Molecular characterization of antimicrobial resistance in clinical *Shigella* isolates during 2014 and 2015: trends in South India

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Abstract

Background ***Shigella*** **species are an important cause of acute diarrheal disease worldwide. This study describes the prevalence of** ***Shigella*** **spp. serotypes and their resistance profile in Vellore, South India from 2014 to 2015.**

Methods From 2014 to 2015, 338 ***Shigella*** **strains were isolated from stool samples at Christian Medical College, Vellore, India. Identification and serotyping was carried out using standard protocols. Antimicrobial susceptibility testing was done against commonly used antibiotics. Multidrug resistance was detected in 157 isolates. A subset of 73 isolates was randomly characterized further for acquired antimicrobial resistance genes in this study.**

Results The resistance profile of the study isolates varied by species and year. ***S. sonnei*** **isolates were 100% resistant to all tested antibiotics in 2014, whereas in 2015, resistance was found for AMP-NAL-TAX-SXT-FIX. The resistance phenotypes among** ***S. flexneri*** **isolates for the year 2014 and 2015 were AMP-SXT-NAL-NOR-FIX-TAX and AMP-NAL-SXT-TAX-NOR-FIX respectively. Screening for antimicrobial resistance genes in** ***S. flexneri*** **found** ***dhfr1A, sulII,*** bla_{OXA}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, ***qnr*****B,** ***qnr*****S and AmpC genes while** ***S. sonnei*** **were found to have only** ***dhfr1A, sulII,*** $bla_{CTX\text{-}M\text{-}1}$ **and** ***qnr*****S genes respectively. Antimicrobial resistance genes were predominantly seen in AMP-SXT-NAL and AMP-SXT-NAL-NOR resistance phenotypes.**

Conclusion ***Shigella*** **prevalence of 4.8% to 4.6% was documented between the years 2014 to 2015 in this study. We show evidence that resistance to commonly used antibiotics continues to increase among** ***Shigella*** **spp. in South India. The presence of** ***qnr*****S and** $bla_{CTX\text{-}M\text{-}15}$ **in the study isolates further indicates the threat of spreading resistance to quinolones and third-generation cephalosporins.**

Keywords ***Shigella*** **prevalence, antimicrobial resistance,** ***qnr*****S,** $bla_{CTX\text{-}M\text{-}1}$, bla_{TEM}, **non-agglutinable** ***Shigella***

Introduction

Shigella species are an important cause of acute diarrheal disease worldwide. The majority of cases occur among children less than five years of age.[1,2] *Shigella* has been generally characterized by serological typing for epidemiological and diagnostic purposes. *Shigella* species include four serogroups: *S. dysenteriae*, *S. flexneri*, *S. sonnei*, and *S. boydii*. Each serogroup contains several serotypes differentiated based on

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the O-antigen structure.[1] All four *Shigella* spp. may cause disease, the most common being *S. sonnei* in developed countries but this species has shown a propensity for intercontinental spread to developing countries as a single and rapidly evolving lineage. In contrast *S. flexneri* is predominant in developing countries, while *S. dysenteriae* and *S. boydii* are rarely isolated.[3] Although shigellosis is primarily self-limiting, antibiotics are recommended for reducing illness duration and for preventing transmission; the current drugs of choice are fluoroquinolones and third generation cephalosporins.[4]

However, antimicrobial resistance (AMR) is an emerging problem among *Shigella* spp., particularly in Asia and Africa.[5] Resistance to first line drugs such as ampicillin and trimethoprim-sulfamethoxazole has become high.[6] Reports on the emergence of ciprofloxacin resistance further narrows the list of effective antimicrobials. Infection due to resistant bacteria can persist longer than infections with susceptible bacteria.[7]

Currently there are no vaccines available for shigellosis due to their large dependence on the prevailing species and serotypes, as only serotype specific immunity has been demonstrated in humans.[8,9] The distribution of *Shigella* serogroups/serotypes and antimicrobial resistance differs over time and place. Due to the scarcity of data especially from developing countries like India, a comprehensive understanding of the epidemiology of *Shigella* spp. is essential to control shigellosis. The present study describes the trends in *Shigella* species, serotypes and their resistance mechanisms, in isolates from patients with diarrhea over the years 2014 and 2015.

Methods

Bacterial strains

Isolation and identification of the organism was carried out using conventional biochemical reactions (urea, citrate, triple sugar iron, indole, motility).[10] All the isolates were serotyped using commercial antisera as per the manufacturer's instructions (Denka Seiken, Tokyo, Japan).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *Shigella* isolates against ampicillin 10 µg (AMP), trimethoprim/sulfamethoxazole 1.25/23.75 µg (SXT), nalidixic acid 30 µg (NAL), norfloxacin 10 µg (NOR), cefotaxime 30 µg (TAX) and cefixime 5 µg (FIX) was performed using Kirby-Bauer disk diffusion method and results were interpreted using breakpoints recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines 2014 and 2015.

Molecular analysis of antibiotic resistance genes

Genomic DNA was extracted with QIAamp DNA mini kit (Qiagen, Hilden, Germany). The presence of β-lactamase genes (bla_{OXA}, bla_{TEM}), ESC genes $bla_{CTX\text{-}M\text{-}1}$, AmpC ($bla_{MOX}$, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC} and bla_{FOX}), trimethoprim resistance gene (*dhfr1a*), sulphonamide resistance gene (*sulII*) and plasmid mediated quinolone resistance (PMQR) (*qnr*A, *qnr*B, *qnr*S) genes were assessed by PCR as described earlier.[11]

Statistical analysis

Comparisons of variables were derived using two-tailed Chi-squared test. A p-value of less than 0.05 was considered significant.

Results

Between 2014 and 2015, a total of 7212 stool specimens were processed, of which 338 were confirmed as *Shigella* spp. and serotyped as follows: *S. flexneri* (n=211), *S. sonnei* (n=90), *S. dysenteriae* (n=12), *S. boydii* (n=8) and non-agglutinable *Shigella* (n=17). Multidrug resistance (MDR) (resistance to 3 or more classes of antibiotics) was detected in 157 isolates. The species distribution over time is shown in Figure 1. *S. flexneri* was the prevalent serogroup found, followed by *S. sonnei*, *S. dysenteriae*, and *S. boydii*. The isolation rate of *S. sonnei* has gradually increased from one year to the next (19.8% to 33.9%). The trends in *S. flexneri* serotypes are described in Figure 2, with *S. flexneri* 2 being the most commonly isolated serotype followed by other serotypes. In addition, the rate of non-typeable *Shigella* was found to be higher in the year 2014 (8.5%) and decreased to 1.2% in 2015 (p=0.002). Of the *Shigella* positive isolates, 60% (202/338) were from children less than or equal to five years of age. *Shigella* infection was found more frequently among males (57%) than

females (43%). Seasonal variation of *Shigella* infection was observed during the study period with a peak isolation rate seen between the months May-July.

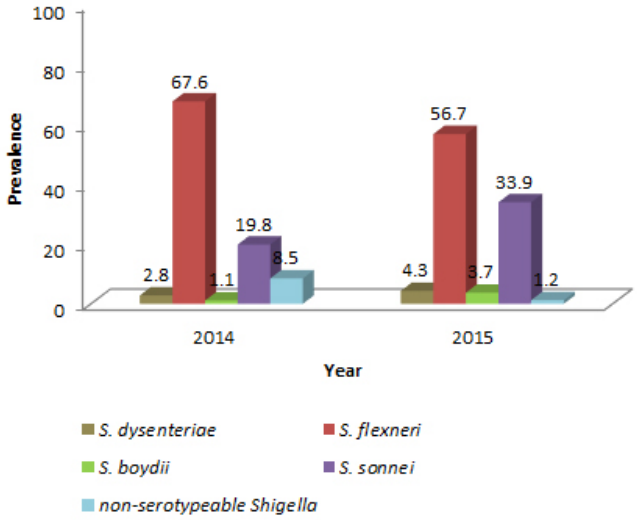


Figure 1. Prevalence of *Shigella* species isolated between the years 2014 and 2015

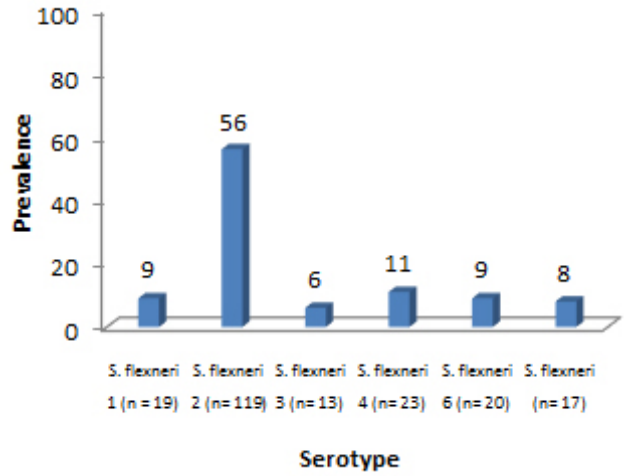


Figure 2. Distribution of *S. flexneri* serotypes isolated over the study period

Overall, multidrug resistance was more frequently detected in *S. flexneri* and *S. sonnei* than in *S. dysenteriae* and *S. boydii*. The antimicrobial resistance trend change was calculated between the species and years. A significant decrease was seen in the norfloxacin resistance profile of *Shigella* spp. during the study period ($p<0.001$) and a notable change was observed for ampicillin resistance between the species *S. flexneri* and *S. sonnei* ($p<0.001$). The resistance profile of other antibiotics remains unchanged between the years and the species (Tables 1 and 2). The resistance rate was found to be higher in patients with less than five years and followed by those greater than fifteen years of age (Figure 3).

A subset of 73 MDR (2014 – *S. flexneri* (n=28), *S. sonnei* (n=1); 2015 – *S. flexneri* (n=35), *S. sonnei* (n=9)) isolates was randomly selected and characterized for the antimicrobial resistance mechanism in this study. Among the selected isolates (n=73), *S. sonnei* from the year 2014 were 100% resistant to all tested antibiotics, whereas in 2015, *S. sonnei* was found to be resistant to AMP (100%), SXT (55%), NAL (100%), TAX (67%) and FIX (33%) and none were found to be resistant to norfloxacin. The resistance rate among *S. flexneri* isolates for the year 2014 and 2015 were AMP (100 and 100%), SXT (100 and 77%), NAL (96 and 97%), NOR (78 and 26%), TAX (18 and 43%) and FIX (28 and 20%) respectively. Further molecular characterization of the isolates showed varied antimicrobial resistance genes. Screening of resistance by PCR in *S. flexneri* found *dhfr1A*, *sulII*, bla_{OXA}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, *qnr*B,S and AmpC genes while *S. sonnei* were found to have only *dhfr1A*, *sulII*, $bla_{CTX\text{-}M\text{-}1}$ and *qnr*S genes respectively. None of the isolates were positive for *qnr*A gene. The results are illustrated in Table 3. Notably a higher number of resistance genes was found in individuals less than five years of age. AMR genes were predominantly seen in the AMP-SXT-NAL and AMP-SXT-NAL-NOR resistance phenotype (data not shown).

Discussion

According to the World Health Organization (WHO), diarrheal disease was considered to be the second leading cause of mortality in children less than five years of age worldwide.[12] Shigellosis represents a major cause of gastroenteritis, especially in low income countries. The isolation rate of *Shigella* varies from 2% to 6% in different studies from India.[11]

Table 1. Comparison of antimicrobial resistance profiles of *S. flexneri* and *S. sonnei* between 2014 and 2015

S. flexneri

Year (*n*)	Phenotype	AMP	SXT	NAL	NOR	TAX	FIX
2014 (119)	R	84	113	111	80	9	12
	S	35	6	8	39	110	107
2015 (92)	R	63	88	85	20	12	9
	S	29	4	7	72	80	83
p value		0.741	0.810	0.802	<0.005	0.187	0.942
Chi (df)		0.1 (210) = 21	0.05 (210) = 10.5	0.06 (210) = 12.6	43 (210) = 9030	1.73 (210) = 363.3	0.005 (210) = 1.05

S. sonnei

Year (*n*)	Phenotype	AMP	SXT	NAL	NOR	TAX	FIX
2014 (35)	R	4	32	35	23	1	3
	S	31	3	0	12	34	32
2015 (55)	R	13	54	54	10	3	4
	S	42	1	1	45	52	51
p value		0.149	0.128	0.423	<0.005	0.561	0.825
Chi (df)		2.08 (89) = 185.1	2.29 (89) = 203.8	0.64 (89) = 56.9	20.8 (89) = 1851	0.33 (89) = 29.3	0.05 (89) = 4.45

R – resistant, **S** – susceptible, **AMP** – ampicillin, **SXT** – trimethoprim/sulfamethoxazole, **NAL** – nalidixic acid, **NOR** – norfloxacin, **TAX** – cefotaxime, **FIX** – cefixime.

Table 2. Comparison of antimicrobial resistance profiles between species: *S. flexneri* vs. *S. sonnei* (2014-2015)

Year	Serotype (*n*)	Phenotype	AMP	SXT	NAL	NOR	TAX	FIX
2014	*S. flexneri* (119)	R	84	113	111	80	9	12
		S	35	6	8	39	110	107
	S. sonnei (35)	R	4	32	35	23	1	3
		S	31	3	0	12	34	32
p value			<0.005	0.435	0.114	0.865	0.322	0.787
Chi (df)			38.6 (153) = 5905	0.61 (153) = 93.3	2.48 (153) = 379.4	0.02 (153) = 3.0	0.98 (153) = 150	0.07 (153) = 11
2015	*S. flexneri* (92)	R	63	88	85	20	12	9
		S	29	4	7	72	80	83
	S. sonnei (55)	R	13	54	54	10	3	4
		S	42	1	1	45	52	51
p value			<0.005	0.412	0.133	0.603	0.141	0.603
Chi (df)			27.7 (146) = 4044	0.67 (146) = 98	2.24 (146) = 327	0.26 (146) = 38	2.16 (146) = 315	0.26 (146) = 38

R – resistant, **S** – susceptible, **AMP** – ampicillin, **SXT** – trimethoprim/sulfamethoxazole, **NAL** – nalidixic acid, **NOR** – norfloxacin, **TAX** – cefotaxime, **FIX** – cefixime.

The present study documents a prevalence of 4.8% to 4.6% between the years 2014 to 2015.

S. flexneri was found to be the predominant serogroup and subtype 2 was the most common. Similar results were reported from Manipal and Puducherry in South India, where >90 percent of the isolates were *S. flexneri*.[4,13] In an earlier study conducted in Vellore, South India (1997-2004) *Shigella* accounted for 5.4%, whereas *S.*

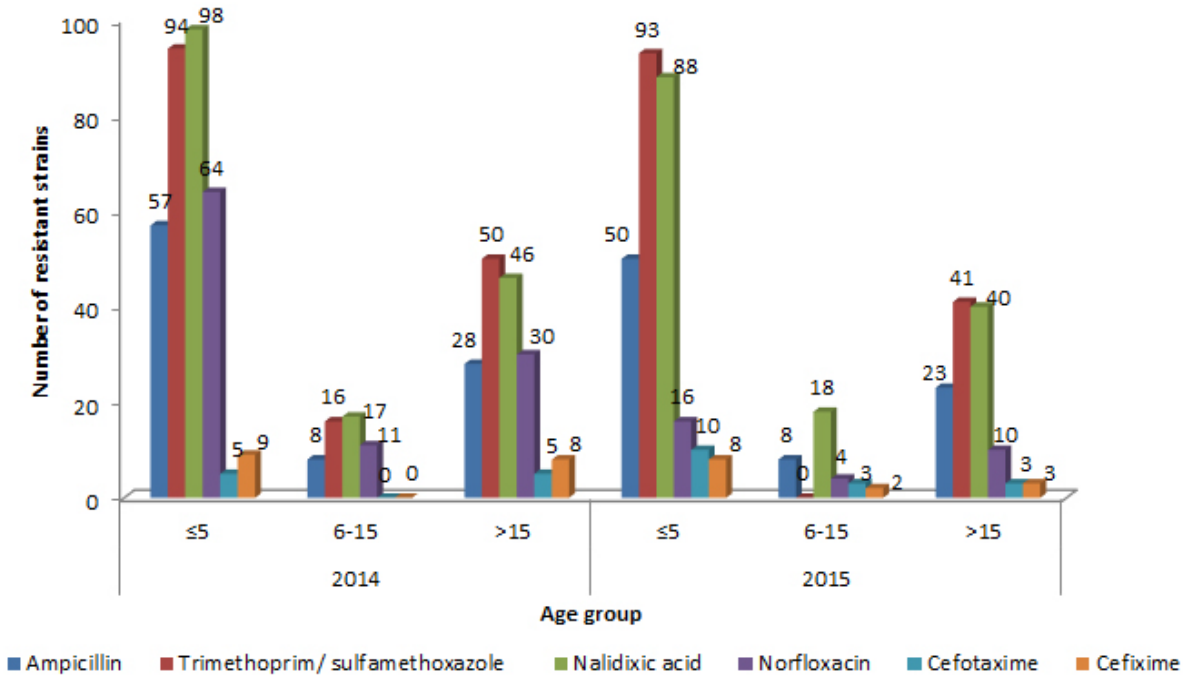


Figure 3. Antimicrobial resistance profile of *Shigella* spp. by age group.

Table 3. Molecular characterization of multidrug resistant *Shigella* spp.

Antibiotic	Year	% resistance (n=73)		Genes	AMR gene prevalence (n=73)	
		S. flexneri	*S. sonnei*		*S. flexneri* % (n=63)	*S. sonnei* % (n=10)
AMP	2014	100	100	bla_{OXA}	43 (12)	0 (0)
AMP	2015	100	100	bla_{OXA}	51 (18)	0 (0)
TMP-SXT	2014	100	100	*dhfr*1A	100 (28)	100 (1)
				sulII	43 (12)	100 (1)
TMP-SXT	2015	77	55	*dhfr*1A	80 (28)	100 (9)
				sulII	77 (27)	56 (5)
NAL	2014	96	100	*qnr*S	14 (4)	100 (1)
NOR		78	100	*qnr*B&S	3 (1)	0
NAL	2015	97	100	*qnr*S	23 (8)	0 (0)
NOR		26	0	*qnr*B&S	0 (0)	0 (0)
TAX	2014	18	100	bla_{TEM}	14 (4)	0 (0)
FIX		28	100	AmpC	7 (2)	0 (0)
				$bla_{CTX\text{-}M\text{-}15}$	7 (2)	100 (1)
TAX	2015	43	67	bla_{TEM}	34 (12)	0 (0)
FIX		20	33	AmpC	20 (7)	0 (0)
				$bla_{CTX\text{-}M\text{-}15}$	3 (1)	11 (1)

2014 – *S. flexneri* (n=28), *S. sonnei* (n=1); 2015 – *S. flexneri* (n=35), *S. sonnei* (n=9)

AMR – antimicrobial resistance, **AMP** – ampicillin, **TMP-SXT** – trimethoprim/sulfamethoxazole, **NAL** – nalidixic acid, **NOR** – norfloxacin, **TAX** – cefotaxime, **FIX** – cefixime.

flexneri was the most common serogroup (57.6%) followed by *S. sonnei* (31%).[14]

In recent years, an epidemiological shift in *Shigella* serogroups was reported. *S. sonnei* is now emerging in regions that used to be dominated by *S. flexneri*.[13,15-17] This shift has been documented in many regions in Asia, Latin America, and the Middle East.[17,18] *S. flexneri* was the most common serotype in the United States in the early 1960s but was replaced by *S. sonnei* during 1964-1968.[19] Similarly, the present study documents a significant increase in the prevalence of *S. sonnei* from 2014 to 2015 with the simultaneous fall of *S. flexneri*. The exact reason behind this phenomenon is unclear.

However, the growing prevalence of *S. sonnei* in developing countries might be due to improvement in the quality of water and sanitation, which prevents the passive immunization derived from *P. shigelloides*, normally found in contaminated water. In addition, the amoeba *A. castellani* acts as a reservoir of *S. sonnei*, which allows their persistence in highly chlorinated environments while *S. flexneri* is not able to grow. Finally, *S. sonnei* has a greater ability to acquire resistance than *S. flexneri*, which provides an added advantage over *S. flexneri*, especially in areas with low antimicrobial usage.[17]

In addition, the incidence of non-typeable *Shigella* spp. seems to be increasing over years worldwide.[13] About 5% of the isolates were found to be non-typeable during the study period. AMR was occasionally reported in the provisional/non-typeable serovars of *Shigella*.[20] In this study 41% of the isolates were found to be MDR (data not shown). A similar trend of surfacing increase in non-typeable *Shigella* strains (13%) was reported in Kolkata.[21]

Worldwide, the majority of *Shigella* infections were found to occur in young children.[13] A multicentric study from six Asian countries demonstrated the incidence of shigellosis with higher rates in patients younger than five years and patients above 40 years of age.[8] The present study also evidences the similar trend in accordance with earlier reports.

In India, AMR was more common in *Shigella* than in other enteric bacteria.[22] Although WHO recommends ceftriaxone, pivmecillinam and azithromycin for shigellosis due to the increase in fluoroquinolone resistant shigellae, the species was found to be resistant to one of the third generation cephalosporins and azithromycin. These have also been reported from other parts of south India.[13] Ceftriaxone resistance was also observed by a multicentre study in eight Asian countries, among *Shigella* isolates.[6]

Generally, ampicillin resistance was encoded by OXA type β-lactamases. Overall, in the current study, 41% of the isolates harbored bla_{OXA} genes. Among those exhibiting co-trimoxazole resistance, *dhfr1A* trimethoprim resistance gene was more prevalent, being considered the most common gene in the genus, followed by the *sulII* gene. Navia et al.[23] demonstrated evidence of the aforementioned results of the current study.

Screening of *Shigella* isolates for the presence of PMQR genes showed 18% and 1% positivity for *qnr*S and co-occurrence of *qnr*B & S respectively. The presence of PMQR genes is a great concern because they can promote mutations within the quinolone resistance determining region (QRDR), which results in fluoroquinolone resistance, but may also disseminate to other species of Enterobacteriaceae.[3] In this study, *qnr* genes were frequently seen in *S. flexneri* isolates than in *S. sonnei*, which could be due to the fact that *qnr*S gene was first detected in a *S. flexneri* serotype 1a in 2002, and later reported from Japan in 2005.[24] At the same time, *qnr*S was the commonly found allele of the *qnr* genes, followed by *qnr*B in *Shigella* spp.[25,26]

Among *Shigella* spp. bla_{TEM} was the most common β-lactamase gene followed by $bla_{CTX\text{-}M\text{-}1}$, which was found to be the most frequent type of cephalosporinase conferring resistance to third generation cephalosporins especially to cefotaxime.[6,11,27] Among the study isolates 22% and 7% were found to carry bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes respectively. Similarly Taneja et al.[6] reported $bla_{CTX\text{-}M\text{-}1}$ (n=10) in *S. flexneri* isolates, thus indicating the consistent prevalence in India. Consequently, there are reports of *Shigella*

spp. carrying different ESBL genes from other Asian countries. In clinical isolates, CTX-M encoding genes are found on plasmids; transposons or comprised cassettes in integrons.[27] In addition, cephalosporin resistance was mediated by plasmid-mediated AmpC β-lactamases. In the present study, 12% of the isolates had AmpC genes. The AMR gene prevalence by species and year are given in Table 3.

This study provides evidence to show that resistance to antibiotics such as ampicillin and trimethoprim-sulfamethoxazole is not surprising among *Shigella* spp. Cephalosporin and quinolones such as norfloxacin are considered to be effective in the management of shigellosis. However, the increasing prevalence of ESBL and PMQR genes may have been an important driving force for selection of resistance to these antimicrobials. Moreover, the antimicrobial resistance pattern differs from place to place and between two regions in the same place.[28]

Many developing countries lack proper antibiotic resistance surveillance. Often changes in the incidence and antimicrobial resistance pattern from time to time make the treatment ineffective for shigellosis. Although the present study provides data only for a small number of isolates, the available information would help regional or national surveillance programs to document the resistance rates and trends of *Shigella* spp. in our locality.

Conclusions

The study highlights the growing importance of *S. sonnei* in a developing country like India, and emphasizes that antimicrobial resistant *Shigella* spp. is a raising problem among enteric pathogens in India. In our study, most of the isolates were resistant to ampicillin, trimethoprim-sulfamethoxazole and nalidixic acid. The presence of ESBL producing genes such as $bla_{\text{CTX-M-1}}$ in the study isolates further indicates the threat of spreading resistance to third-generation cephalosporins, as cephalosporins are used for treatment in children, where quinolones are usually not recommended. Notably, an increasing number of non-agglutinable *Shigella* highlights the need for effective surveillance of circulating *Shigella* species and serotypes. Furthermore, monitoring changing trends in antibiotic resistance over periods of time is necessary for effective treatment.

Authors' contributions statement: DP analyzed, interpreted data and wrote the manuscript. SA helped write the manuscript, analyzed and interpreted data. DP and RG carried out bench work, generated data. BV, KW and VP critically revised and approved the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest: All authors – none to declare.

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