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

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Zhen Zhu  
College of Life Science and Food Engineering, Hebei University of Engineering

Mingze Cao  
Hebei University of Engineering

Weiwei Wang  
Chinese Academy of Agricultural Sciences Lanzhou Institute of Husbandry and Pharmaceutical Sciences

Guanhui Liu  
Hebei University of Engineering

Xuzheng Zhou  
Chinese Academy of Agricultural Sciences Lanzhou Institute of Husbandry and Pharmaceutical Sciences

Bing Li  
Chinese Academy of Agricultural Sciences Lanzhou Institute of Husbandry and Pharmaceutical Sciences

Yuxiang Shi  
Hebei University of Engineering

Jiyu Zhang  
jiyuzhang123@163.com  
Lanzhou Institute of husbandry and Pharmaceutical Sciences of CAAS  
*Corresponding Author*

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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 (bioMerieux Vitek, Marcy-I’ 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**

| 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           | 4 (66.67%) | 0        | 0          |
| VT2                   |               |             |             |            |          |            |
| ipaH + virA +ipaBCD-ial-sen-set1A-set1B- | 5 (9.26%)     | 1 (20%)     | 0           | 0          | 4 (50%)  | 0          |
| VT3                   |               |             |             |            |          |            |
| ipaH + virA +ipaBCD-ial-sen-set1A-set1B- | 3 (5.56%)     | 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          |
| VT6                   |               |             |             |            |          |            |
| ipaH + virA +ipaBCD-ial-sen-set1A-set1B+ | 24 (44.44%)   | 0           | 24 (92.31%) | 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 (ipaH1.4, ipaH2.5,
ipaH4.5, ipaH7.8 and ipaH9.8) 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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**Authors' information**

College of Life Science and Food Engineering, Hebei University of Engineering, Hanshan
District, Handan, China

Key Laboratory of New Animal Drug Project of Gansu Province, Key Laboratory of Veterinary Pharmaceutical Development of Ministry of Agriculture, Lanzhou Institute of Husbandry and Pharmaceutical Sciences of CAAS, Jiangouyan, Qilihe District, Lanzhou, PR China

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Figures

| Strain name | Serotype | Serotype | virulence genes | VT |
|-------------|----------|----------|-----------------|----|
| TYSF1412001 | 2a       | virA     | ipaH           | 6  |
| GBSF1412056 | 2a       | ipaH     | virA           | 6  |
| GBSF1501026 | 2a       | ipaH     | ipaBCD         | 6  |
| GBSF1501071 | Xv       | ipaH     | ipaBCD         | 4  |
| GYSF1501076 | 6        | ipaH     | lal            | 2  |
| QYSF1501088 | 6        | ipaH     | lal            | 4  |
| XBSF1501093 | 2b       | ipaH     | lal            | 4  |
| GBSF1501105 | 2a       | ipaH     | lal            | 6  |
| SBSF1501123 | 4a       | ipaH     | lal            | 6  |
| QYSF1502130 | 6        | ipaH     | lal            | 4  |
| GBSF1502176 | 2a       | ipaH     | lal            | 6  |
| GYSF1502197 | 6        | ipaH     | lal            | 2  |
| SBSF1502219 | 4a       | ipaH     | lal            | 1  |
| XBSF1502236 | 2b       | ipaH     | lal            | 4  |
| GBSF1503241 | 2a       | ipaH     | lal            | 6  |
| GYSF1503270 | 1a       | ipaH     | lal            | 2  |
| GBSF1503288 | 1a       | ipaH     | lal            | 4  |
| GBSF1505314 | 2a       | ipaH     | lal            | 6  |
| SBSF1505331 | 2a       | ipaH     | lal            | 5  |
| GBSF1506340 | Xv       | ipaH     | lal            | 3  |
| GBSF1507358 | 1a       | ipaH     | lal            | 4  |
| GBSF1509369 | 2a       | ipaH     | lal            | 6  |
| GBSF1510375 | 2a       | ipaH     | lal            | 6  |
| GBSF1510390 | 2a       | ipaH     | lal            | 6  |
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.
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.
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.
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

This is a list of supplementary files associated with the primary manuscript. Click to download.

Table S1.docx
Table S2.docx