Molecular characterization of multidrug-resistant *Shigella* species isolated from epidemic and endemic cases of shigellosis in India

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Shigella species represent one of the growing numbers of antimicrobial-resistant bacteria in developing countries. Fluoroquinolone-resistant strains of *Shigella dysenteriae* type1 and *Shigella flexneri* type 2a emerged in India during 2002 and 2003, respectively. Sixty strains of *Shigella* from different parts of India were analysed for antimicrobial susceptibility, the presence of the *qnr* plasmid, mutations in the quinolone resistance determining regions (QRDRs), fluoroquinolone accumulation, and the presence of other genes encoding resistance to various antimicrobials. Fluoroquinolone-resistant strains had mutations in *gyrA* and *parC* genes and had an active efflux system. They were also resistant to several other antimicrobials but were susceptible to azithromycin and ceftriaxone. The majority of the strains harboured genes encoding resistance to ampicillin (97 %), tetracycline (95 %), streptomycin (95 %) and chloramphenicol (94 %). PFGE analysis revealed clonality among strains of *S. dysenteriae* types 1 and 5, *S. flexneri* type 2a and *Shigella boydii* type 12.

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INTRODUCTION

Shigellosis remains an important public health problem in developing countries with *Shigella sonnei* in Europe and the US and *Shigella flexneri* in Asian and African countries being of epidemiological importance. Antimicrobial therapy is advocated for shigellosis to shorten the duration of illness (Salam & Bennish, 1991). However, in Asia and Africa, antimicrobial resistance is an emerging problem among *Shigella* species (von Seidlein *et al.*, 2006) and treatment options are becoming limited globally (Salam & Bennish, 1991; Kariuki & Hart, 2001). The World Health Organization has recommended that ciprofloxacin should be considered a first-line antibiotic for the treatment of shigellosis, and the use of nalidixic acid is not encouraged, even in areas where it is still effective against *Shigella* (WHO, 2004). Similar to the prevalence of different serotypes, antimicrobial-resistance patterns of strains also differ from country to country and even within the same country (Pazhani *et al.*, 2005; von Seidlein *et al.*, 2006), which may be due to the spread of resistant clones as found for multidrug-resistant strains of *Shigella dysenteriae* type 1 in eastern parts of India (Pazhani *et al.*, 2004). In this study, we have investigated the mechanisms of antibiotic resistance and clonal relatedness of *Shigella* strains isolated from epidemic and endemic cases of shigellosis in different parts of India.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; QRDR, quinolone resistance determining region.

The GenBank/EMBL/DDBJ accession numbers for the $bla_{CTX\text{-}M\text{-}3}$ sequence of *S. boydii* type 1, the *aac(6′)-Ib-cr* sequence of *S. flexneri* type 3b, the *aac(6′)-Ib-cr* sequence of *S. boydii* type 1 and the *gyrA* and *parC* sequences for *S. boydii* type 1 are EF077620, EF501990, EF501993, EF077618 and EF077619, respectively.

METHODS

Bacterial strains. We examined 60 strains of *Shigella* species (20 *S. dysenteriae*, 16 *S. flexneri*, 7 *Shigella boydii* and 17 *S. sonnei*) isolated from dysentery outbreaks from different parts of India and sporadic hospitalized cases of shigellosis in Kolkata and Goa between 2001 and 2004. Strains were confirmed as *Shigella* spp. by standard biochemical tests (WHO, 1987) and serotyped using commercially available antisera (Denka Seiken).

Antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed by a disc diffusion method in accordance with National Committee of Clinical Laboratory Standards guidelines (NCCLS, 2004) for ampicillin (10 μg), co-trimoxazole (25 μg),

tetracycline (30 μg), chloramphenicol (30 μg), streptomycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), ofloxacin (5 μg), ceftriaxone (30 μg) and azithromycin (15 μg) (Becton Dickinson). *Escherichia coli* ATCC 25922 was used for quality control in each batch of tests. MICs of nalidixic acid, ciprofloxacin, norfloxacin, ofloxacin and azithromycin were determined for selected strains by the E-test (AB Biodisk).

Plasmid isolation and Southern hybridization. Plasmids were isolated from the representative quinolone- and fluoroquinolone-resistant *Shigella* strains using QIAGEN tip 100. Plasmids were transferred to Hybond-N^+ nylon membrane (Amersham Pharmacia) by a capillary method (Sambrook *et al.*, 1989). The membrane was hybridized with a DIG-labelled *qnr* probe, which was amplified from a *qnr*-positive strain (J53; p^{MG252}) with published primers (Wang *et al.*, 2003). For hybridization, a DIG-labelling and detection kit was used (Boehringer Mannheim). All strains were also screened with the *qnr* probe by colony hybridization.

PCR amplification

Amplification of the quinolone resistance determining regions (QRDRs). QRDRs of *gyrA* and *parC* genes were amplified as reported previously (Chu *et al.*, 1998). For each strain, 10 ng of the chromosomal DNA was used in the PCR assay.

Screening of antimicrobial-resistance genes. PCR was performed to detect genes encoding resistance to ampicillin (bla_{TEM}), gentamicin (*aadB*), streptomycin (*aadA1*, *strA*), kanamycin (*aphA1-Ia*), chloramphenicol (*catA1*), tetracycline [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(Y)] and β-lactams (bla_{OXA-1}, bla_{OXA-7}, bla_{SHV}, bla_{PSE-4} and $bla_{CTX-M-3}$) and plasmid mediated quinolone resistance (*qnr*) as published for other organisms (Maidhof *et al.*, 2002; Maynard *et al.*, 2003; Robicsek *et al.*, 2006). The newly designed primers FMEF (5′-GCA ACG CAA AAA CAA AGT TAG G-3′) and FMER (5′-GTG TTT GAA CCA TGT ACA-3′) were used to detect *aac(6′)-1b* variants. All assays were carried out as single PCR assays except that for the *qnr* gene, which was performed in a multiplex format, targeting all the three variants of *qnrA*, *qnrB* and *qnrS*. Template DNA was prepared by boiling the cultures grown in Luria–Bertani (LB) broth medium (Difco) for 10 min, rapidly cooled on ice followed by brief centrifugation at 5000 r.p.m.; the supernatant was retained for PCR assays.

Nucleotide sequencing of the PCR products. PCR products were purified with a QIAquick PCR purification column (Qiagen), and sequencing reactions were carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Nucleotide sequencing was performed in both directions with the same PCR primers used for the amplification of the target genes in an automatic sequencer (ABI Prism 3200; Applied Biosystems). Contig sequences were edited with DNASTAR (Lasergene) and compared in BLAST of the NCBI database.

Fluoroquinolone accumulation assay. Fluoroquinolone-sensitive and -resistant strains of *Shigella* were grown to mid-exponential phase in LB (OD_{600} 0.4), harvested, and suspended in 0.2 M MOPS/Tris buffer (pH 7.0) to an OD_{600} of 20 ml^{-1}. Cells were energized with 0.2 % glucose for 20 min and fluoroquinolones were added at a concentration of 10 μg ml^{-1}. Aliquots of this mixture were taken and suspended in 1 ml 100 mM glycine/HCl (pH 3.0), shaken for 1 h at room temperature, and the amount of released fluoroquinolone was determined spectrofluorometrically with excitation at 277 nm and emission at 448 nm. Experiments were performed in triplicate after the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to the assay mixture, as an inhibitor of the proton-motive force, at a final concentration of 100 μM.

PFGE. DNA fingerprinting was carried out by PFGE with the restriction enzyme *Xba*I (Takara) according to a standard procedure (CDC, 2000). PFGE run conditions were generated by the autoalgorithm mode of the CHEF Mapper system with a size range of 30–600 kb (Bio-Rad). After gel electrophoresis, gels were stained with ethidium bromide for 30 min and destained for 30 min with distilled water. The gel images were digitalized for computer-aided analysis (Gel Doc 2000; Bio-Rad).

Cluster analysis. PFGE gel images were retrieved and aligned to generate composite images containing the banding profiles of all the strains. The images were analysed with Diversity Database fingerprinting software (version 2.2.0; Bio-Rad) to determine the relatedness of the strains. Bands ranging from 48.5 to 600 kb were considered for the construction of dendrograms. Degrees of homology were determined by comparison of the Dice coefficient, and clustering correlation coefficients were calculated by an unweighted pair-group method with arithmetic averages (UPGMA). A dendrogram showing the hierarchical representation of the level of linkage between the strains was drawn to predict the degree of clonal relatedness.

RESULTS AND DISCUSSION

Antimicrobial resistance

Table 1 shows the breakdown of the serotypes of the 60 *Shigella* strains, their antimicrobial-resistance profiles and resistance gene complement. *S. dysenteriae* type 1 strains (n=17) were uniformly resistant to all the tested antimicrobials, except for azithromycin and ceftriaxone. *S. dysenteriae* type 1 strains HU8 and BCH518 isolated during 1988 and 1995 from a dysentery outbreak and sporadic infections, respectively, had similar resistance profiles. The two *S. dysenteriae* type 5 strains were susceptible to ampicillin, fluoroquinolone, azithromycin and ceftriaxone and were resistant to co-trimoxazole, tetracycline, chloramphenicol, nalidixic acid and streptomycin. Except for two strains (NK2685 and NK2683), all the tested *S. flexneri* strains (n=16) were resistant to co-trimoxazole, tetracycline and streptomycin. Three strains of *S. boydii* serotype 12 and the majority (94 %) of the *S. sonnei* strains had an identical resistance pattern (co-trimoxazole, tetracycline, nalidixic acid and streptomycin). The MIC for azithromycin-resistant *S. flexneri* type 3b (NK2788) and *S. boydii* type 1 (G24371) was 192 and 128 μg ml^{-1}, respectively. None of the other *Shigella* strains proved to be resistant to azithromycin and ceftriaxone, in contrast to a recent report from Bangladesh of resistance to these agents among *Shigella* species (Rahman *et al.*, 2004).

Fluoroquinolone resistance and resistance mechanisms

Ciprofloxacin, norfloxacin and ofloxacin are broad-spectrum fluoroquinolone agents that have excellent activity against most enteric pathogens. Clinical studies have underlined their safe use in adults and children (Bhattacharya *et al.*, 1997; Salam *et al.*, 1998). In this study, 30 % of the *Shigella* strains were resistant to

Table 1. Antimicrobial-resistance genes and profiles of *Shigella* strains

Serotype*	Strain	Resistance gene	Resistance profile†
S. dysenteriae type 1 (2)	HU 8, BCH 518	bla_{OXA-1}, *tet*(B), *catA1*, *strA*	A Co T C Na S
S. dysenteriae type 1 (16)		bla_{OXA-1}, *tet*(B), *catA1*, *strA*	A Co T C Na Nf Cf Of S
S. dysenteriae type 5 (2)	NK2440, NK2454	*tet*(A), *catA1*, *strA*	Co T C Na S
S. flexneri 1a	NK2685	–	–
S. flexneri 1b	NK2293	bla_{OXA-1}, *catA1*, *tet*(B), *strA*	A Co T Na S
S. flexneri 1b	NK2683	*catA1*, *tet*(B)	Co T C
S. flexneri 2a	NK2227	bla_{OXA-1}, *catA1*, *tet*(B), *aadA1*	A Co T C Na S
S. flexneri 2a	NK2674	bla_{OXA-1}, *catA1*, *tet*(B), *aadA1*	A Co T C S
S. flexneri 2a	NK2774	bla_{OXA-1}, *catA1*, *tet*(B), *strA*	A Co T C Na S
S. flexneri 2a	NK2475	bla_{OXA-1}, *tet*(B), *aadA1*	A Co T S
S. flexneri 2a	NK2555	*catA1*, *tet*(B), *aadA1*	Co T C Na S
S. flexneri 2a	NK2640	*catA1*, *tet*(B), *aadA1*	Co T C S
S. flexneri 2b	NK2226	*tet*(B), *aadA1*	Co T Na S
S. flexneri 2b	NK2399	bla_{OXA-1}, *catA1*, *tet*(B), *strA*	A Co T C S
S. flexneri 2b	NK2630	*tet*(B), *strA*	A Co T C Na S
S. flexneri 3a	NK2220	bla_{OXA-1}, *catA1*, *tet*(B), *strA*	A Co T C Na S
S. flexneri 6	NK2407	bla_{OXA-1}, *tet*(B), *catA1*, *aadA1*	A Co T C S
S. flexneri 3a	NK2668	*catA1*, *tet*(B), *strA*	Co T C Na S
S. flexneri 3b	NK2788	bla_{OXA-1}, bla_{TEM-1}, *tet*(AB), *strA*, *aac(6′)-Ib-cr*	A Co T Na Cf Nf Of Az S
S. boydii 1	NK2379	*aadA1*	Co S
S. boydii 1	G24371	bla_{OXA-1}, *tet*(AB), $bla_{CTX-M-3}$, *aadA1*, *aac(6′)-Ib-cr*	A Co T C Na Nf Cf Of Cr AZ S
S. boydii 2	NK1919	*tet*(B), *catA1*, *aadA1*	Co T C S
S. boydii 11	NK2310	–	Co S
S. boydii 12 (2)	NK1900, NK3701	*tet*(A), *strA*	Co T Na S
S. boydii 12	I14400	*tet*(B), *aadA1*	Co T Na S
S. sonnei (12)		*tet*(A), *strA*	Co T Na S
S. sonnei (2)	NK1825, NK1949	*strA*	Co T Na S
S. sonnei (2)	NK2077, NK2251		Co T Na S
S. sonnei (1)	NK 2183	bla_{OXA-1}, *tet*(AB), *catA1*, *strA*, *aadA1*	A Co T C S

*Number of strains is indicated in parentheses.
†A, Ampicillin; Co, co-trimoxazole; T, tetracycline; C, chloramphenicol; Na, nalidixic acid; Nf, norfloxacin; Cf, ciprofloxacin; Of, ofloxacin; Cr, ceftriaxone; Az, azithromycin; S, streptomycin.

fluoroquinolones and a *S. boydii* serotype 1 strain (G24371) was resistant to each of the four compounds tested (Table 2). Due to the unrestricted use of fluoroquinolones in Kolkata for the treatment of diarrhoea and other infectious diseases, resistance to these drugs has been reported among enteric pathogens (Garg *et al.*, 2001; Sinha *et al.*, 2004). In India, this trend has been increasing year on year since 2002 among *Shigella* species (Pazhani *et al.*, 2005). Fluoroquinolone resistance has also been identified in *Shigella* isolates in many Asian countries (MoezArdalan *et al.*, 2003; Talukder *et al.*, 2004; von Seidlein *et al.*, 2006) and Canada (CCDR, 2005). At present, fluoroquinolone-resistant *S. dysenteriae* type 1 and *S. flexneri* 2a strains remain susceptible to azithromycin and ceftriaxone, which have been reported to be effective against shigellosis in many countries (Khan *et al.*, 1997; Ashkenazi *et al.*, 2003), although cephalosporin resistance has been reported from Spain (Vila *et al.*, 1994) and Argentina (Radice *et al.*, 2001).

S. dysenteriae type 1 strains isolated from sporadic cases of dysentery from Kolkata (BCH518, NK2678 and H16576) and Goa (12567) and outbreak cases from Kolkata, Siliguri, Aizwal and Chandigarh (D2, 21, AZ11 and 115, respectively) were tested for mutations in the *gyrA* and *parC* genes. For comparison, we included nalidixic acid-resistant and fluoroquinolone-susceptible *S. dysenteriae* type 1 strains isolated during 1988 (HU8) and 1995 (BCH518). All fluoroquinolone-resistant strains had a uniform mutation in GyrA at position 83 (replacement of serine with leucine), and the majority of strains had a second mutation at position 87, with replacement of aspartic acid with either glycine or asparagine (Table 2). However, the *S. dysenteriae* type 1 strain BCH518, isolated during 1995, had a single mutation in GyrA at position 83, while strain HU8, isolated in Tripura during 1988, had no mutation in *gyrA* and *parC* and showed reduced susceptibility to nalidixic acid although it was susceptible to fluoroquinolones (Table 1). However, *S. flexneri* NK2788, *S. boydii* G24371 and a *S. dysenteriae* type 1 strain from the Aizwal outbreak showed amino acid replacement at position 87 (Asp→Asn). To our knowledge, this is the first report of *S. boydii* having a

Table 2. Fluoroquinolone resistance and amino acid substitutions in the QRDR of *Shigella* strains

Strain	Year	Place	MIC (μg ml^{-1})*				Amino acid substitution		
			Na	Cf	Nf	Of	GyrA		ParC
HU8 *S. dysenteriae* type 1	1988	Tripura outbreak	1	0.08	0.027	0.047	Ser_{83}	Ser_{87}	Ser_{80}
BCH518 *S. dysenteriae* type 1	1995	Kolkata sporadic case	>256	0.094	0.19	0.038	Ser_{83}→Leu	Asp_{87}	Ser_{80}
NK2678 *S. dysenteriae* type 1	2002	Kolkata sporadic case	>256	4	12	12	Ser_{83}→Leu	Asp_{87}→Gly	Ser_{80}→Ile
H16576 *S. dysenteriae* type 1	2002	Kolkata sporadic case	>256	6	16	8	Ser_{83}→Leu	Asp_{87}→Gly	Ser_{80}→Ile
D2 *S. dysenteriae* type 1	2002	Diamond Harbor outbreak	>256	4	12	8	Ser_{83}→Leu	Asp_{87}→Gly	Ser_{80}→Ile
21 *S. dysenteriae* type 1	2002	Siliguri outbreak	>256	6	16	12	Ser_{83}→Leu	Asp_{87}→Gly	Ser_{80}→Ile
12567 *S. dysenteriae* type 1	2002	Goa sporadic case	>256	4	8	12	Ser_{83}→Leu	Asp_{87}→Gly	Ser_{80}→Ile
AZ11 *S. dysenteriae* type 1	2003	Aizwal outbreak	>256	3	6	16	Ser_{83}→Leu	Asp_{87}→Asn	Ser_{80}→Ile
115 *S. dysenteriae* type 1	2003	Chandigarh outbreak	>256	3	8	8	Ser_{83}→Leu	Asp_{87}→Gly	Ser_{80}→Ile
NK2379 *S. boydii* type 1	2002	Kolkata sporadic case	1	0.006	0.032	0.047	Ser_{83}	Ser_{87}	Ser_{80}
G24371 *S. boydii* type 1	2001	Kolkata sporadic case	>256	200	500	>32	Ser_{83}→Leu	Asp_{87}→Asn	Ser_{80}→Ile
NK2788 *S. flexneri* type 3b	2002	Kolkata sporadic case	>256	200	250	>32	Ser_{83}→Leu	Asp_{87}→Asn	Ser_{80}→Ile
C15320 *S. flexneri* type 3b	1997	Kolkata sporadic case	1.25	0.012	0.023	0.064	Ser_{83}	Ser_{87}	Ser_{80}
NK2017 *S. sonnei*	2002	Kolkata sporadic case	>256	0.064	0.014	0.012	Ser_{83}→Leu	Ser_{87}	Ser_{80}

*Na, Nalidixic acid; Cf, ciprofloxacin; Nf, norfloxacin; Of, ofloxacin.

mutation in *gyrA*. All the fluoroquinolone-resistant strains had a single mutation in ParC at position 80 (replacement of serine with isoleucine). In a nalidixic acid-resistant *S. sonnei* strain (NK2017), a mutation was identified at position 83 (replacement of serine with leucine). Fluoroquinolone-resistant strains of *S. boydii* (G24371), *S. flexneri* (NK2788) and a representative *S. dysenteriae* type 1 (12567) strongly exhibited fluoroquinolone efflux (Table 3). The steady state accumulation of norfloxacin and ciprofloxacin was two- to fourfold lower in the resistant strains compared to that in the case of the sensitive strain C152 (Table 3). This suggests that the lower accumulation of fluoroquinolones can also account for the resistance of these strains. After the disruption of the efflux pump with the proton-motive force uncoupler CCCP, the accumulation was almost at the same level in all the tested strains. This clearly suggests that efflux pumps are one of the factors responsible for the development of resistance.

S. dysenteriae type 1 strains from South Asia and Canada had uniform mutations in *gyrA* and *parC* (Talukder *et al.*, 2004; CCDR, 2005). A *S. dysenteriae* strain (BCH518) which was susceptible to fluoroquinolones but resistant to nalidixic acid had an identical mutation in *gyrA*, similar to that of a 1995 strain isolated in Kolkata (Ahamed *et al.*, 1999). Similarly, mutations in *gyrA* of *S. flexneri* and *S. sonnei* were identical to those reported from other studies (Jeong *et al.*, 2003; Navia *et al.*, 2005). In shigellae, nalidixic acid resistance is not only due to mutations in the QRDR region, but also to an active efflux system (Ahamed *et al.*, 1999). Novel mechanisms for quinolone and fluoroquinolone resistance in members of the *Enterobacteriaceae* are emerging all the time (Perichon *et al.*, 2007; Yamane *et al.*, 2007), but high-level fluoroquinolone resistance in *S. dysenteriae* due to a proton-motive force-dependent efflux system was reported almost a decade ago in strains from Kolkata which were devoid of any *gyrA* mutations (Ghosh *et al.*, 1998).

Table 3. Accumulation of norfloxacin and ciprofloxacin in clinical isolates of *Shigella* strains

Strain	Serotype	Accumulation of norfloxacin* [μg (mg cells)$^{-1}$]		Accumulation of ciprofloxacin* [μg (mg cells)$^{-1}$]	
		Before addition of CCCP	After addition of CCCP	Before addition of CCCP	After addition of CCCP
S. dysenteriae C152	1	0.220±0.003	0.320±0.004	0.053±0.007	0.079±0.001
S. dysenteriae 12567	1	0.096±0.006	0.310±0.001	0.026±0.009	0.076±0.002
S. boydii NK2379	1	0.250±0.009	0.350±0.001	0.057±0.008	0.084±0.002
S. boydii G24371	1	0.066±0.005	0.330±0.002	0.012±0.003	0.081±0.002
S. flexneri C15320	3b	0.260±0.005	0.340±0.007	0.059±0.008	0.082±0.001
S. flexneri NK2788	3b	0.087±0.001	0.320±0.002	0.019±0.001	0.080±0.003

*Data represent the means±standard deviations of three determinations.

Plasmid-mediated quinolone resistance due to DNA gyrase protection by a protein from the pentapeptide repeat family called Qnr has recently been described in many clinical isolates of several species (Martinez-Martinez *et al.*, 2003; Jonas *et al.*, 2005). Indeed, clinical strains of *S. flexneri* type 2b from Japan were found to carry a transferable plasmid, which had 56 % amino acid identity with Qnr (Hata *et al.*, 2005). Based on the amino acid sequence, three subtypes of *qnr*, i.e. *qnrA*, *qnrB* and *qnrS*, and six variants each in *qnrA* and *qnrB* and two in *qnrS* have also been reported (Nordmann & Poirel, 2005; Cattoir *et al.*, 2007). In this study, none of the strains harboured *qnr* or its alleles. A novel ciprofloxacin-modifying enzyme (aminoglycoside acetyltransferase) encoding gene *aac(6′)-Ib-cr* was found in members of the *Enterobacteriaceae* resistant to fluoroquinolones (Park *et al.*, 2006). We have identified the *aac(6′)-Ib-cr* gene in *S. boydii* type 1 (G24371) and *S. flexneri* 3b (NK 2788) strains; to our knowledge, this gene has not been reported previously in these *Shigella* species.

Resistance to other antimicrobials and resistance genes

All the *S. dysenteriae* type 1 strains harboured $bla_{\text{OXA-1}}$, *catA1*, *tet*(B) and *strA* genes, encoding resistance to ampicillin, chloramphenicol, tetracycline and streptomycin, respectively (Table 1). *tet*(B) was more common (90 %) than *tet*(A) (10 %) in *S. dysenteriae*. Irrespective of serotypes, *S. flexneri* strains harboured *tet*(B) as well as $bla_{\text{OXA-1}}$, and in strain NK2788, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$ and *tet*(A) genes were detected (Table 1). Group 9 CTX-M β-lactamase has been reported in *S. boydii* (Vasilev *et al.*, 2007). In this study, $bla_{\text{CTX-M-3}}$ was found in a *S. boydii* type 1 strain (G24371) and, to our knowledge, this is the first report of this enzyme in this serotype. The majority of *S. sonnei* strains harboured *strA* (88 %) and *tet*(A) (76 %) genes rather than *aadA1* and *tet*(B) (6 % each). Genes encoding resistance to kanamycin (*aph1a*), gentamicin (*aadB*) and tetracycline [*tet*(C), *tet*(D), *tet*(E) and *tet*(Y)] were not found (data not shown). The chromosomal multi-antibiotic resistance locus of *S. flexneri* type 2a (Rajakumar *et al.*, 1997) was identified, supporting its common occurrence among several serotypes of *S. flexneri* (Casalino *et al.*, 1994; Thong *et al.*, 2002). We found 97 % and 3 % of ampicillin-resistant *Shigella* strains harbouring $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1}}$ genes, respectively. The predominance of $bla_{\text{OXA-1}}$ in *Shigella* has been reported from many countries (Maraki *et al.*, 1998; Siu *et al.*, 2000; Huang *et al.*, 2005). Similar to the reports from Mexico and Brazil (Martinez-Salazar *et al.*, 1986; Peirano *et al.*, 2005), we found a high frequency of *tet*(B) among *S. flexneri* strains, and in common with some South American *Shigella* strains (Peirano *et al.*, 2005), the presence of the chloramphenicol-resistance gene *catA1*, which encodes chloramphenicol *O*-acetyltransferase, and streptomycin resistance was confirmed in *S. flexneri* strains with either *strA* or *aadA1* genes or both (Table 1).

PFGE analysis

It was necessary to determine whether the frequency of antimicrobial resistance and its determinants was due to the widespread occurrence of specific clones. Two *S. dysenteriae* type 5 strains (NK2440 and NK2454) had identical *Xba*I restriction patterns by PFGE, but were different from serotype 1 strains, which had similar patterns (Fig. 1), which are akin to the previously reported PFGE profile (Pazhani *et al.*, 2004). Sixteen strains representing different serotypes of *S. flexneri* showed extensive variation in PFGE profile (Fig. 2) but six strains of type 2a were identical and clustered closest to two strains of serotype 2b. The remainder of the *S. flexneri* serotypes were distinct in DNA profile. Of the seven *S. boydii* strains, three belonging to serotype 12 were closely related in DNA profile while the remainder were distinct (Fig. 3). Eleven of the 17 *S. sonnei* strains were identical in DNA pattern and two strains clustered closely to this group, being different by two to three bands (Fig. 4). This underlines the findings by others of the clonal nature of *S. sonnei* (Alcoba-Florez *et al.*, 2005; Mammina *et al.*, 2005).

Although PFGE has proved to be a powerful tool for the discrimination of strains and identification of clonal lineages in several bacterial species, in some *S. boydii* serotypes it may not be as indicative of absolute strain relatedness. Woodward *et al.* (2005) reported that strains of *S. boydii* serotypes 1, 18, 19 and 20 from Canada gave highly similar *Xba*I macrorestriction patterns, which suggests that some strains that express different lipopolysaccharide antigen epitopes share a common genetic background. Other serotypes were genetically heterogeneous. The population structure of this species therefore

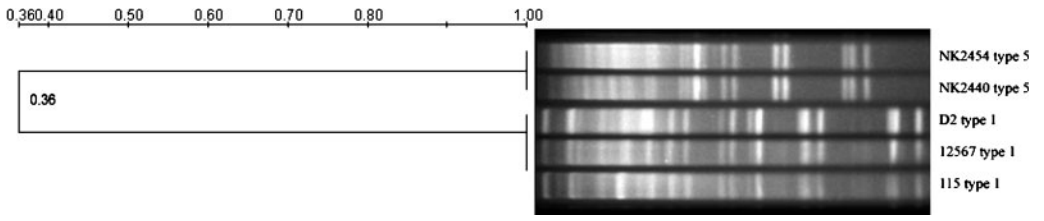

Fig. 1. *Xba*I PFGE profile of *S. dysenteriae* strains and dendrogram with percentage similarity.

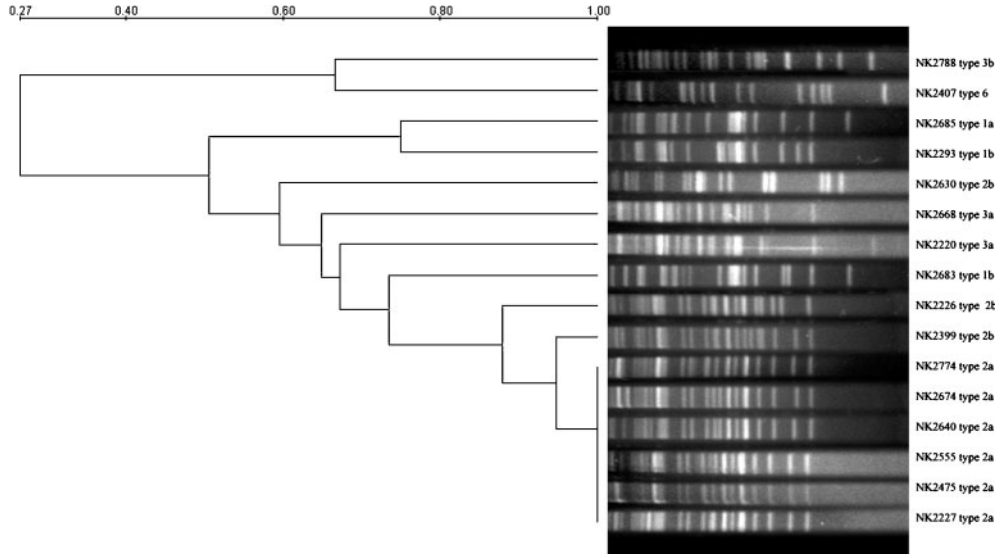

Fig. 2. *Xba*I PFGE profile of *S. flexneri* strains and dendrogram with percentage similarity.

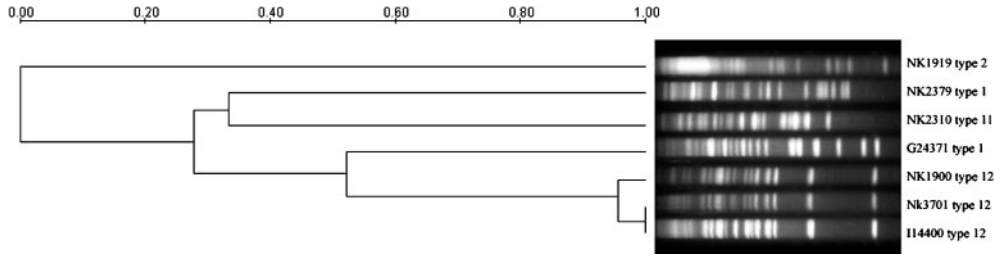

Fig. 3. *Xba*I PFGE profile of *S. boydii* strains and dendrogram with percentage similarity.

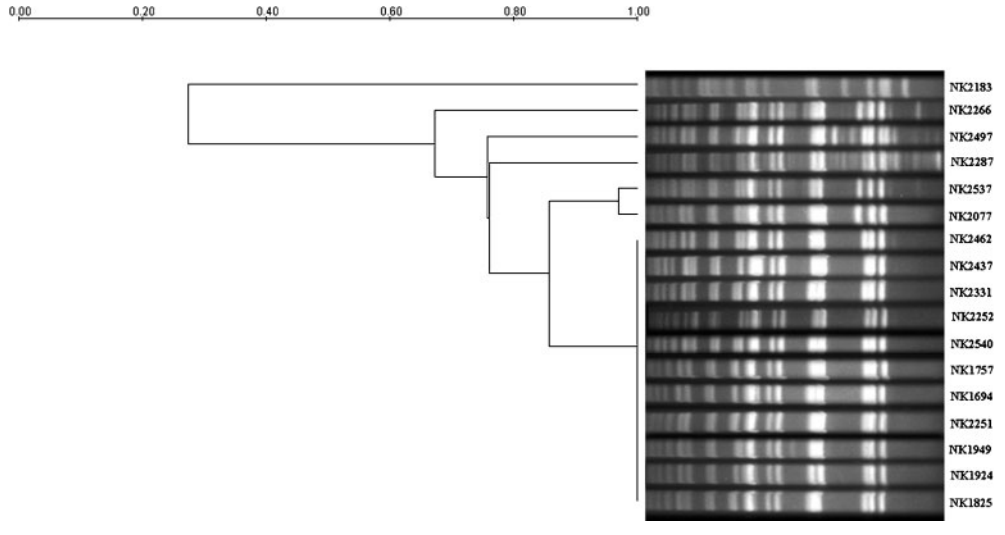

Fig. 4. *Xba*I PFGE profile of *S. sonnei* strains and dendrogram with percentage similarity.

warrants further investigation with complementary molecular tools such as multilocus sequence typing.

In conclusion, we have identified multidrug resistance among several serotypes of *Shigella* species isolated from acute diarrhoeal patients. Irrespective of the serogroup/serotype, most of the strains carried similar genes encoding resistance to specific antimicrobials. It is evident that fluoroquinolone resistance is spreading across different serogroups of *Shigella* species as they all carried mutations in the *gyrA* gene. With few exceptions, multidrug-resistant *Shigella* strains belonged to distinct clones.

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