaH* gene, the *ial* gene is not common. The *ipaH* gene is only located on the *inv* plasmid, and compared with chromosome gene, the stability of IPAH plasmid to storage/subculturing is poor [6–8]. Our results show that the *ial* gene has a high invasiveness in the isolates studied. Therefore, it should be noted that the *ial* gene is involved in the invasion of intestinal cells and that the higher positive rate of this gene in *S. flexneri* might indicate stronger aggressiveness.

The *Shigella* enterotoxins *ShET-1* and *ShET-2* as alter electrolyte and water transport for small intestine can cause diarrhea and dehydration [22]. *ShET-1* is located on chromosomes encoding *set1* (A and B subunit) genes, which is almost exclusively found in several *S. flexneri* serotype 2 isolates and is rarely found in other serotypes [30]. Consistent with previous studies, our study showed that *set1A* and *set1B* were only detected in the *S. flexneri* 2a strain. It is known that the plasmid encoding *ShET-2* (encoded by *sen*) is an enterotoxin hemolysin that causes an inflammatory response during the invasion of *Shigella* [12, 22]. It's reported that there is a close relationship among *sen*, *set* enterotoxins and bloody diarrhea [22].And it implied that *sen* and *set*

enterotoxins is the pathogentic factor of bloody diarrhea. However, unlike *ShET-1*, *ShET-2* could be harbored by other species of *Shigella.*

Molecular characterization of strains is significant for epidemiological studies. However, rare reports are available to systematically understand the molecular characteristics of *S. flexneri* isolated from animals. Recently, several useful genotyping tools with higher discriminatory power than traditional tests, which containing MLST [31], PFGE [32] and MLVA [33], have been applied to explore and analysis the characteristic of *Shigella* isolates. The method of analysis on relationship of phylogeny as an important part to support strain isolation is based on the difference of strain genetics.

MLST is an important source of sequence data for relative genetics, providing a tool for exploring molecular evolutionary methods among bacteria [34]. With the key elements of 15 housekeeping genes and analysis of the EcMLST database, the advantage of MLST is that the comparison of data from different laboratories. Our results suggested that the predominant ST was ST 100, which has before been found in human *S. flexneri* isolates [35, 36]. To be specific, isolates belonging to the same serotype often showed one ST type, indicating the low discriminative ability in closely related strains within a specific serotype due to the high sequence conservation of the housekeeping genes.

Compared with the MLST profiles, MLVA and PFGE may be forceful tools that can provide a satisfactory level of discrimination. Whereas the function of MLVA in phylogenetic analysis of different bacterial species or serotypes are poorly targeted [37]. Nevertheless, MLVA is an ordinarily used typing tool that has been used for establishing genetic relatedness and performing phylogenetic analysis among strains of monomorphic species. In our study, with approximately 20% similarity, the 54 *S. flexneri* isolates were divided into 39 different MTs and clustered into 5 groups. Though applied in a limited collection of *S. flexneri* isolates, this study indicates the high discriminatory power of the MLVA method for subtyping strains with the same serotype.

With the strong function and widespread use, PFGE is also an applicable typing tool available in the laboratory for discriminating several enteric bacteria, such as *Shigella*. PFGE has a high degree of intra- and interlaboratory reproducibility when standardized protocols are followed [38]. Thirty-one low homophyly and unique PFGE patterns confirmed the existence of diverse *S. flexneri* clones and the usefulness of PFGE in local epidemiological studies.

Conclusion

This study demonstrated that spontaneous prevalent *S. flexneri* in cows shelter the same virulence factors as the prevalent isolates in humans. Therefore, these isolates are a potential threat to public safety. To systematically understand *S. flexneri*, PFGE, MLVA and MLST methods were applied to hereditary characterize the 54 isolates. MLVA based on 8 VNTR loci discriminated the 54 isolates into 39 different MTs, PFGE based on *Not*I digestion ambiguous the 54 isolates into 31 PTs, while MLST based on 15 housekeeping genes differentiated the 54 isolates into 7 STs, with 1 ST (ST227) being novel. Although MLST provided suitable discrimination in S. flexneri subtyping, PFGE and MLVA might both 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. flexneri*.

Methods

Bacterial isolates and bacteriological examination

Animal-based active surveillance was conducted in 3321 diarrhea calves from five provinces in northwestern China from 2014 to 2016. A total of 54 isolates of *S. flexneri*, including six serotypes, were isolated and analyzed. Biochemical tests were performed on *S. flexneri* using API20E test strips (bioMerieux Vitek, Marcy-l' Etoile, France) according to the manufacturer's recommendations.

Preparation of DNA templates

The DNA templates for PCR (virulence factors, MLST, MLVA) were directly extracted from bacterial colonies using the boiled lysate method, as previous report [39].

Detection of virulence factors

All 54 strains were tested by PCR for the presence of 8 virulence-associated genes, including *ipaH*, *ipaBCD*, *virA*, *ial*, *stx*, *set1A*, *set1B*, and *sen* according to published procedures [40-42]. PCR reactions were performed according to published protocols and the primers sequences were listed in Table S1.

Multilocus sequence typing (MLST)

All isolates were subjected to MLST according to the protocols described in the EcMLST database (http://www.shigatox.net/ecmlst). The PCR products were bidirectionally sequenced, and the sequences of the 15 housekeeping genes were edited by using SeqMan 7.0. Each unique allele was assigned a different number, and the allelic profile (string of fifteen allelic loci) was used to define each isolate's sequence type (ST) [43]. Clustering and minimum spanning tree (MST) analyses were used to infer relationships among the isolates using the fingerprint analysis software BioNumerics (version 7.1).

Multiple-locus variable number tandem repeat analysis (MLVA)

MLVA analysis of 8 VNTR loci (SF3, SF4, SF6, SF7, SF8, SF9, SF10 and SF25) was performed using a previously described method [33]. The forward primer for each primer set was labelled at its 5' end with an ABI compatible dye: HEX, 6'-FAM, TAMRA, and ROX (Table S2). In these cases, the loci were individually amplified, with each 20 μL PCR mixture containing 1 μL each primer, 1 μL DNA template, 10 μL Taq MasterMix (Takara, Japan) and deionized water to a final volume of 20 μL. PCR was performed with a denaturing step of 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s and a final extension at 72°C for 5 min at the final step.

The PCR products were analyzed by capillary electrophoresis on an ABI Prism 3730 XL Genetic Analyzer with the GeneScan 500 LIZ Size Standard as previously described [44]. The number of repeat units for each allele was converted from the length of the amplicon. The copy number of each VNTR locus was subjected to cluster analysis using the MST algorithm and the categorical coefficient provided in the BioNumerics software. Each unique allelic string was designated a unique MLVA type (MT). A dendrogram was constructed by UPGMA clustering based on categorical coefficient analysis [33,45].

Pulsed field gel electrophoresis (PFGE)

DNA fingerprinting was performed by PFGE with the restriction enzyme *Not*I (TaKaRa; Japan) according to the international standards set by the CDC. PFGE images were photographed with a Universal Hood II (Bio-Rad; USA) and analyzed with BioNumerics using the Dice similarity coefficient, unweighted pair-group method with the arithmetic mean (UPGMA) and 1.0% band position tolerance. A PFGE type (PT) was defined as a pattern with one or more DNA bands different from other patterns.

Abbreviations

VTs: Virulence gene profile types; BTs: MLVA: Multiple-locus variable number tandem repeat analysis; VNTR: Variable Number of Tandem Repeats; PFGE: Pulsed-field gel electrophoresis; MLST: Multilocus sequence typing.

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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Tables

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

Biotype		Total (n=54)	Isolates					
			1a (n=5)	2a (n=26)	2b (n=4)	4a(n=6)	6 (n=8)	Xv (n=5)
BT1	glucose+, mannose+, arabinose-, melibiose+	2 (3.70%)	0	2(7.69%)	0	0	0	0
BT2	glucose+, mannose+, arabinose+, melibiose-	9 (16.67%)	0	1(3.85%)	0	0	8(100%)	0
BT3	glucose+, mannose-, arabinose+, melibiose+	5(9.26%)	0	1(3.85%)	0	4(66.67%)	0	0
BT4	glucose+, mannose+, arabinose+, melibiose+	38(70.37%)	5 (100%)	22(84.62%)	4(100%)	2(33.33%)	0	5(100%)

Table 2 Statistical the rate of each virulence genes types in *S.flexneri isolates.*

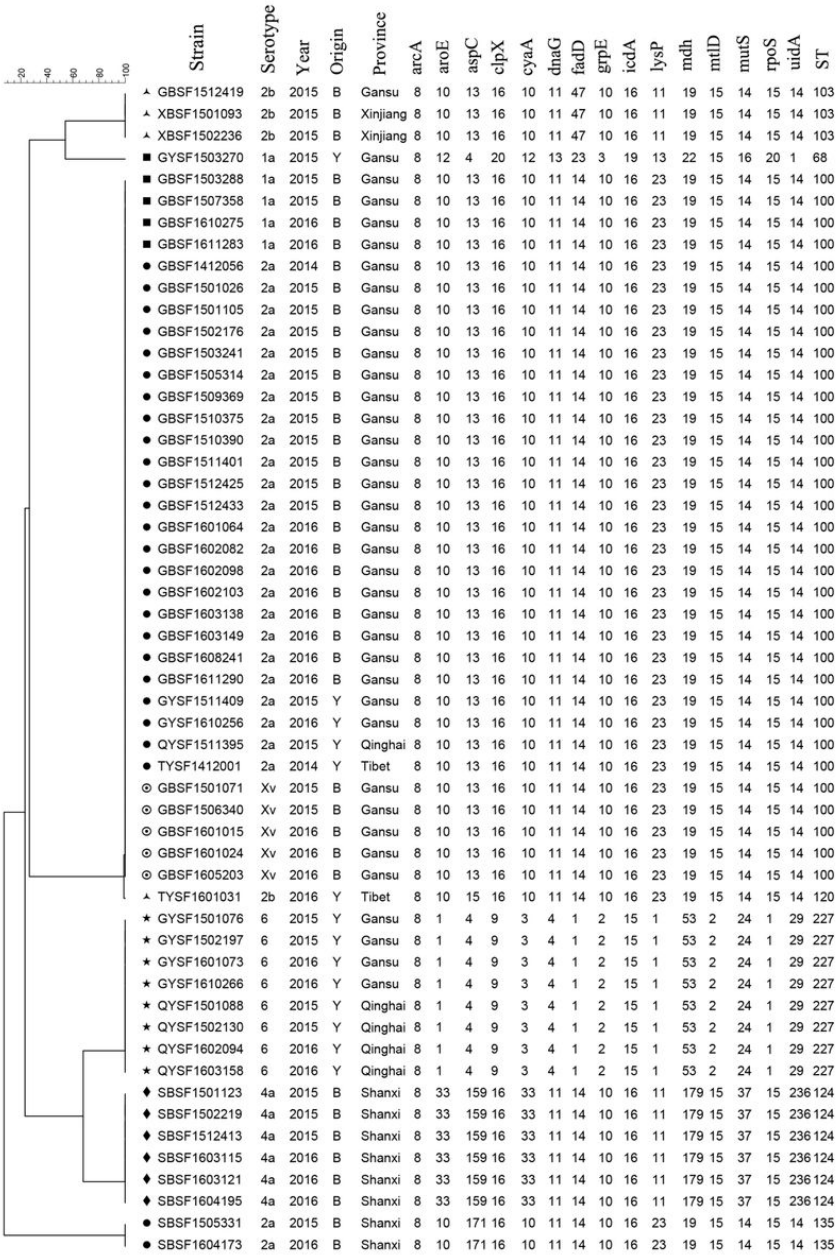
Virulence genes types		Total (n=54)	Serotype distribution					
			1a (n=5)	2a (n=26)	2b (n=4)	4a (n=6)	6 (n=8)	Xv (n=5)
VT1	*ipaH+virA+ipaBCD-ial-sen-set1A-set1B-*	4 (7.41%)	0	0	0	4 (66.67%)	0	0
VT2	*ipaH+virA+ipaBCD+ial-sen-set1A-set1B-*	5 (9.26%)	1 (20%)	0	0	0	4 (50%)	0
VT3	*ipaH+ virA+ipaBCD+ial-sen+set1A-set1B-*	3 (5.56%)	0	0	0	0	0	3 (60%)
VT4	*ipaH+ virA+ipaBCD+ial+sen+set1A-set1B-*	16 (29.63%)	4 (80%)	0	4 (100%)	2 (33.33%)	4 (50%)	2 (40%)
VT5	*ipaH+virA+ipaBCD+ial+sen-set1A+set1B+*	2 (3.7%)	0	2 (7.69%)	0	0	0	0
VT6	*ipaH+virA+ipaBCD+ial+sen+set1A+set1B+*	24 (44.44%)	0	24 (92.31%)	0	0	0	0

Figures

Strain name	Serotype	virulence genes							VT
		ipaH	*virA*	*ipaBCD*	*ial*	*sen*	*set1A*	*set1B*	*stx*	
TYSF1412001	2a									6
GBSF1412056	2a									6
GBSF1501026	2a									6
GBSF1501071	Xv									4
GYSF1501076	6									2
QYSF1501088	6									4
XBSF1501093	2b									4
GBSF1501105	2a									6
SBSF1501123	4a									1
QYSF1502130	6									4
GBSF1502176	2a									6
GYSF1502197	6									2
SBSF1502219	4a									1
XBSF1502236	2b									4
GBSF1503241	2a									6
GYSF1503270	1a									2
GBSF1503288	1a									4
GBSF1505314	2a									6
SBSF1505331	2a									5
GBSF1506340	Xv									3
GBSF1507358	1a									4
GBSF1509369	2a									6
GBSF1510375	2a									6
GBSF1510390	2a									6
QYSF1511395	2a									6
GBSF1511401	2a									6
GYSF1511409	2a									6
SBSF1512413	4a									4
GBSF1512419	2b									4
GBSF1512425	2a									6
GBSF1512433	2a									6
GBSF1601015	Xv									4
GBSF1601024	Xv									3
TYSF1601031	2b									4
GBSF1601064	2a									6
GYSF1601073	6									2
GBSF1602082	2a									6
QYSF1602094	6									4
GBSF1602098	2a									6
GBSF1602103	2a									6
SBSF1603115	4a									1
SBSF1603121	4a									4
GBSF1603138	2a									6
GBSF1603149	2a									6
QYSF1603158	6									4
SBSF1604173	2a									5
SBSF1604195	4a									1
GBSF1605203	Xv									3
GBSF1608241	2a									6
GYSF1610256	2a									6
GYSF1610266	6									2
GBSF1610275	1a									4
GBSF1611283	1a									4
GBSF1611290	2a									6
Total		54	54	50	42	43	26	26	0	
Rate		**100%**	**100%**	**92.59%**	**77.78%**	**79.63%**	**48.15%**	**48.15%**	**0**	

Figure 1

Statistical analysis of the presence of virulence factors in S. flexneri isolates. Red=present; Blue=absent.



Strain	Serotype	Year	Origin	Province	arcA	aroE	aspC	clpX	cyaA	dnaG	fadD	grpE	icdA	lysP	mdh	mtlD	mutS	rpoS	uidA	ST
GBSF1512419	2b	2015	B	Gansu	8	10	13	16	10	11	47	10	16	11	19	15	14	15	14	103
XBSF1501093	2b	2015	B	Xinjiang	8	10	13	16	10	11	47	10	16	11	19	15	14	15	14	103
XBSF1502236	2b	2015	B	Xinjiang	8	10	13	16	10	11	47	10	16	11	19	15	14	15	14	103
GYSF1503270	1a	2015	Y	Gansu	8	12	4	20	12	13	23	3	19	13	22	15	16	20	1	68
GBSF1503288	1a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1507358	1a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1610275	1a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1611283	1a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1412056	2a	2014	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1501026	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1501105	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1502176	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1503241	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1505314	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1509369	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1510375	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1510390	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1511401	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1512425	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1512433	2a	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1601064	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1602082	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1602098	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1602103	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1603138	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1603149	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1608241	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1611290	2a	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GYSF1511409	2a	2015	Y	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GYSF1610256	2a	2016	Y	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
QYSF1511395	2a	2015	Y	Qinghai	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
TYSF1412001	2a	2014	Y	Tibet	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1501071	Xv	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1506340	Xv	2015	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1601015	Xv	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1601024	Xv	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
GBSF1605203	Xv	2016	B	Gansu	8	10	13	16	10	11	14	10	16	23	19	15	14	15	14	100
TYSF1601031	2b	2016	Y	Tibet	8	10	15	16	10	11	14	10	16	23	19	15	14	15	14	120
GYSF1501076	6	2015	Y	Gansu	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
GYSF1502197	6	2015	Y	Gansu	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
GYSF1601073	6	2016	Y	Gansu	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
GYSF1610266	6	2016	Y	Gansu	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
QYSF1501088	6	2015	Y	Qinghai	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
QYSF1502130	6	2015	Y	Qinghai	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
QYSF1602094	6	2016	Y	Qinghai	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
QYSF1603158	6	2016	Y	Qinghai	8	1	4	9	3	4	1	2	15	1	53	2	24	1	29	227
SBSF1501123	4a	2015	B	Shanxi	8	33	159	16	33	11	14	10	16	11	179	15	37	15	236	124
SBSF1502219	4a	2015	B	Shanxi	8	33	159	16	33	11	14	10	16	11	179	15	37	15	236	124
SBSF1512413	4a	2015	B	Shanxi	8	33	159	16	33	11	14	10	16	11	179	15	37	15	236	124
SBSF1603115	4a	2016	B	Shanxi	8	33	159	16	33	11	14	10	16	11	179	15	37	15	236	124
SBSF1603121	4a	2016	B	Shanxi	8	33	159	16	33	11	14	10	16	11	179	15	37	15	236	124
SBSF1604195	4a	2016	B	Shanxi	8	33	159	16	33	11	14	10	16	11	179	15	37	15	236	124
SBSF1505331	2a	2015	B	Shanxi	8	10	171	16	10	11	14	10	16	23	19	15	14	15	14	135
SBSF1604173	2a	2016	B	Shanxi	8	10	171	16	10	11	14	10	16	23	19	15	14	15	14	135

Figure 2

MLST clustering tree of S. flexneri isolates isolated from 2014 to 2016 from diarrhea calves. The 54 isolates were analyzed using a 15 allele MLST as described in the Materials and Methods.

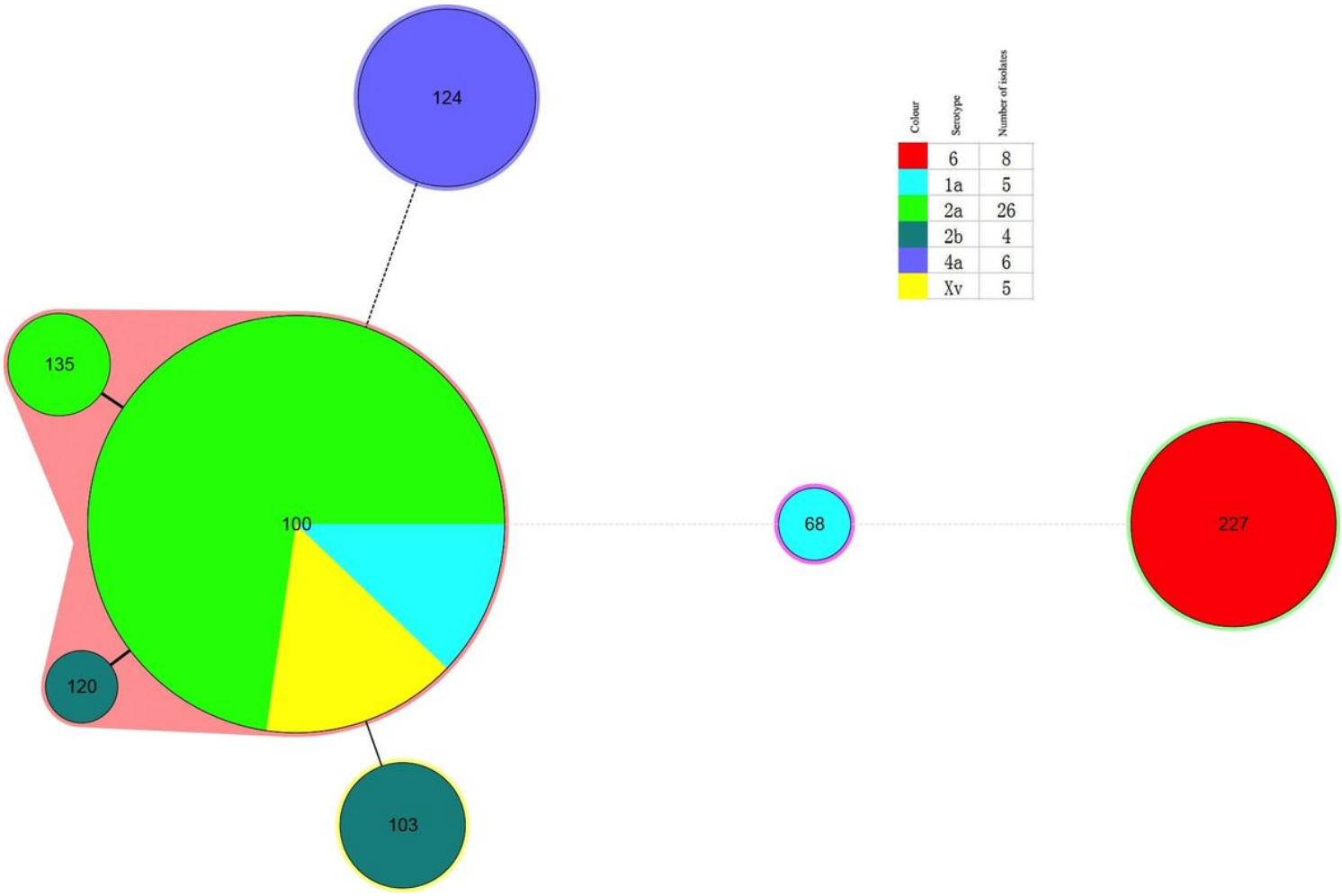
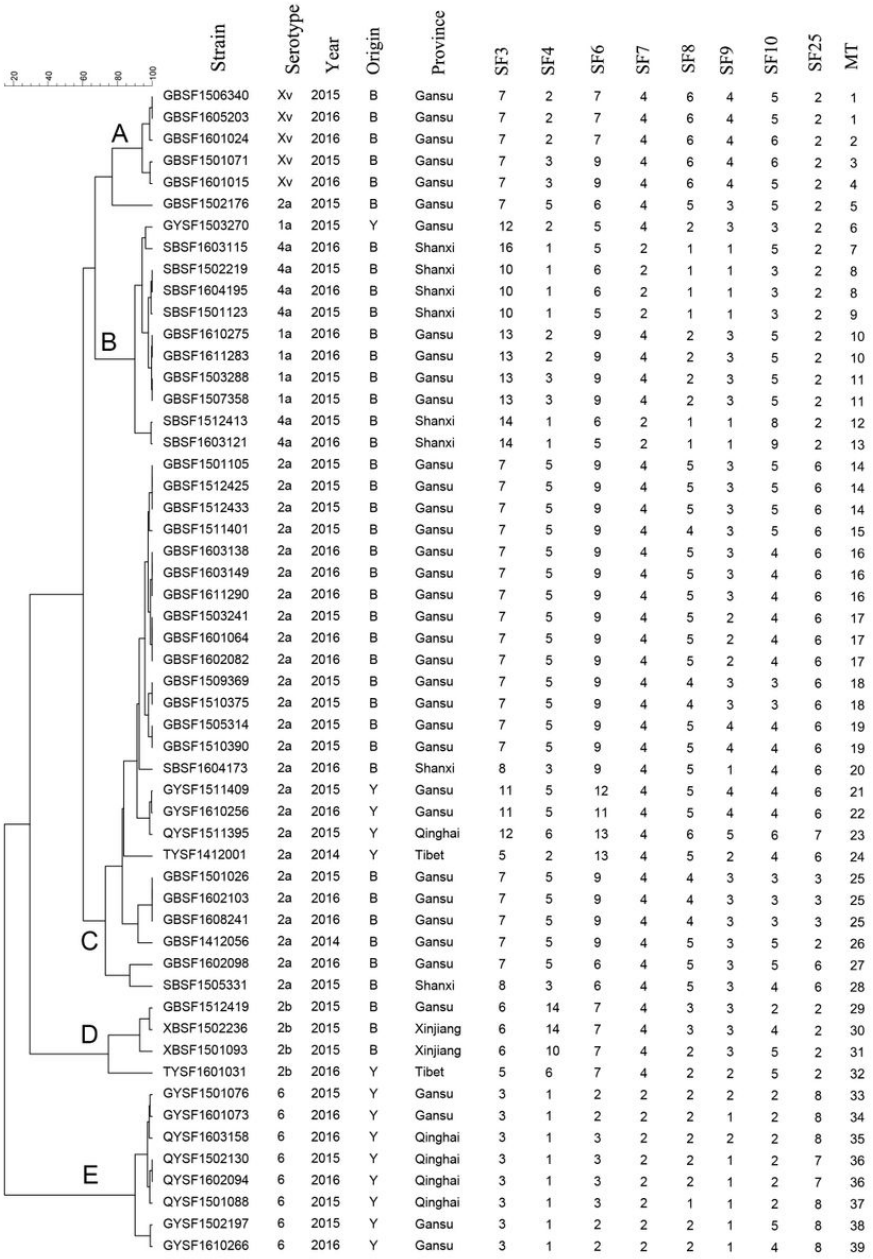


Figure 3

Minimum spanning tree of the 54 S. flexneri isolates from diarrhea calves based on multilocus sequence typing (MLST). The minimum spanning tree was constructed using the 7 identified STs obtained from the 54 isolates using BioNumerics Software. Each circle corresponds to a single ST. The shadow zones in different colors correspond to different serotypes. The size of the circle is proportional to the number of isolates, and the color within the circles represents the serotype of the isolates. The corresponding color, serotype, number of isolates and background information are shown to the right of the minimum spanning tree.



Strain	Serotype	Year	Origin	Province	SF3	SF4	SF6	SF7	SF8	SF9	SF10	SF25	MT
GBSF1506340	Xv	2015	B	Gansu	7	2	7	4	6	4	5	2	1
GBSF1605203	Xv	2016	B	Gansu	7	2	7	4	6	4	5	2	1
GBSF1601024	Xv	2016	B	Gansu	7	2	7	4	6	4	6	2	2
GBSF1501071	Xv	2015	B	Gansu	7	3	9	4	6	4	6	2	3
GBSF1601015	Xv	2016	B	Gansu	7	3	9	4	6	4	5	2	4
GBSF1502176	2a	2015	B	Gansu	7	5	6	4	5	3	5	2	5
GYSF1503270	1a	2015	Y	Gansu	12	2	5	4	2	3	3	2	6
SBSF1603115	4a	2016	B	Shanxi	16	1	5	2	1	1	5	2	7
SBSF1502219	4a	2015	B	Shanxi	10	1	6	2	1	1	3	2	8
SBSF1604195	4a	2016	B	Shanxi	10	1	6	2	1	1	3	2	8
SBSF1501123	4a	2015	B	Shanxi	10	1	5	2	1	1	3	2	9
GBSF1610275	1a	2016	B	Gansu	13	2	9	4	2	3	5	2	10
GBSF1611283	1a	2016	B	Gansu	13	2	9	4	2	3	5	2	10
GBSF1503288	1a	2015	B	Gansu	13	3	9	4	2	3	5	2	11
GBSF1507358	1a	2015	B	Gansu	13	3	9	4	2	3	5	2	11
SBSF1512413	4a	2015	B	Shanxi	14	1	6	2	1	1	8	2	12
SBSF1603121	4a	2016	B	Shanxi	14	1	5	2	1	1	9	2	13
GBSF1501105	2a	2015	B	Gansu	7	5	9	4	5	3	5	6	14
GBSF1512425	2a	2015	B	Gansu	7	5	9	4	5	3	5	6	14
GBSF1512433	2a	2015	B	Gansu	7	5	9	4	5	3	5	6	14
GBSF1511401	2a	2015	B	Gansu	7	5	9	4	4	3	5	6	15
GBSF1603138	2a	2016	B	Gansu	7	5	9	4	5	3	4	6	16
GBSF1603149	2a	2016	B	Gansu	7	5	9	4	5	3	4	6	16
GBSF1611290	2a	2016	B	Gansu	7	5	9	4	5	3	4	6	16
GBSF1503241	2a	2015	B	Gansu	7	5	9	4	5	2	4	6	17
GBSF1601064	2a	2016	B	Gansu	7	5	9	4	5	2	4	6	17
GBSF1602082	2a	2016	B	Gansu	7	5	9	4	5	2	4	6	17
GBSF1509369	2a	2015	B	Gansu	7	5	9	4	4	3	3	6	18
GBSF1510375	2a	2015	B	Gansu	7	5	9	4	4	3	3	6	18
GBSF1505314	2a	2015	B	Gansu	7	5	9	4	5	4	4	6	19
GBSF1510390	2a	2015	B	Gansu	7	5	9	4	5	4	4	6	19
SBSF1604173	2a	2016	B	Shanxi	8	3	9	4	5	1	4	6	20
GYSF1511409	2a	2015	Y	Gansu	11	5	12	4	5	4	4	6	21
GYSF1610256	2a	2016	Y	Gansu	11	5	11	4	5	4	4	6	22
QYSF1511395	2a	2015	Y	Qinghai	12	6	13	4	6	5	6	7	23
TYSF1412001	2a	2014	Y	Tibet	5	2	13	4	5	2	4	6	24
GBSF1501026	2a	2015	B	Gansu	7	5	9	4	4	3	3	3	25
GBSF1602103	2a	2016	B	Gansu	7	5	9	4	4	3	3	3	25
GBSF1608241	2a	2016	B	Gansu	7	5	9	4	4	3	3	3	25
GBSF1412056	2a	2014	B	Gansu	7	5	9	4	5	3	5	2	26
GBSF1602098	2a	2016	B	Gansu	7	5	6	4	5	3	5	6	27
SBSF1505331	2a	2015	B	Shanxi	8	3	6	4	5	3	4	6	28
GBSF1512419	2b	2015	B	Gansu	6	14	7	4	3	3	2	2	29
XBSF1502236	2b	2015	B	Xinjiang	6	14	7	4	3	3	4	2	30
XBSF1501093	2b	2015	B	Xinjiang	6	10	7	4	2	3	5	2	31
TYSF1601031	2b	2016	Y	Tibet	5	6	7	4	2	2	5	2	32
GYSF1501076	6	2015	Y	Gansu	3	1	2	2	2	2	2	8	33
GYSF1601073	6	2016	Y	Gansu	3	1	2	2	2	1	2	8	34
QYSF1603158	6	2016	Y	Qinghai	3	1	3	2	2	2	2	8	35
QYSF1502130	6	2015	Y	Qinghai	3	1	3	2	2	1	2	7	36
QYSF1602094	6	2016	Y	Qinghai	3	1	3	2	2	1	2	7	36
QYSF1501088	6	2015	Y	Qinghai	3	1	3	2	1	1	2	8	37
GYSF1502197	6	2015	Y	Gansu	3	1	2	2	2	1	5	8	38
GYSF1610266	6	2016	Y	Gansu	3	1	2	2	2	1	4	8	39

Figure 4

Relationship of S. flexneri isolates isolated from diarrhea calves based on MLVA. Isolates were analyzed using an eight VNTR loci MLVA scheme. The dendrogram was constructed using UPGMA. The corresponding MLVA type with the copy numbers of the eight VNTRs, serotype, and background information are shown to the right of the dendrogram.

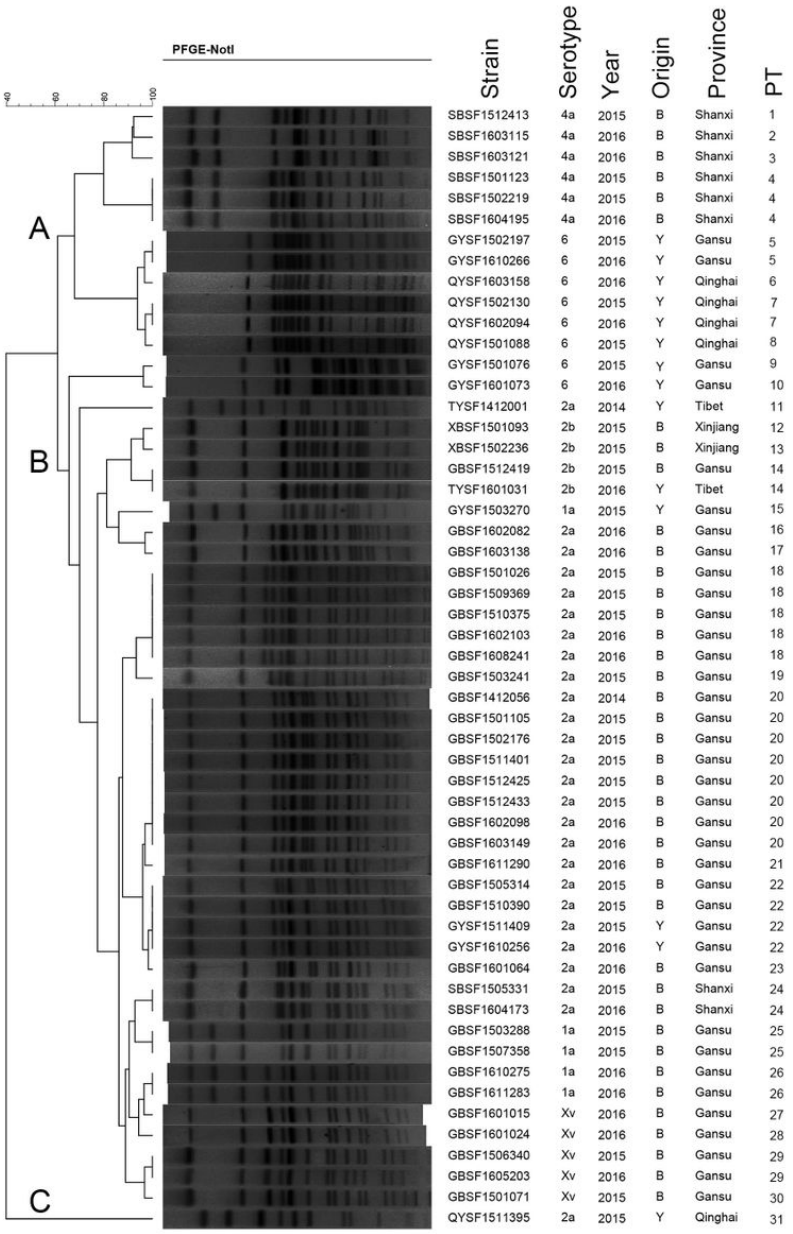


Figure 5

Dendrogram of 54 NotI-digested S. flexneri isolates based on the cluster analysis of PFGE patterns. The dendrogram was constructed using the UPGMA clustering method. The corresponding antibiotic resistance profile, PFGE pattern and background information for each strain are listed on the right side of the dendrogram.

Supplementary Files

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- supplement11.docx
- supplement12.docx
- supplement13.docx