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Short Communication

IncFII plasmid carrying antimicrobial resistance genes in *Shigella flexneri*: Vehicle for dissemination

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ABSTRACT

Objectives: Plasmids harbouring antimicrobial resistance determinants in clinical strains are a significant public-health concern worldwide. The present study investigated such plasmids in clinical isolates of *Shigella flexneri*.

Methods: A total of 162 *Shigella* isolates were obtained from stool specimens in the year 2015. Among the 70 multidrug-resistant (MDR) *Shigella* spp., 27 *S. flexneri* isolates were randomly selected for further characterisation. Antimicrobial resistance genes (ARGs) and plasmid incompatibility (Inc) types were analysed.

Results: IncFII plasmids were found in 63% (17/27) of the studied *S. flexneri* isolates. ARGs such as *dhfr1a* (81%), *sulII* (74%), bla_{OXA} (74%), bla_{TEM} (33%), bla_{AmpC} (30%), *qnrS* (15%) and *qnrB* (4%) were identified by PCR, whereas bla_{CTX-M} was not detected. Next-generation sequencing of a representative *S. flexneri* IncFII-type plasmid (pSF470) revealed the presence of bla_{TEM1-B}, bla_{DHA-1}, *qnrB10*, *mphA*, *sulI*, *sulII*, *strA*, *strB* and *tetR* ARGs along with the *intI1* integrase gene. In addition, pMLST analysis showed that the replicon belonged to F2:A-:B- type.

Conclusions: This study helps to know the prevalent plasmid types in MDR *Shigella* isolates and will improve our understanding of resistance dissemination among enteric bacteria. ARGs in plasmids further highlight the importance of such studies in enteric bacteria.

1. Introduction

Shigella spp. are aetiological agents of acute intestinal infections. Depending on the virulence potential of the strain and the immune status of the individual, shigellosis can progress to severe disease. Interestingly, clones with high virulence and multidrug resistance have spread globally, with plasmids playing a major role in conferring these characteristics [1].

Antimicrobial resistance (AMR) is a growing problem in *Shigella* spp., particularly to clinically important antimicrobial agents in clinical practice such as ciprofloxacin, ceftriaxone/cefixime and azithromycin. Initially, sulfonamides and tetracycline were used for the treatment of shigellosis, followed by ampicillin, trimethoprim/sulfamethoxazole (SXT) and nalidixic acid. These drugs are no longer recommended unless susceptibility is known owing to the emergence of resistant strains [2,3]. The ability of the organism to acquire various antimicrobial resistance genes (ARGs) through mobile genetic elements such as plasmids, transposons, integrons and insertion sequence (IS) elements has been a major factor in the transmission of multidrug-resistant (MDR) strains [4].

In particular, conjugative plasmids play a significant role in the evolution of pathogenic bacteria, which can transmit resistance readily through horizontal gene transfer and thus facilitate interspecies and intraspecies dissemination. Plasmids are classified as incompatibility (Inc) groups when two plasmids are unable to propagate steadily in the same host [5]. Up to now 27 different plasmid incompatibility groups have been recognised in the Enterobacteriaceae family [6]. The IncF plasmids represent one of the most prevalent incompatibility types involved in the transfer of resistance determinants among Enterobacteriaceae and have been reported worldwide [1,6]. The current study was undertaken to characterise IncF-type plasmids carrying ARGs among MDR *Shigella flexneri* isolated from clinical specimens in India.

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2. Materials and methods

2.1. Bacterial isolates

A total of 162 *Shigella* spp. isolates were obtained from stool specimens in the year 2015 at Christian Medical College (Vellore, India). Phenotypic identification of isolates was carried out using standard protocols [7]. Serotyping was done by the slide agglutination method using commercial antisera (Denka Seiken Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions [8].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *Shigella* isolates to ampicillin (10 μg), trimethoprim/sulfamethoxazole (SXT) (1.25/23.75 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), cefotaxime (30 μg), cefixime (5 μg) and azithromycin (15 μg) was performed by the Kirby–Bauer disk diffusion method and the results were interpreted using breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) [9]. The quality control strains used were *Escherichia coli* ATCC 35218 and *E. coli* ATCC 25922.

2.3. Analysis of antimicrobial resistance genes

Total DNA was isolated from selected *Shigella* isolates using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). Isolates were analysed for the presence of ARGs, including β-lactamases (bla_{OXA} and bla_{TEM}), extended-spectrum cephalosporin resistance gene (bla_{CTX-M}), AmpC β-lactamases (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC} and bla_{FOX}), sulphonamide resistance genes (*dhfr1a* and *sulII*) and plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB* and *qnrS*) as described previously [10]. The *mphA* gene encoding macrolide resistance was also analysed [11]. Known positives were used as controls for all of the targets in every run.

2.4. Plasmid isolation and PCR

Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN, Helsinki, Finland).

Plasmid incompatibility groups were determined by PCR-based replicon typing (PBRT) using primers described previously [12,13]. Amplification was performed in one multiplex (FIA, FIB, FIC and FII) and one monoplex (FIIA) PCR for recognition of the replicons.

2.5. Next-generation sequencing

A single isolate was selected for sequencing based on the ARG profile in order to study the genetic arrangement of the genes in the plasmid. The target plasmid was excised from the agarose gel and was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI) as per the manufacturer's instructions. Briefly, the excised gel was dissolved by adding membrane binding solution, was vortexed and was incubated at 60 °C until completely dissolved. The dissolved gel mixture was then added to the minicolumn assembly, was washed twice with membrane wash solution and was finally eluted with nuclease-free water.

Sequencing was performed with the purified plasmid DNA using Ion Torrent™ (PGM; Life Technologies, Carlsbad, CA) with 400-bp read chemistry. Raw sequences were assembled de novo using Assembler SPAdes v.5.0.0.0 embedded in Torrent Suite Server v.5.0.3. Annotation was performed in PATRIC, the bacterial bioinformatics database and analysis resource [14], and using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP).

Downstream analysis was done using Prokka [15] and the Center for Genomic Epidemiology server (http://www.cbs.dtu.dk/services), where resistance gene profiles were identified using ResFinder 3.0 [16]. Plasmid screening was performed using PlasmidFinder 1.3 [17]. Furthermore, plasmid types were identified using the plasmid multilocus sequence typing (pMLST) v.1.4 web tool based on the FAB typing scheme [17]. PATRIC was used in downstream analysis, where ARGs in the Antibiotic Resistance Genes Database (ARDB) and Comprehensive Antibiotic Resistance Database (CARD) were screened. PATRIC was also used to map the genetic arrangements in the sequenced plasmid. The sequence has been submitted to GenBank with the accession no. **NGVZ00000000**.

3. Results

3.1. Antimicrobial resistance in multidrug-resistant Shigellae

Of 162 *Shigella* spp. isolated during the study period, *S. flexneri* was the predominant type (56.7%), followed by *Shigella sonnei* (33.9%), *Shigella dysenteriae* (4.3%), *Shigella boydii* (3.7%) and non-agglutinable *Shigella* (1.2%). Multidrug resistance was detected in 70 *Shigella* isolates, of which *S. flexneri* was the most common species, followed by *S. sonnei*. A subset of 27 MDR *S. flexneri* isolates were further characterised for the presence of ARGs and IncF plasmids. The isolates were highly resistant to ampicillin, SXT and nalidixic acid.

3.2. Analysis of antimicrobial resistance genes in Shigella flexneri isolates

Screening for ARGs showed that SXT resistance attributed to *dhfr1a* and *sulII* genes was found in 81% (22/27) and 74% (20/27) of isolates, respectively. The β-lactamase genes bla_{OXA} (74%; 20/27) and bla_{TEM} (33%; 9/27) were also identified. AmpC β-lactamase genes were detected in 30% (8/27) of isolates, of which 26% (7/27) had bla_{DHA} and 4% (1/27) had both bla_{DHA} and bla_{ACC} genes. For PMQR, 15% of isolates (4/27) were positive for *qnrS* and 4% (1/27) were positive for *qnrB*. The *mphA* gene was also identified in one isolate (4%) (Table 1).

3.3. PCR screening for plasmid detection and sequencing

In PBRT, 17 isolates were found positive, belonging to the IncFII-type plasmid, whereas all other isolates ($n = 10$) tested negative for IncF plasmids. The results are shown in Table 1. Sequencing of plasmid pSF470 isolated from *S. flexneri* isolate Sh470 revealed the existence of ARGs such as bla_{TEM-1B}, bla_{DHA-1}, *qnrB10*, *mphA*, *sulI*, *sulII*, *strA*, *strB* and *tetR*. Plasmid conjugative transfer (*tra*) genes, replication protein (*rep*), integrase (*intI1*), transposase (*Tn*) and IS elements were also identified.

The sequential gene arrangement is depicted in Fig. 1. The plasmid type was confirmed to be an IncFII replicon with 100% identity to the reference sequences in the database using PlasmidFinder. In addition, pMLST analysis revealed that the FII replicon belongs to F2:A-:B- FAB type.

4. Discussion

The present study investigated the occurrence of an IncF-type plasmid carrying AMR determinants in clinical MDR *S. flexneri* isolates. Horizontal gene transfer is a key mechanism responsible for antimicrobial resistance development in bacteria where mobile

Table 1
Antimicrobial resistance gene (ARG) profile of multidrug-resistant *Shigella flexneri* isolates.

Isolate ID	Resistance profile	ARGs								IncF PCR
		dhfr1A	*sulII*	bla_{OXA}	bla_{TEM}	bla_{CTX-M}	bla_{AmpC}	*qnr*	*mphA*	
Sh470[a]	AMP/SXT/NAL/NOR/CIP/CTX/CFM/AZM	–	+	–	+	–	DHA	*qnrB*	+	IncFII
Sh1481	AMP/SXT/NAL/NOR/CIP/CTX/CFM/AZM	+	+	+	+	–	–	*qnrS*	–	IncFII
Sh1247	AMP/SXT/NAL/NOR/CIP (MS)/CTX/CFM	+	+	–	+	–	–	*qnrS*	–	IncFII
Sh906	AMP/SXT/NAL/NOR/CIP/CTX/CFM	+	+	+	–	–	–	–	–	IncFII
Sh1387	AMP/SXT/NAL/CIP/AZM	+	+	+	–	–	–	–	–	IncFII
Sh1909	AMP/SXT/NAL/CIP/CTX	+	+	–	–	–	–	*qnrS*	–	IncFII
Sh3433	AMP/SXT/NAL/CIP/CTX	+	–	+	–	–	–	–	–	IncFII
Sh2126	AMP/SXT/NAL/CIP	+	+	+	+	–	–	–	–	IncFII
Sh2157	AMP/SXT/NAL/CFM	–	+	–	+	–	DHA, ACC	–	–	IncFII
Sh1653	AMP/SXT/NAL	+	+	+	+	–	–	*qnrS*	–	IncFII
Sh1972	AMP/SXT/NAL	+	–	+	+	–	DHA	–	–	IncFII
Sh2132	AMP/SXT/NAL	+	+	+	–	–	DHA	–	–	IncFII
Sh1582	AMP/SXT/NAL	+	–	+	–	–	DHA	–	–	IncFII
Sh1554	AMP/SXT/NAL	+	+	+	–	–	–	–	–	IncFII
Sh1667	AMP/SXT/NAL	+	–	+	–	–	–	–	–	IncFII
Sh2615	AMP/SXT/NAL	+	+	–	–	–	–	–	–	IncFII
Sh1170	AMP/SXT/NAL	+	–	+	–	–	–	–	–	IncFII
Sh1607	AMP/SXT/NAL/NOR/CTX/CFM	+	+	+	–	–	–	–	–	–
Sh1538	AMP/SXT/NAL/NOR/CIP/AZM	–	–	+	–	–	DHA	–	–	–
Sh2016	AMP/SXT/NAL/CIP/AZM	+	+	+	–	–	–	–	–	–
Sh1763	AMP/SXT/NAL/CIP/AZM	–	+	–	+	–	–	–	–	–
Sh1464	AMP/SXT/NAL/CIP/AZM	+	+	+	–	–	–	–	–	–
Sh1906	AMP/SXT/NAL/NOR	+	+	+	+	–	DHA	–	–	–
Sh2188	AMP/SXT/NAL/NOR	–	+	+	–	–	DHA	–	–	–
Sh1455	AMP/SXT/NAL/CIP (MS)	+	+	+	–	–	–	–	–	–
Sh1390	AMP/SXT/NAL	+	–	+	–	–	–	–	–	–
Sh1182	AMP/SXT/NAL	+	+	–	–	–	–	–	–	–

AMP, ampicillin; SXT, trimethoprim/sulfamethoxazole; NAL, nalidixic acid; NOR, norfloxacin; CIP, ciprofloxacin; CTX, cefotaxime; CFM, cefixime; AZM, azithromycin; MS, moderately susceptible; *dhfr*, *sul*, SXT resistance genes; OXA, TEM, CTX-M, extended-spectrum β-lactamases; DHA, ACC, AmpC β-lactamases; *qnr*, plasmid-mediated quinolone resistance gene; *mphA*, azithromycin resistance gene.
[a] Isolate sequenced.

genetic elements can be transferred between bacteria of the same or different species [1,18].

Among the 17 isolates with an identified replicon type, all were shown to be the IncFII type. The IncF group is reported to be the major incompatibility group involved in the concurrent transfer of AMR and virulence genes, which increases co-selection and probably leads to the emergence or outbreaks of virulent and MDR clones [1]. IncF plasmids are low-copy-number plasmids, often carrying more than one replicon. Mainly, these Inc group plasmids have limited host range to the genera of Enterobacteriaceae, whereas the IncP, IncA/C and IncQ plasmids showed a wide range of hosts [19]. Besides, IncF plasmids are reported to carry $bla_{CTX-M-15}$ (β-lactamase gene), *aac(6′)-Ib*-cr (fluoroquinolone resistance) and PAI (pathogenicity island for virulence) [1,20]. An earlier report suggested that certain resistance genes were associated with a specific plasmid back bone [6]. For instance, the bla_{TEM-1} gene, mobilised by the *Tn*3 transposon, often coexists with the $bla_{CTX-M-15}$ gene on the same plasmid. However, the $bla_{CTX-M-15}$ gene was not identified in plasmid pSF470 in the current study.

Whereas the *qnrB* gene associated with extended-spectrum β-lactamase (ESBL) genes was identified previously in *E. coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* in Korea found on an IncF plasmid replicon FIIA [19]. However, here we identified the *qnrB* gene in F2:A-:B-, representing the FII replicon type without FIA and FIB replicons by the notion A–/B–. Notably, the occurrence of ARGs was found to be higher in this particular replicon type than in other types [6]. In contrast, Dutta et al. investigated the AMR, virulence and plasmid profile in 13 serologically atypical provisional serovars of *Shigella* isolates and showed the presence of an IncFIIA plasmid type in all isolates [13].

To the best of our knowledge, this is the first report on Inc typing of plasmids in MDR *S. flexneri* from India. A similar finding has been previously reported by Villa et al. in *Salmonella* and *E. coli* [12]. In this study, isolates harbouring IncFII-type plasmids were phenotypically resistant to three or more antibiotics and also had more than one ARG genotypically.

The presence of ARGs is not always associated with a resistant phenotype or plasmids, as these genes could be non-expressive. For β-lactamases, the study isolates harboured the bla_{OXA} and/or bla_{TEM} gene, except for three isolates. Resistance to SXT was correlated with the presence of either the *dhfr1a* and/or *sulII* gene, except for one isolate. All *qnr*-positive isolates had plasmids, however all other genes are present either with or without plasmids. Isolates that are phenotypically resistant but had no genes may be due to other resistance mechanisms. In addition, PCR assays may not identify new resistance gene variants or new resistance mechanisms, and identification of different genes by individual PCR is costly, which limits its use in the detection of AMR.

Studying the transmission of resistance is of great interest, and most studies focus on plasmids and resistance determinants independently. However, analysis of these characteristics together will provide a better understanding of their association in the development of resistance particularly in enteric bacteria where horizontal gene transfer plays a major role in the dissemination of resistance between genera or even within species.

In this study, analysis was focused on the IncF-type plasmid among MDR *S. flexneri* isolates, the predominant serogroup in India. A limitation of this study is that only a single isolate was sequenced and analysed. Plasmid analysis studies in enteric bacteria such as *Shigella* spp., which continues to be a leading cause of morbidity, will help to enhance our knowledge on the development and spread of AMR.

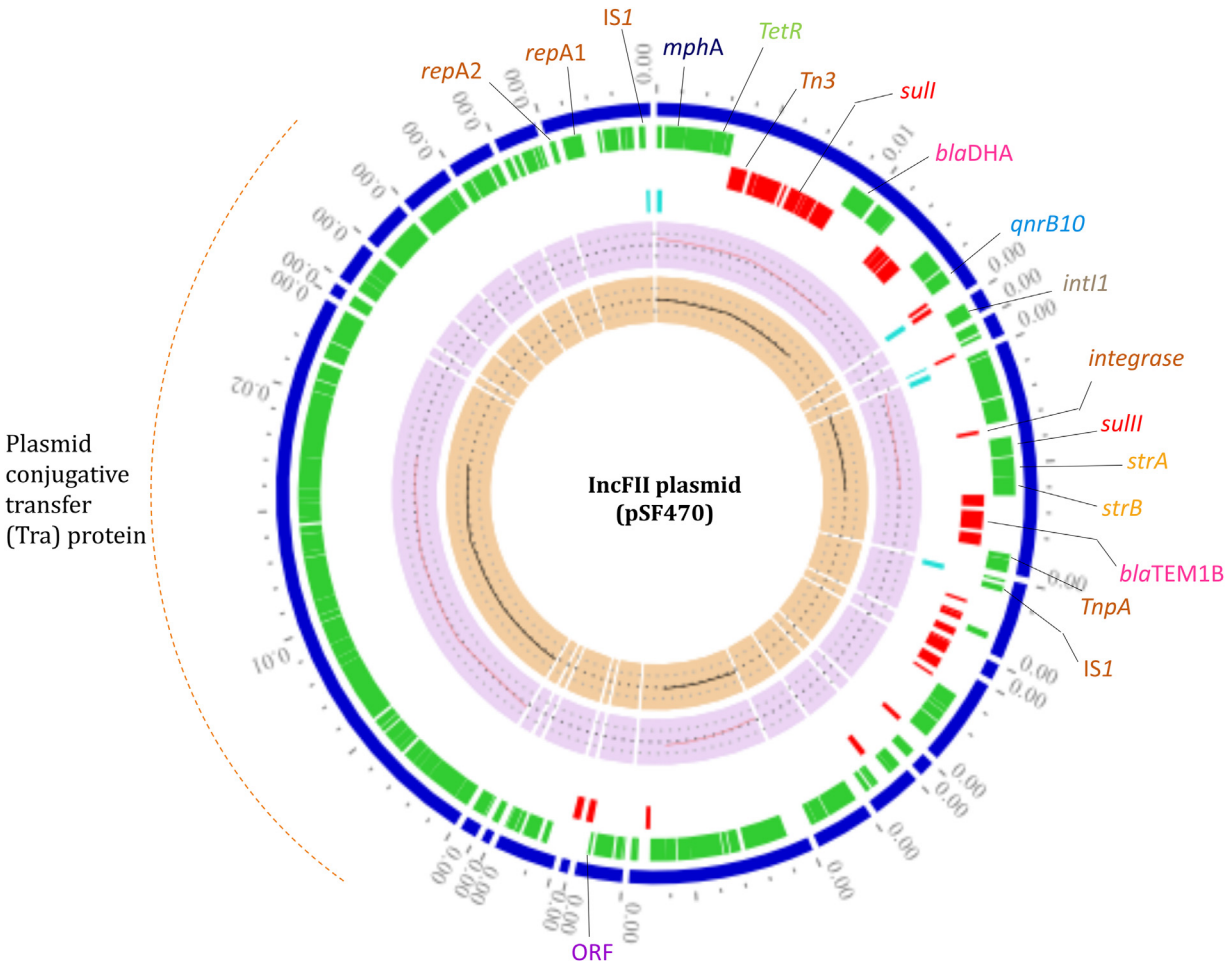

Fig. 1. Circular representation of IncFII plasmid pSF470. The outer blue ring represents the contigs of the plasmid. The next two rings (coloured green and red) show the coding sequences (CDSs) on the forward and reverse strand of the plasmid. Light blue denotes repeat region. The two inner rings represent the GC content and GC skew graph. IS*1*, insertion sequence; ORF, open reading frame; *sulI*/*sulII*, genes encoding sulphonamide resistance; *strA*/*strB*, genes encoding aminoglycoside resistance; $bla_{\text{TEM-1B}}$, $bla_{\text{DHA-1}}$, genes encoding β-lactam resistance; *mphA*, gene encoding macrolide resistance; *tetR*, gene responsible for tetracycline resistance; *qnrB10*, plasmid-mediated quinolone resistance gene; *intI1*, class 1 integrase; *rep*, replication protein; *tnpA*, transposase.

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Competing interests

None declared.

Ethical approval

Not required.

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