PATHOGENS AND GLOBAL HEALTH
2019, VOL. 113, NO. 4, 173–179
https://doi.org/10.1080/20477724.2019.1632062

Virulence gene profiles of *Shigella* species isolated from stool specimens in India: its association with clinical manifestation and antimicrobial resistance

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ABSTRACT
Shigella is the major cause of bacillary dysentery worldwide, especially in developing countries. There are several virulence factors essential for the organism to be virulent which are generally present in the virulence plasmid and on chromosomal pathogenicity islands. The present study was undertaken to determine the virulence gene profile of *Shigella spp* isolated from a clinical specimen and to study their significant association with common clinical symptoms and antimicrobial resistance. Sixty *Shigella* whole genome sequences, including 22 *S. flexneri*, 14 *S. sonnei*, 17 *S. boydii* and 7 *S. dysenteriae* were analyzed for the presence of virulence genes. The gene found predominantly in this study were *ipa*H (90%) followed by *sig*A (83%), and *lpf*A (78%) respectively. The virulence genes were significantly higher in *S. flexneri*, particularly in serotype 2 compared to *S. sonnei*. Interestingly, a significant association was observed between *sig*A gene and fever whereas *sep*A and *sig*A were found to be associated with diarrhea. Among the studied *Shigella* isolates, the presence of virulence genes was found higher in isolates resistant to more than three antibiotic classes. The present work revealed the varying incidence of virulence determinants among different *Shigella* serogroups and shows their contribution to disease severity.

KEYWORDS
Shigella spp; virulence; *sep*A; *sig*A; *lpf*A; *ipa*H

Introduction

Shigella is a pathogen restricted to humans and a common cause of diarrhea in developing countries. Among the four *Shigella* serogroups, *S. flexneri* is the most prevailing species in developing nations followed by *S. sonnei*, while *S. boydii* and *S. dysenteriae* are less frequently isolated [1]. The spectrum of the disease varies from mild to severe infections.

Although shigellosis is self-limiting, use of antibiotics reduces the duration of illness and thus reduces the person to person transmission. Recently, WHO has recommended ciprofloxacin as the first choice for treating dysentery in adults and children, and azithromycin, cefixime or ceftriaxone as the second choice. Trimethoprim-sulfamethoxazole can be considered as an alternative second-line drug but generally is recommended only when the susceptibility is known or based on the local surveillance data [2].

Several virulence factors have been reported to be associated with the pathogenesis of shigellosis, which may be located either in the chromosome or plasmid. The virulence *inv* plasmid is an essential virulence determinant of *Shigella spp* which encodes the molecular machinery necessary for tissue invasion and intracellular survival [3]. The key factor for *Shigella* pathogenesis is the ability to invade and colonize intestinal epithelial cells [4]. The epithelial cell penetration and host response modification for cell to cell dissemination are generally mediated by an invasion-associated locus (*ial*), which is located on a plasmid and the invasion plasmid antigen H (*ipa*H) genes present in both plasmid and chromosome [5].

In addition, *Shigella spp* also produces distinct enterotoxins genes such as ShET-1, which encodes *set1*A and *set*1B genes located on the chromosome are found to be responsible for watery diarrhea. ShET-2, encoded by *sen* gene that are located on the virulence plasmid are believed to be involved in the invasion process [5,6]. Another important virulence factor related to *S. dysenteriae* is the presence of *stx* toxin which is released only during cell lysis [7].

There are very few studies that exist worldwide on the molecular characterization of *Shigella* virulence factors, and such reports are scarce in India. The present study was undertaken to determine the virulence gene profile of *Shigella spp* isolated in India and to study their association with common clinical symptoms and antimicrobial resistance.

Materials and methods

Isolate identification and serotyping

Sixty *Shigella* isolates were included in this study. These isolates were non-duplicate stool specimen obtained

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from patients suspected with enteric infection, collected as a part of routine diagnostics at the department of clinical microbiology, Christian Medical College, Vellore, India during the year 2011–2017. The stool specimen was inoculated on to the selective media such as deoxycholate citrate agar (DCA), xylose lysine deoxycholate agar (XLD) and on the MacConkey agar. The colonies that exhibit the characteristics of *Shigella spp* were selected and identified to the species level by biochemical tests [8] and serotypes were determined with commercially available polyclonal and monoclonal-specific antisera (Denka Seiken, Tokyo, Japan). Patient details were obtained through an electronic database maintained in the hospital and included for further analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of isolates was performed using Kirby-Bauer disc diffusion method against ampicillin (10 µg), trimethoprim/sulfamethoxazole (1.25/ 23.75 µg), nalidixic acid (30 µg), norfloxacin (10 µg), cefotaxime (30 µg) and cefixime (5 µg). The results were interpreted using breakpoints endorsed by the Clinical Laboratory Standards Institute (CLSI) guidelines 2017 [9]. Quality control strains used were *Escherichia coli* ATCC 35218 and *Escherichia coli* ATCC 25922 for the antibiotics tested.

Whole genome sequencing (WGS)

Genomic DNA was extracted using the QiaSymphony DNA extraction platform (Qiagen) as per manufacturer's instruction. The WGS was performed using Ion Torrent (PGM, Life technologies) with 400-bp read chemistry (Life Technologies, Carlsbad, CA) as described earlier [10]. The genome data was assembled *de novo* using AssemblerSPAdes v.5.0.0.0 embedded in Torrent suite server v.5.0.5. Sequences were annotated using PATRIC, the bacterial bioinformatics database and analysis resource (http://www.patricbrc.org) [11], and the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (http://www.ncbi.nlm. nih.gov/genomes/static/ Pipeline.html) [12].

The whole genome data was analyzed for the presence of virulence genes using an open access tool, VirulenceFinder 1.5 (https://cge.cbs.dtu.dk//services/ VirulenceFinder/) with the 90% threshold for identity and with 60% of minimum length coverage, where reads were mapped to the reference sequence in the database [13]. Species-specific virulence genes were also analyzed through PATRIC database. These whole genome shotgun sequences were deposited in DDBJ/ ENA/GenBank. This study also includes 20 *Shigella* genomes retrieved from our previous study for analysis [10].

Statistical analysis

Comparisons of variables were derived using two-tailed Chi-squared test. A p-value of <0.05 was considered statistically significant. The significance levels for important virulence genes were determined between the two predominant serogroups (*S. flexneri* and *S. sonnei*) and for the most common clinical symptoms of *Shigella*.

Results

Sixty *Shigella* isolates, including *S. flexneri* (n = 22), *S. sonnei* (n = 14), *S. boydii* (n = 17) and *S. dysenteriae* (n = 7) were analyzed in this study.

Antimicrobial susceptibility

Of the isolates tested, 63% were multi-drug resistant (MDR), resistant to three or more antibiotic classes. Mostly, the isolates were resistant to ampicillin (70%), trimethoprim/sulfamethoxazole (90%) and nalidixic acid (72%). While 33%, 27% and 20% resistance was observed for norfloxacin, cefotaxime and cefixime, respectively. One isolate was susceptible to all tested antimicrobials.

Analysis of WGS results

The prevalence of virulence genes among different *Shigella* serogroups is shown in Table 1. All isolates were positive for *ipa*H gene. The virulence genes found predominantly in the study isolates other than *ipa*H were *sig*A (83%), and *lpf*A (78%). The enterotoxin gene such as ShET-1 (28% *set*1A, 23% *set*1B) and ShET-2 (72% *sen*B) were identified. The ShET-1 gene was mostly seen in *S. flexneri* isolates. The genes including *vir*F, *iha, sep*A, *ipa*D, *ial, gad, celb, cap*U and *pic* were also identified in 63%, 40%, 27%, 63%, 57%, 5%, 15%, 57% and 18% of isolates, respectively. A high heterogeneity in the combination of virulence genes was observed.

The presence of virulence genes was compared in the most prevalent serogroups. The virulence genes were significantly higher (p < 0.005) in *S. flexneri* isolates when compared to *S. sonnei*. (Table 1). Most of the virulence genes were found in all four serogroups. However, the *sep*A and *pic* genes were identified only in *S. flexneri* isolates, while *gad* gene was found in *S. boydii* and *S. sonnei* isolates. Similarly, *iha* gene was seen only in *S. dysenteriae* and *S. boydii* isolates. Whereas, *S. sonnei* harbored the *celb* gene but all other serogroups were negative for this gene. While 3 *S. flexneri* isolates were negative for all gene except *ipa*H and *lpf*A genes. The distribution of virulence genes among *S. flexneri* serotypes were given in the Table 2.

Table 1. Distribution of virulence genes among 60 *Shigella* isolates *n* (%).

	*vir*F	*iha*	*sep*A	*set*1A	*set*1B	*sen*B	*ipa*D	*ipa*H	*ial*	*gad*	*celb*	*cap*U	*sig*A	*pic*	*lpf*A
S. dysenteriae (*n* = 7)	5 (100)	5 (100)	0 (0)	0 (0)	1 (14)	7 (100)	7 (100)	7 (100)	7 (100)	0 (0)	0 (0)	7 (100)	7 (100)	0 (0)	7 (100)
S. boydii (*n* = 17)	17 (100)	17 (100)	0 (0)	1 (6)	1 (6)	17 (100)	16 (94)	17 (100)	15 (88)	1 (6)	0 (0)	15 (88)	14 (82)	0 (0)	7 (41)
S. flexneri (*n* = 22)	16 (73)	0 (0)	16 (73)	15 (68)	12 (54)	5 (23)	15 (68)	22 (100)	7 (32)	0 (0)	0 (0)	11 (50)	15 (68)	11 (50)	21 (95)
S. sonnei (*n* = 14)	0 (7)	0 (0)	0 (0)	1 (7)	1 (7)	14 (100)	0 (0)	14 (100)	5 (36)	2 (14)	9 (64)	1 (7)	14 (100)	0 (0)	14 (100)
Flexneri vs Sonnei (p-value)	<0.001	NS	<0.001	<0.003	<0.028	<0.001	<0.001	NS	NS	NS	<0.001	<0.015	NS	<0.014	NS
Total	38 (63)	24 (40)	16 (27)	17 (28)	14 (23)	43 (72)	38 (63)	60 (100)	34 (57)	3 (5)	9 (15)	34 (57)	50 (83)	11 (18)	47 (78)

NS – not significant, $p < 0.05$ was considered significant

Table 2. Prevalence of virulence genes among *S. flexneri* serotypes studied *n* (%).

Virulence genes	*S. flexneri* 1 (*n* = 3)	*S. flexneri* 2 (*n* = 11)	*S. flexneri* 4 (*n* = 3)	*S. flexneri* (untypeable) (*n* = 5)
*vir*F	2 (67)	10 (91)	2 (67)	2 (40)
iha	0 (0)	0 (0)	0 (0)	0 (0)
*sep*A	2 (67)	9 (82)	3 (100)	2 (40)
*set*1A	0 (0)	10 (91)	3 (100)	2 (40)
*set*1B	0 (0)	8 (73)	2 (67)	2 (40
*sen*B	2 (67)	1 (9)	1 (33)	1 (20)
*ipa*D	1 (33)	10 (91)	2 (67)	2 (40)
*ipa*H	3 (100)	11 (100)	3 (100)	5 (100)
ial	2 (67)	3 (27)	0 (0)	2 (40)
gad	0 (0)	0 (0)	0 (0)	0 (0)
celb	0 (0)	0 (0)	0 (0)	0 (0)
*cap*U	2 (67)	6 (54)	2 (67)	1 (20)
*sig*A	0 (0)	10 (91)	3 (100)	2 (40)
pic	0 (0)	6 (54)	2 (67)	3 (60)
*lpf*A	3 (100)	10 (91)	3 (100)	5 (100)

Additionally, common clinical features of *Shigella* infection (fever, abdominal pain, vomiting, diarrhea and hospitalization) were compared with the presence of common virulence genes to study their association (Table 3). The most common complaint was fever (38%), followed by diarrhea (33%), vomiting (8%) and abdominal pain (7%). Remarkably, 45% of the patients had a history of hospitalization in this study. A significant association was observed for *ipa*H with the presence of abdominal pain and vomiting, and similarly for *the sig*A gene with fever. Whereas *sep*A and *sig*A were found to be associated with diarrhea ($p < 0.005$). The other symptoms were, however, not significantly associated with any of the virulence genes. Also, analyzing the occurrence of clinical manifestations among the different *Shigella* species showed that the symptoms were not associated with any serogroup in the present study (data not shown). Among the studied *Shigella* isolates, the presence of virulence genes was found to be higher in isolates resistant to more than three antibiotic classes (Figure 1). The accession numbers of the isolates are provided in Table 4.

Discussion

Among the pathogens causing dysentery, *Shigella* continues to be the major etiological agent, especially in developing countries. The pathogenicity of *Shigella* is associated with the presence of several virulence determinants that are associated with invasion of the colonic epithelium and cell to cell dissemination [5]. The present study investigated the virulence attributes in all four serogroups of *Shigella*.

Increased resistance is sometimes associated with increased virulence in bacteria [14]. Several studies support this hypothesis that highly pathogenic bacterial strains would need antibiotic treatment to reduce the severity of symptoms and are probably subjected to the selective pressure of antibiotic exposure. Similarly, the association of virulence and antimicrobial resistance among the study isolates revealed that the number of

Table 3. Association of common virulence genes with certain symptoms of shigellosis.

		Fever			Abdominal pain		p-value	Diarrhea			Vomiting			Hospitalization		
Virulence genes		Yes	No	p-value	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value
*sep*A	Positive	4	19	NS	2	2	NS	2	14	***p*< 0.05***	0	5	NS	6	21	NS
	Negative	12	25		14	42		18	26		16	39		10	23	
*set*1A	Positive	5	18	NS	1	3	NS	4	16	NS	1	4	NS	9	18	NS
	Negative	12	25		16	40		13	27		16	39		8	25	
*set*1B	Positive	3	20	NS	0	4	NS	2	18	NS	1	4	NS	5	22	NS
	Negative	11	26		14	42		12	28		13	42		9	24	
*sen*B	Positive	19	4	NS	4	0	NS	17	3	NS	5	0	NS	21	6	NS
	Negative	24	13		39	17		26	14		38	17		22	11	
*ipa*H	Positive	23	37	NS	4	56	***p*< 0.05***	20	40	NS	5	55	***p*< 0.05***	27	33	NS
	Negative	0	0		0	0		0	0		0	0		0	0	
ial	Positive	13	10	NS	2	2	NS	11	9	NS	3	2	NS	17	10	NS
	Negative	21	16		32	24		23	17		31	24		17	16	
*sig*A	Positive	16	34	***p*< 0.05***	3	1	NS	13	37	***p*< 0.05***	4	1	NS	22	5	NS
	Negative	7	3		47	9		7	3		46	9		28	5	
pic	Positive	2	21	NS	0	4	NS	1	19	NS	0	5	NS	4	23	NS
	Negative	9	28		11	45		10	30		11	44		7	26	

NS – not significant, * significant difference

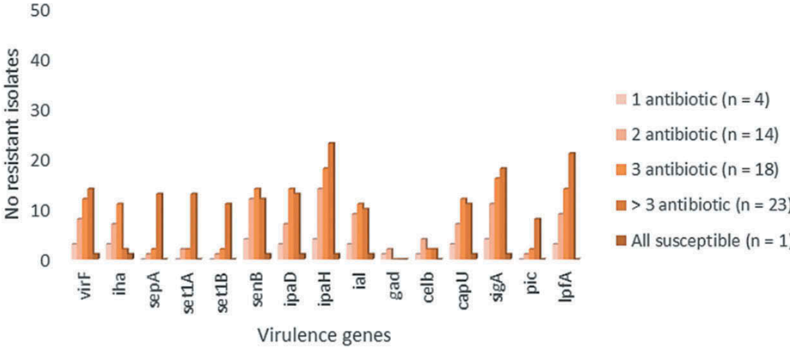

Figure 1. Distribution of virulence genes with varying antimicrobial resistance pattern among the study isolates.

virulence genes was higher in isolates resistant to three or more antimicrobials which in concordance with the previous study [15]. This could be due to the increased prevalence of MDR *Shigella* strains. Further proves the fact that virulence and antimicrobial resistance are well associated.

An earlier study showed that *S. flexneri* 2a was found to possess more virulence factors than other serogroups [16]. Similarly, on the analysis of 60 *Shigella* whole genome sequences of different serogroups, *S. flexneri* was found associated with an increased number of virulence genes particularly serotype 2a. Among the invasion-associated genes, *ipa*H was predominantly seen followed by *ipa*D and *ial* genes. Both *ial* and *ipa*H were reported to be responsible for epithelial cell penetration and cell to cell dissemination [17]. These genes were seen in all serogroups except *S. sonnei*, in which *ipa*D was absent.

The *ipa*H gene is generally present as multiple copies, seven on chromosomes, of which five are located in *ipa*H-islands which are acquired through phage-mediated lateral gene transfer and five on large plasmids [6,18]. The presence of *the ipa*H gene in all the studied isolates could be due to these high copy numbers. Besides, an association of *ipa*H gene with symptoms, abdominal pain and vomiting was observed in the present study which is in concordance with the previous report where the *ipa*H gene was found to be associated with fever, vomiting and dehydration in the infected children [6]. Further, *ipa*D, is part of the Ipa family required for *Shigella* invasion, plays an important role in Type III secretion systems (T3SSs), which are the common virulence factors among Gram-negative bacteria. In *Shigella*, T3SS is assembled when the environmental conditions are appropriate for invasion, secretion is initiated only after its contact to the host cell [19].

There is another gene, *vir*F located on the virulence plasmid (pINV) which activates the transcription of *ics*A and *vir*B genes, prompting the full expression of *Shigella* invasion program [20]. This gene was identified in 38 of the 60 *Shigella* isolates tested. Also, the absence of *the vir*F gene in 22 isolates showed the lack of virulence plasmid, which could be due to the loss of plasmid during the culture.

Toxins are some of the major virulence factors produced by bacteria. *stx* is the toxin gene, exclusively produced by *S. dysenteriae* type 1 and rarely by other *Shigella* serogroups. The role of *stx* in shigellosis is still not clear as this is not essential for invasion [21]. However, the present study does not include *S. dysenteriae* type 1 and hence the gene was not identified in the study isolates.

Shigella spp also produces an enterotoxin (*set*1A/B) and *sen*) and exotoxin called Shiga toxin [7]. Cruz et al. reported that *set*1B subunit belongs to ShET-1 is the

Table 4. Details of the *Shigella* isolates analyzed in this study.

Isolate ID	year	Age/Sex	Organism	AST pattern	Accession
FC1882	2014	47/F	*S. boydii*	SXT-NAL	*MDDI00000000
FC1764	2014	21/F	*S. boydii*	AMP-SXT	*MDDH00000000
FC1661	2014	3/F	*S. boydii*	SXT-NAL-FIX	*MDGW00000000
FC2833	2014	1/F	*S. boydii*	ALL SUSCEPTIBLE	*MDJL00000000
FC1567	2012	1/M	*S. boydii*	AMP-SXT-NAL	*MIIV00000000
FC2117	2012	8/M	*S. boydii*	AMP-SXT	*MINP00000000
FC2125	2012	2/M	*S. boydii*	SXT-NAL-NX	*MINQ00000000
FC2175	2012	24/M	*S. boydii*	SXT	*MINR00000000
FC2710	2014	0/M	*S. boydii*	AMP-SXT-NAL (MS)	*MINU00000000
FC1139	2011	1/M	*S. flexneri*	AMP-SXT	*MECX00000000
FC1172	2011	3/M	*S. flexneri*	AMP-SXT-NAL-NX (MS)	*MDJI00000000
FC1056	2015	75/F	*S. dysenteriae*	NAL-TAX	*MECW00000000
FC1708	2012	2/M	*S. dysenteriae*	SXT	*MIIX00000000
FC1737	2013	3/M	*S. dysenteriae*	NAL	*MIIY00000000
FC2531	2013	74/F	*S. dysenteriae*	AMP-NAL-TAX	*MINS00000000
FC2541	2013	31/F	*S. dysenteriae*	AMP-NAL-TAX	*MINT00000000
FC2383	2014	26/F	*S. boydii*	AMP-SXT-NAL	*MDJK00000000
FC1544	2014	2/M	*S. dysenteriae*	AMP-SXT-NAL	*MECT00000000
FC3196	2015	60/M	*S. dysenteriae*	AMP-SXT-NAL	*MINV00000000
FC288	2016	1/F	*S. sonnei*	AMP-SXT-NAL-NX	NGWI00000000
FC1373	2016	4/F	*S. sonnei*	AMP-SXT-NAL-NX	NGWH00000000
FC1417	2016	24/M	*S. flexneri 4*	AMP-SXT-NAL-NX-TAX-FIX	NGWG00000000
FC1846	2014	1/M	*S. boydii*	AMP-SXT-NAL-TAX-FIX	NGWF00000000
FC2615	2015	5/M	*S. boydii*	AMP-SXT-NAL	NGWE00000000
FC906	2015	0/M	*S. flexneri 2*	AMP-SXT-NAL-NX-TAX-FIX	NGWD00000000
FC1182	2015	1/M	*S. flexneri 1*	AMP-SXT-NAL	NGWC00000000
FC1772	2014	0/M	*S. sonnei*	AMP-SXT-NAL-NX-TAX-FIX	NGWB00000000
FC1659	2015	3/M	*S. flexneri 2*	SXT-NAL	NGWA00000000
FC470	2015	6/M	*S. flexneri 2*	AMP-SXT-NAL-NX-TAX-FIX	NGVZ00000000
FC1247	2015	3/F	*S. flexneri 2*	AMP-SXT-NAL-NX-TAX-FIX	NGVY00000000
FC1607	2015	21/F	*S. flexneri 4*	AMP-SXT-NAL-NX-TAX-FIX	NGVX00000000
FC1481	2015	1/M	*S. flexneri 4*	AMP-SXT-NAL-NX-TAX-FIX	NGVW00000000
FC3278	2015	3/F	*S. sonnei*	AMP-SXT-NAL	NMYB00000000
FC1244	2015	65/F	*S. sonnei*	SXT-NAL	NMYA00000000
FC3433	2015	4/F	*S. flexneri 2*	AMP-SXT-NAL-TAX	NMXZ00000000
FC653	2017	9/M	*S. sonnei*	AMP-SXT	NMXY00000000
FC1170	2015	45/F	*S. flexneri 2*	AMP-SXT-NAL	NMXX00000000
FC1824	2014	45/M	*S. flexneri 2*	AMP-SXT-NAL	NMXW00000000
FC601	2017	18/F	*S. flexneri 1*	AMP-SXT	NMXV00000000
FC3209	2016	1/M	*S. sonnei*	SXT-NAL-NX	NMXU00000000
FC666	2017	12/M	*S. boydii*	SXT	NMXT00000000
FC1747	2016	48/m	*S. sonnei*	SXT-NAL	NMXS00000000
FC15	2017	69/F	*S. sonnei*	AMP-SXT-NAL-NX-TAX-FIX	NMXR00000000
FC401	2017	2/M	*S. flexneri 1*	AMP-SXT-NAL-NX	NMXQ00000000
FC420	2017	2/M	*S. flexneri 2*	AMP-SXT-NAL-NX	NMXP00000000
FC248	2017	20/F	*S. flexneri*	AMP-SXT-NAL-NX	NMXO00000000
FC1642	2017	1/F	*S. boydii*	SXT	PDYE00000000
FC1655	2017	2/M	*S. boydii*	AMP-SXT-TAX-FIX	PDYD00000000
FC1676	2017	3/F	*S. boydii*	AMP-SXT	PDYC00000000
FC1706	2017	31/F	*S. sonnei*	SXT	PDYB00000000
FC1628	2017	11/M	*S. sonnei*	SXT	PDYA00000000
FC1667	2017	31/F	*S. sonnei*	NAL	PDXZ00000000
FC1717	2017	1/M	*S. boydii*	AMP-SXT	PDXY00000000
FC1653	2017	2/F	*S. sonnei*	SXT	PDXX00000000
FC1677	2013	1/M	*S. sonnei*	AMP-SXT-NAL-TAX-FIX	PDXW00000000
FC1405	2011	4/M	*S. flexneri*	AMP-SXT-NAL-NX	PDXV00000000
FC2101	2016	2/M	*S. flexneri 2*	AMP-SXT-NAL-TAX-FIX	PDXU00000000
FC2414	2016	0/M	*S. flexneri 2*	AMP-SXT-NX	PDXT00000000
FC1954	2016	0/M	*S. flexneri 2*	AMP-SXT-NAL-NX	PDXS00000000
FC1180	2011	3/M	*S. flexneri*	AMP-SXT-NAL-NX (MS)	*MDJJ00000000

AMP – ampicillin, SXT – trimethoprim/sulfamethoxazole, NAL – nalidixic acid, NX – norfloxacin, TAX – cefotaxime, FIX – cefixime.* isolates from previous study [10]

potential aggravating factor for dehydration in shigellosis and detected only in *S. flexneri* isolates [6]. In this study, *set*1A/B was predominantly seen among the *S. flexneri* isolates. Whereas, *sen* gene which was known to be responsible for inducing bloody diarrhea was identified in all serogroups in concurrence with the previous study by Lluque et al. [16].

Furthermore, all *set*1A/B positive isolates also presented with *sig*A gene, which is a *Shigella* IgA-like protease homolog (*sig*A) that belongs to the members of cytotoxic class 1 serine protease autotransporters of Enterobacteriaceae (SPATEs). This class also includes plasmid-encoded toxin gene (*pet*) and secreted autotransporter toxin gene (*sat*) [22]. In this study, *sig*A was identified in all four *Shigella* serogroups while *sat* and *pet* genes were absent in the study isolates. Besides the symptom fever was significantly associated with the presence of *sig*A gene. However, the class 2 members are non-cytotoxic and include the protein involved in mucosal colonization (*pic*) and tissue invasion (*sep*A).

The *pic* and *sep*A genes were more prevalent in *S. flexneri* isolates, which is in concordance with the previous report [23].

Moreover, the *set*1A/B gene is reported to be present in the complementary strand of the *pic* gene. However, the presence of *set*1A or *set*1B in the absence of *pic*, absence of *set*1A/B genes and the presence of all three genes are observed in the study isolates. The combination of *set*1A + *set*1B + *pic* was present in 8 isolates. The differences observed between the presence of these genes have previously been reported in the studies [16,18,24]. The probable reason for this scenario could be due to inactive/truncated *pic* gene that results in the absence of *set*1A/B genes [16,18].

Notably, the presence of both *sep*A and *sig*A genes significantly correlated with the presence of diarrhea. A comparable result was observed for *sep*A gene with diarrhea in enteroaggregative *E. coli* in another study [22]. A recent study showed that the *sen* gene had a significant association with hospitalization and bloody diarrhea [5]. Similarly, the presence of *sen* gene was found higher in patients with hospitalization in the present study.

The limitation of the present study is the lack of invasion efficiency analysis to ensure whether the clinical isolates retained their virulence plasmid and truly invasive. Also, further study is needed to confirm the virulence gene expressions with other techniques like quantitative RT-PCR or Western blots analysis.

In conclusion, the present work revealed the heterogeneity of virulence determinants in *Shigella* serogroups. *S. flexneri* was found to have more numbers of virulence genes. The study revealed that particular symptoms were found to be associated with the presence of certain virulence factors. These findings enhance our understanding on the contribution of virulence genes in disease severity. Although, a larger sample size with clinical metadata and outcome is necessary to provide a greater insight into it.

Acknowledgments

The authors gratefully acknowledge the Institutional Review Board of the Christian Medical College, Vellore (83-i/11/13) for approving the study and providing lab space and facilities.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The study was funded by Indian Council of Medical Research (ICMR), New Delhi (Ref. No: AMR/TF/55/13ECDII dated 23/10/2013).

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