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

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Research

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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.

Methods: From 2014 to 2016, 54 *S. flexneri* isolates were collected from diarrhea, and their biochemical characteristics were determined according to API20E and virulence factors via PCR. The molecular characteristics of the isolates were studied by MLST, MLVA and PFGE.

Results: Fifty-four *S. flexneri* isolates possessed four typical biochemical characteristics of *Shigella*. The prevalences of *ipaH*, *virA*, *ipaBCD*, *ial*, *sen*, *set1A*, and *set1B* were 100%, 100%, 77.78%, 79.63%, 48.15% and 48.15%, respectively. None of the studied strains possessed the *stx* gene. Regarding the differences in virulence factor distributions, the 54 *S. flexneri* isolates fell into seven gene profile types. Among these VTs, VT4 and VT6 were the most common, accounting for 74.07% of all VTs. MLVA based on 8 VNTR loci discriminated the isolates into 39 different MTs, PFGE based on *NotI* digestion divided the 54 isolates into 31 PTs, and MLST based on 15 housekeeping genes differentiated the isolates into 7 STs, with 1 ST (ST227) being novel.

Conclusion: Our findings provide baseline information on the distribution of virulence genes in and the molecular characteristics of *S. flexneri* collected from diarrheal calves, which is a potential threat to public safety. These data will be important for addressing clinical and epidemiological issues regarding Shigellosis.

Background

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

The pathogenesis of shigellosis is related to various virulence factors located in the chromosome or large virulent inv plasmids [6]. These different virulence factors are associated with the colonization of intestinal cells and intracellular invasion, which may lead to clinical manifestations, such as intestinal inflammatory responses and watery diarrhea [1]. Chromosomal DNA and/or heavy plasmid DNA encoding the *ipaH* gene (invasion plasmid antigen H) allows bacterial cell-to-cell movement and dissemination within epithelial cells of the intestine, while the plasmid-encoded gene *ial* (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 present in all *S. flexneri* 2a. *Shigella* enterotoxin 2 (ShET-2), encoded by the gene *sen*, is located on a large plasmid associated with the virulence of *Shigella* and is found in many, but not all, *Shigella* of different serotypes and in enteroinvasive *Escherichia coli* (EIEC) [11, 12]. ShET-1 and ShET-2, in addition to their enterotoxic activity, play important roles in the transport of electrolytes and water in the intestine [12]. *VirA* is

located on large virulent plasmids and acts as a virulence determinant in intercellular spreading and invasion [13]. On the other hand, the type III secretion system (T3SS), which is responsible for bacterial entry, is 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]. 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, reports of *Shigella* infection in new hosts, including monkeys, rabbits, calves, fish, chickens and piglets, have emerged [4, 17–21]. In recent years, our group isolated *S. dysenteriae*, *S. flexneri*, and *S. sonnei* from cows and conducted a series of studies. Although *Shigella* is becoming a huge threat to animals, there is limited information on the genetic background of local strains. Therefore, the objective of the study was to molecularly genotype and determine the genetic relatedness diversity of local *S. flexneri* strains using MLST, MLVA and PFGE.

Materials And 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 (bioMérieux 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. Briefly, a single colony from an overnight culture at 37 °C on LB agar was suspended in 30 µL sterile molecular grade water and boiled at 100 °C for 10 min. The sample was immediately cooled on ice for 5 min and centrifuged at 13,000 g at 4 °C for 10 min. The supernatant, containing DNA, was transferred into a fresh tube for use [22].

Detection of virulence factors

All *S. flexneri* isolates were examined for the presence of seven virulence factors, including invasion plasmid antigen H (ipaH), invasion plasmid antigen genes (ipaBCD), inactivates rab proteins (virA), invasion associated locus (ial), Shiga toxin gene (stx), and *Shigella* enterotoxin genes (set1A, set1B, and sen), by PCR according to published procedures [23–25]. The primers for these virulence factors are listed in Table S1. Amplification products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide.

Multilocus Sequence Typing (MLST)

MLST analysis of 15 housekeeping genes was performed for each isolate according to the protocols described on the EcMLST website (<http://www.shigatox.net/ecmlst>). The PCR amplification conditions of the 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, Japan). 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) [26]. 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 [27]. 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 [28]. 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 [27, 29].

Pulsed field gel electrophoresis (PFGE)

DNA fingerprinting was performed by PFGE with the restriction enzyme NotI (TaKaRa; Japan) according to the international standards set by the CDC. The *Salmonella enterica* serotype Braenderup strain H9812 was digested with the restriction enzyme XbaI and used as a molecular size marker for this analysis. 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 pulsotype (PT) was defined as a pattern with one or more DNA bands different from other patterns.

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.

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%)

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%).

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

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. *NotI*-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, as an important invasive enteric infectious pathogen, may appear in different sporadic, epidemic, and pandemic forms [3], which remains the hallmark etiology of inflammatory diarrhea and dysentery and presents a serious challenge to public health, especially in developing countries and regions with substandard hygiene and poor quality water supplies [30]. All four species of *Shigella* can cause shigellosis, but *S. flexneri* is the most prevalent bacterial agent in shigellosis [31]. The conventional hosts of this pathogen are constrained in primates; however, its host range has been expanded to many animals in recent decades [4], which presents a challenge for controlling *Shigella* infection.

The pathogenesis of *Shigella* is attributed to the organism's ability to invade, replicate and spread intercellularly within the colonic epithelium. Virulence factors are responsible for the invasion of virulent *Shigella* strains into intestinal epithelial cells, causing dysentery and other intestinal clinical signs in hosts [32], and its pathogenesis is often multifactorial and coordinately regulated [33]. Virulence factors have become a significant marker of pathogenic bacteria.

According to the virulence factors examined, the *Shigella* isolates used in the present study had vast genetic diversity. The results of our study revealed that *ipaH* and *virA* were found in each strain. Previous reports have shown 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, the *ipaH* gene is often an appealing target for use as a diagnostic tool to detect *Shigella*, even in the absence of the plasmid [34]. *VirA* was initially thought to play a role in *Shigella* invasion; however, structural analysis showed that *VirA* lacks the suggested papain-like protease activity for tubulin cleavage. *VirA* belongs to a family of GTPase-activating proteins, and evidence indicates that

it is involved in the lysis of the single membrane entry vacuole. A previous study has shown that VirA is often harbored in *Shigella* and is an important determinant for bacterial penetration into host cells and actin nucleation at one end of the bacterium [9, 35].

Among those virulence factors, T3SS is essential for host cell invasion and intracellular survival, whereas IpaB, IpaC, and IpaD are key factors of virulent *Shigella* [9, 36, 37]. Unlike the *ipaH* gene, the *ial* gene is not common. The *ipaH* gene is only located on the *inv* plasmid, which is less stable to storage/subculturing than chromosomal genes [6–8]. Our results show the high invasive capacity of *ial* genes among the studied isolates. 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 can alter electrolyte and water transport in the small intestine, which can cause diarrhea and dehydration [30]. ShET-1 is located on chromosomes and encodes the *set1* (A and B subunit) gene, which is almost exclusively found in several *S. flexneri* serotype 2 isolates and is rarely found in other serotypes [38]. In agreement with previous studies, our study showed that *set1A* and *set1B* were only detected in the *S. flexneri* 2a strain. The plasmid encoding ShET-2 (encoded by *sen*) is known to be an enterotoxin hemolysin that elicits an inflammatory response during *Shigella* invasion [12, 30]. As reported, both *sen* and *set* enterotoxins are significantly associated with bloody diarrhea [30]. However, unlike ShET-1, ShET-2 could be harbored by different species of *Shigella*.

Molecular characterization of strains is important for epidemiological studies. Fewer data, however, 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, such as MLST [39], PFGE [40] and MLVA [27], have been used to characterize *Shigella* isolates. These methods are primarily based on the difference of strain genetics according to the analysis of phylogenetic relationships.

MLST is an important source of sequence data for comparative genetics, providing a tool for exploring molecular evolutionary methods among bacteria [41]. Based on the 15 housekeeping genes and analysis of the EcMLST database, MLST allows the comparison of data from different laboratories. Our results suggested that the predominant ST was ST 100, which has previously been found in human *S. flexneri* isolates [42, 43]. 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 powerful tools that can provide a satisfactory level of discrimination. MLVA may not be appropriate as a subtyping tool for phylogenetic analysis of different bacterial species or serotypes [44]. Nevertheless, MLVA is a commonly used typing tool that has been used for establishing genetic relatedness and performing phylogenetic analysis among strains of monomorphic species. In our study, the 54 *S. flexneri* isolates were discriminated into 39 different MTs and clustered into 5 groups with approximately 20% similarity. 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.

PFGE is also a powerful and broadly 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 [45]. Thirty-one low similarity 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 natural prevalent *S. flexneri* in cows harbored 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 genetically characterize the 54 isolates. MLVA based on 8 VNTR loci discriminated the 54 isolates into 39 different MTs, PFGE based on NotI digestion divided 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*.

Declarations

Ethics approval and consent to participate

Permission for accessing specific locations, information regarding the number of samples harvested, and an associated permit number for calves were not required, and no endangered or protected species were involved or harmed during this 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.

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Figures

Strain name	Serotype	virulence genes							VT
		<i>ipaH</i>	<i>virA</i>	<i>ipaBCD</i>	<i>ial</i>	<i>sen</i>	<i>set1A</i>	<i>set1B</i>	
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.

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 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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