Molecular profiling of antimicrobial resistance and integron association of multidrug-resistant clinical isolates of *Shigella* species from Faisalabad, Pakistan

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Abstract: Bacillary dysentery, common in developing countries, is usually caused by *Shigella* species. A major problem in shigellosis is the rapid emergence of multidrug-resistant strains. This is the first detailed molecular study on drug resistance of *Shigella* isolates from the Faisalabad region of Pakistan. Ninety-five *Shigella* isolates obtained after screening of 2500 stool samples were evaluated for in vitro resistance to commonly used antimicrobial agents; the presence or absence of 20 of the most relevant drug resistance genes; and the prevalence of integrons 1, 2, and 3. *Shigella flexneri* was found to be the most prevalent and most resistant species. Collectively, high resistance was found towards ampicillin (96.84%), tetracycline (93.68%), streptomycin (77.89%), and chloramphenicol (72.63%). Significant emerging resistance was detected towards the modern frontline drugs ciprofloxacin (12.63%), cefradine (17.89%), ceftriaxone (20.00%), cefoperazone (22.10%), and cefixime (28.42%). Prevalence rates for bla_{TEM}, $bla_{CTX\text{-}M}$, *gyrA*, *gyrB*, *qnrS*, *aadA1*, *strAB*, *tetA*, *tetB*, *catA*, and *catP* were 78.94%, 12.63%, 20.00%, 21.05%, 21.05%, 67.36%, 42.10%, 12.63%, 53.68%, 33.68%, and 25.26%, respectively. Class 2 integrons (42.10%) were more common in the local isolates. Simultaneous detection of class 1 and 2 integrons in some isolates and a rapidly emerging resistance to modern frontline drugs are the major findings of this study.

Key words: drug resistance, *Shigellae*, genetic elements.

Résumé : La dysenterie bacillaire, fréquente dans les pays en voie de développement, est habituellement causée par l'espèce *Shigella*. Un problème majeur de la shigellose consiste en l'émergence rapide de souches multi-résistantes aux médicaments. Il s'agit de la première étude moléculaire détaillée de la résistance aux médicaments d'isolats de *Shigella* provenant de la région de Faisalabad au Pakistan. Quatre-vingt-quinze isolats de *Shigella* obtenus après dépistage de 2500 échantillons de selles ont été évalués au plan de la résistance in vitro à des agents antimicrobiens d'utilisation courante, de la présence ou absence des 20 gènes de résistance les plus pertinents, et de la prévalence des intégrons 1, 2 et 3. *Shigella flexneri* s'est avérée l'espèce la plus fréquente et la plus résistante. Globalement, une forte résistance à l'ampicilline (96,84 %), à la tétracycline (93,68 %), à la streptomycine (77,89 %) et au chloramphénicol (72,63 %) a été trouvée. Une résistance en émergence significative contre les médicaments de pointe modernes ciprofloxacine (12,63 %), cefradine (17,89 %), ceftriaxone (20,00 %), cefoperazone (22,10 %) et cefixime (28,42 %) a été détectée. Les taux de prévalence de bla_{TEM}, $bla_{CTX\text{-}M}$, *gyrA*, *gyrB*, *qnrS*, *aadA1*, *strAB*, *tetA*, *tetB*, *catA* et *catP* étaient respectivement de 78,94 %, 12,63 %, 20,00 %, 21,05 %, 21,05 %, 67,36 %, 42,10 %, 12,63 %, 53,68 %, 33,68 % et 25,26 % respectivement. Les intégrons de classe 2 (42,10 %) étaient les plus fréquents dans les isolats locaux. La détection simultanée des intégrons de classe 1 et 2 dans quelques isolats et l'émergence rapide de la résistance aux médicament de pointe modernes sont les principaux faits saillants de cette étude.

Mots-clés : résistance aux drogues, *Shigella*, éléments génétiques.

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Introduction

Shigellae are enteropathogens that cause diarrhoea and bacillary dysentery in humans. Diarrhoea is the fourth most common infectious disease in the world and is second on the list as a cause of loss of productive life years (disability-adjusted life year) due to premature mortality and morbidity (Talukder et al. 2006). The genus *Shigella* is divided into 4 groups: A (*Shigella dysenteriae*), B (*Shigella flexneri*), C (*Shigella boydii*), and D (*Shigella sonnei*); and into 47 serotypes on the basis of "O" polysaccharide antigen (Zafar et al. 2005). Treatment of shigellosis is a combination of rehydration and antibiotic therapy. The traditionally used drugs

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trimethoprim–sulphamethoxazole, ampicillin, and chloramphenicol (Ahmed et al. 2006) show highly variable geographical patterns in terms of efficacy (Kuo et al. 2008). Most of the isolates from developing countries are resistant to these drugs and serve as reservoirs for drug resistance genes (Hens et al. 2005). Fluoroquinolones such as norfloxacin and ciprofloxacin are considered among the few drugs that are still effective against shigellosis (Sharma et al. 2010); the latter is the drug of choice for treating patients with bloody diarrhoea irrespective of the patient's age (Zafar et al. 2005).

Shigellae were among the first organisms shown to harbour transferable antibiotic resistance elements, and their acquisition of genes is reflected in the emergence of multidrug resistance (Peng et al. 2009). Little data are available regarding the distribution of drug-resistant isolates of *Shigella* in Pakistan. However, a study in Karachi reported high resistance to routinely used antibiotics: co-trimoxazole (87.75%), ampicillin (55.5%), and nalidixic acid (39%) (Zafar et al. 2005). Quinolones and cephalosporins have been considered efficacious in the treatment of shigellosis (Sabir and Zafar 2005). Previous reports from Pakistan are based solely on sensitivity determination by the disc diffusion method on clinical isolates of *Shigella.* To our knowledge this is the first report shedding light on the involvement of various drug resistance genes and the presence of integrons in local clinical isolates of *Shigella*.

Materials and methods

Collection of samples

Diarrhoeal patients admitted in the local hospitals with all or any 3 of the classical symptoms (abdominal pain and (or) cramps, high fever, vomiting, blood, pus and mucus in the stool, seizures) were included in this study after obtaining informed consent from the patients and approval from the institutional ethical committee. A total of 2500 stool samples were collected from hospitalised patients with diarrhoea and (or) dysentery, irrespective of age and sex, in Faisalabad region (population of more than 10 million), prior to the start of antimicrobial therapy: 1900 samples during 2009–2010 and 600 samples during 2011. Samples were collected in glycerol saline buffer (dipotassium phosphate 22.7 mmol/L (3.1 g/L), monopotassium phosphate 7 mmol/L (1 g/L), phenol red (0.003 g/L), sodium chloride 72 mmol/L (4.2 g/L)), immediately placed at 4 °C, and transported to the laboratory within 48 h.

Isolation and biochemical identification

After transportation to laboratory, 1 mL of sample was inoculated in 5 mL of selenite medium (Oxoid, Hampshire, UK) and incubated overnight at 37 °C with shaking. A loopful of culture was streaked on MacConkey agar plates (Merck, Darmstadt, Germany) and placed at 37 °C overnight. The suspected colonies of *Shigella* (non-lactose fermenting, white, transparent, convex, and nearly 2 mm in diameter) were subcultured on fresh MacConkey agar plates to get pure colonies. For biochemical identification, colonies were inoculated on triple sugar iron agar slants (Oxoid), and the results were interpreted according to Saeed et al. (2009). Biochemical identification was followed by inoculation on API 20 E (bioMérieux, Marcy l'Etoile, France) for further segregation.

Serology

Species identification was performed with polyvalent antisera (Pro-lab Diagnostics, Richmond Hill, Ontario, Canada) and results were interpreted according to the manufacturer's guidelines.

DNA extraction

Total genomic DNA from the *Shigella* isolates was extracted from overnight cultures in tryptic soy broth (Oxoid) by the conventional phenol–chloroform method. DNA integrity was checked on 1% agarose gel, purity was determined by A_{260}/A_{280} ratio, and quantification was done with a fluorometer (DyNA Quant 200, Hoefer, San Francisco, California, USA).

Confirmation of *Shigella*

Shigella isolates were confirmed by polymerase chain reaction (PCR) targeting the genus-specific *uidA* gene (486 bp) (Saeed et al. 2009) coding β-glucuronidase, using previously reported primers (Table 1). Each 100 μL of the reaction mixture contained 20 ng of template DNA, 1.5 mmol/L $MgCl_2$, 18 μmol/L each dNTP, 0.8 μmol/L each primer, 5 U of *Taq* polymerase, and distilled water to make the volume. The thermal cycler conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 7 min.

Antimicrobial susceptibility testing by disc diffusion method

The confirmed *Shigella* isolates were tested for antimicrobial resistance by the disc diffusion method on Antibiotic Sulfonamide Sensitivity agar (Merck). Eight major antimicrobial groups were covered by using discs of 13 most commonly used drugs: (*i*) trimethoprim–sulphamethoxazole (TMP); (*ii*) tetracycline (TE); (*iii*) nalidixic acid and ciprofloxacin (CIP) (quinolones); (*iv*) gentamicin, streptomycin (S), and amikacin (aminoglycosides); (*v*) chloramphenicol (C) (phenolic derivative); (*vi*) cefradine (CE), cefixime (CFM), ceftriaxone, and cefoperazone (CES) (cephalosporins); (*vii*) ampicillin (AM) (penicillin); and (*viii*) aztreonam (ATM) (monobactam). The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (National Committee for Clinical Laboratory Standards 2004).

PCR for antimicrobial genes

The PCR assays were performed targeting 20 genes associated with resistance to the above drugs using previously reported primers (Table 1). The antimicrobial drugs along with their targeted genes are listed in Table 2. As no datum was available from Pakistan, the gene selection was done on the basis of the highest reported prevalence worldwide. PCR conditions were the same as for the *uidA* gene. Similarly, the thermal cycler conditions for *strAB* (Tamang et al. 2007); bla_{TEM}, bla_{OXA}, *catP*, *catA*, *tetB* (Saeed et al. 2009); $bla_{CTX\text{-}M}$ (Zong et al. 2008); *qnrD*, *aac(6')-1b-cr* (Pu et al. 2009); *sul2* (Chu et al. 2001); and *sul1* (Sundström et al. 1988) genes were the same as for the *uidA* gene. However, for bla_{SHV} (Zong et al. 2008), the annealing temperature was kept at 52 °C for 40 s; for *aadA1*, *aac(3)-1*, and *tetA* (Saeed et al. 2009), 50 °C for 30 s; for *gyrA* (Saeed et al. 2009) and *gyrB* (Chau et al. 2007), 60 °C and 62 °C for 1 min, respectively; for *qnrB* (Robicsek et al. 2006) and *qnrA* (Jacoby et al. 2006), 53 °C for 45 s; for *qnrS* (Whichard et al. 2007),

Table 1. Primers used in the study.

Gene	Primer sequence (5′–3′)	Amplicon size (bp)	Reference
uidA	ATCACCGTGGTGACGCATGTCGC	486	Saeed et al. 2009
	CACCACGATGCCATGTTCATCTGC		
bla$_{TEM}$	ATGAGTATTCAACTTTCCGTGT	876	Saeed et al. 2009
	TTACCAATGCTTAATCAGTGACG		
gyrA	TACCGTCATAGTTATCCACGA	342	Saeed et al. 2009
	GTACTTTACGCCATGAACGT		
gyrB	CAAACTGGCGGACTGTCAGG	345	Chau et al. 2007
	TTCCGGCATCTGACGATAGA		
qnrA	GATAAAGTTTTTCAGCAAGAGG	543	Jacoby et al. 2006
	ATCCAGATCGGCAAAGGTTA		
qnrB	GATCGTGAAAGCCAGAAAGG	469	Robicsek et al. 2006
	ACGATGCCTGGTAGTTGTCC		
qnrD	CGAGATCAATTTACGGGGAATA	656	Pu et al. 2009
	AACAAGCTGAAGCGCCTG		
qnrS	ATGGAAACCTACAATCATAC	492	Whichard et al. 2007
	AAAAACACCTCGACTTAAGT		
aac(6')-1b-cr	GCAACGCAAAAACAAAGTTAGG	560	Pu et al. 2009
	GTGTTTGAACCATGTACA		
catP	CCTGCCACTCATCGCAGT	623	Saeed et al. 2009
	CACCGTTGATATATCCC		
catA	CGCCTGATGAATGCTCATCCG	457	Saeed et al. 2009
	CCTGCCACTCATCGCAGTAC		
tetA	GTAATTCTGAGCACTGTCGC	956	Saeed et al. 2009
	CTGCCTGGACAACATTGCTT		
tetB	CTCAGTATTCCAAGCCTTTG	416	Saeed et al. 2009
	CTAAGCACTTGTCTCCTGTT		
bla$_{OXA}$	ATGAAAAACACAATACATATCAACTTCGC	820	Saeed et al. 2009
	GTGTGTTTAGAATGGTGATCGCATT		
bla$_{CTX-M}$	ATGTGCAG[Y]ACCAGTAARGTKATGGC	593	Zong et al. 2008
	TGGGTRAARTARGTSACCAGAA[Y]CAGCGG		
bla$_{SHV}$	CGCCGGGTTATTCTTATTTGTCGC	1015	Zong et al. 2008
	TCTTTCCGATGCCGCCGCCAGTCA		
aadA1	TGATTTGCTGGTTACGGTGAC	284	Saeed et al. 2009
	CGCTATGTTCTCTTGCTTTTG		
aac(3)-1	ACCTACTCCCAACATCAGCC	157	Saeed et al. 2009
	ATATAGATCTCACTACGCGC		
strAB	ATGGTGGACCCTAAAACTCT	891	Tamang et al. 2007
	CGTCTAGGATCGAGACAAAG		
sul1	CTTCGATGAGAGCCGGCGGC	437	Sundström et al. 1988
	GCAAGGCGGAAACCCGCGCC		
sul2	TCAACATAACCTCGGACAGT	707	Chu et al. 2001
	GATGAAGTCAGCTCCACCT		
intI1	ATCATCGTCGTAGAGACGTCGG	893	Saeed et al. 2009
	GTCAAGGTTCTGGACCAGTTGC		
intI2	GCAAATGAAGTGCAACGC	467	Saeed et al. 2009
	ACACGCTTGCTAACGATG		
intI3	GCAGGGTGTGGACAGATACG	760	Saeed et al. 2009
	ACAGACCGAGAAGGCTTATG		

48 °C for 45 s. In each case, 2 negative controls were included — 1 consisting of DNA of a drug-sensitive bacterium and the other without any template DNA.

Sequencing

Sequencing of the amplified products of *gyrA* and *gyrB* genes was done through a vendor (Macrogen Inc., Korea).

PCR for detection of integrons

Integrons 1, 2, and 3 were detected by targeting *intI1*, *intI2*, and *intI3* (Saeed et al. 2009) genes, respectively, using the primer sets already reported (Table 1). Each 100 μL of the reaction mixture contained 20 ng of template DNA, 1.5 mmol/L $MgCl_2$, 18 μmol/L each dNTP, 0.8 μmol/L each primer, 5 U *Taq* polymerase, and distilled water to make the

Table 2. Relative occurrence of various drug-resistance-related genes.

Group	Drugs	Phenotypic resistance	Related genes	Genotypic resistance
Penicillins	Ampicillin	92 (96.84%)	bla_{TEM}	75 (78.94%)
Cephalosporins	Cefradine	17 (17.89%)	*blt*	0
	Cefixime	27 (28.42%)	bla_{OXA}	0
	Ceftriaxone	19 (20.00%)	bla_{CTX-M}	12 (12.63%)
	Cefoperazone	21 (22.10%)	bla_{SHV}	0
Monobactam	Aztreonam	12 (12.63%)		
Quinolones	Nalidixic acid	63 (66.31%)	*gyrA*	19 (20.00%)
	Ciprofloxacin	12 (12.63%)	*gyrB*	20 (21.05%)
			parE	0
			parC	0
			qnrA	0
			qnrB	0
			qnrD	0
			qnrS	20 (21.05%)
			aac(6')-1b-cr	0
Aminoglycosides	Gentamicin	16 (16.84%)	*aac(3)-1*	0
	Streptomycin	74 (77.89%)	*aadA1*	64 (67.36%)
	Amikacin	23 (24.20%)	*strAB*	40 (42.10%)
Sulphonamide	Trimethoprim – sulphamethoxazole	92 (96.84%)	*sul1*	28 (29.47%)
			sul2	48 (50.52%)
Tetracyclins	Tetracycline	89 (93.68%)	*tetA*	12 (12.63%)
			tetB	51 (53.68%)
Phenolic derivatives	Chloramphenicol	69 (72.63%)	*catA*	32 (33.68%)
			catP	24 (25.26%)

volume. The thermal cycler conditions for *intI1* and *intI2* were as follows: 96 °C for 30 s, 55 °C for 1 min, and 70 °C for 3 min, followed by 25 cycles of 96 °C for 15 s, 55 °C for 30 s, and 70 °C for 3 min. Conditions for *intI3* were 94 °C for 5 min, followed by 30 cycles of 94 °C for 2 min, 57 °C for 1 min, and 72 °C for 1.5 min. A final extension at 72 °C for 7 min was performed.

A multiplex PCR for *intI1* and *intI2* genes was performed for the confirmation of the isolates having both the genes simultaneously under the same thermal cycler conditions as mentioned for *intI1* and *intI2*. Each 100 μL of the reaction mixture contained 20 ng of DNA, 1.5 mmol/L $MgCl_2$, 70 μmol/L each dNTP, 1 μmol/L each primer, 10 U of *Taq* polymerase, and distilled water to make the volume.

Restriction analysis of amplicons for confirmation of integrons

*Nsb*I and *Eco*521 were used for restriction of class 1 integron amplicons, whereas *Nsb*I only was used for class 2 integrons (Hens et al. 2005). In each case, 30 μL of restriction mixture contained 1 μL (10 U) of restriction enzyme, 8 μL of PCR amplified product, 3 μL of enzyme buffer, and 18 μL of double-distilled water. Restriction mixtures were incubated at 37 °C for 45 min and results were interpreted after electrophoresis on 2% agarose gel.

Statistical analysis

Two-way analysis of variance was used to compare the activity of different antibiotics used against 4 *Shigella* spp., and comparison among these species for resistance to different antibiotics was done separately. Difference was considered statistically significant if the P value was ≤ 0.05.

Results

Biochemical and molecular detection of *Shigella* isolates

Out of 2500 stool samples, 95 (3.8%) were positive for *Shigella* biochemically. All these isolates were confirmed by PCR targeting the *uidA* gene. Serological identification showed 47 isolates as *S. flexneri*, 28 as *S. dysenteriae*, 12 as *S. sonnei*, and 8 as *S. boydii*.

Antimicrobial resistance profile by disc diffusion method

The drug resistance patterns by disc diffusion method (after taking the mean of 3 values) are summarized in Table 3. Of 95 isolates, 92 (96.84%) were found to be multidrug resistant (resistant to at least 3 structurally different antimicrobial drugs). *Shigella flexneri*, the most prevalent serotype in Pakistan, mostly showed the drug resistance pattern TMP, TE, and AM, whereas the second commonest serotype, *S. dysenteriae*, was mostly representing the C, TMP, TE, AM, S, and CES pattern. Statistical comparison among 4 species showed *S. flexneri* as significantly resistant to the antimicrobials as compared with other species ($P < 0.05$).

This study revealed that aztreonam, ciprofloxacin, and cephalosporins are drugs of choice for the treatment of shigellosis (irrespective of the infecting species) by showing significant antimicrobial activity ($P < 0.05$) as compared with other selected drugs; however, rapidly emerging resistance to ciprofloxacin and cephalosporin was observed (Table 2).

Table 3. Drug resistance pattern of *Shigella* species by disc diffusion method.

Drug	*S. flexneri* (47 (49.47%))	*S. dysenteriae* (28 (29.47%))	*S. sonnei* (12 (12.63%))	*S. boydii* (8 (8.42%))
Ampicillin	47 (100.00%)	28 (100.00%)	9 (75.00%)	8 (100.00%)
Cefradine	12 (25.53%)	4 (14.28%)	1 (8.33%)	0
Cefixime	16 (34.04%)	8 (28.57%)	1 (8.33%)	2 (25.00%)
Ceftriaxone	13 (27.65%)	4 (14.28%)	0	2 (25.00%)
Cefoperazone	16 (34.04%)	4 (14.28%)	2 (16.66%)	1 (12.50%)
Aztreonam	8 (17.02%)	2 (7.14%)	1 (8.33%)	1 (12.50%)
Nalidixic acid	39 (82.97%)	13 (46.42%)	8 (66.66%)	3 (37.50%)
Ciprofloxacin	6 (12.76%)	2 (7.14%)	2 (16.66%)	2 (25.00%)
Streptomycin	43 (91.48%)	22 (78.57%)	1 (8.33%)	8 (100.00%)
Gentamicin	6 (12.76%)	3 (10.71%)	4 (33.33%)	3 (37.50%)
Amikacin	12 (25.53%)	5 (17.85%)	2 (16.66%)	4 (50.00%)
Trimethoprim–sulphamethoxazole	47 (100.00%)	28 (100.00%)	9 (75.00%)	8 (100.00%)
Tetracycline	47 (100.00%)	28 (100.00%)	8 (66.66%)	6 (75.00%)
Chloramphenicol	35 (74.40%)	20 (71.42%)	8 (66.66%)	6 (75.00%)

Note: Statistical analysis by ANOVA (analysis of variance) for variable number of isolates of different species showed *S. flexneri* more prevalent and resistant to the antimicrobial drugs than the other species.

Detection of antimicrobial resistance genes

The frequency of detection of bla_{TEM} (most reported gene related to penicillin resistance) was 75 (78.94%) isolates. All the isolates harbouring this gene were also resistant to ampicillin by disc diffusion method. For cephalosporin resistance, the bla_{OXA} and bla_{SHV} genes were not detected but 12 (12.63%) isolates showed amplification of $bla_{CTX\text{-}M}$. Eight of these isolates were resistant to cephalosporin by disc diffusion method.

The *gyrA* gene was detected in 19 (20.00%) isolates; only 17 were found sensitive phenotypically to fluoroquinolones. Although the *gyrB* gene was detected in 20 (21.05%) isolates, only 8 were phenotypically resistant. In 4 isolates (4.21%), both *gyrA* and *gyrB* genes were amplified, and 2 out of these 4 isolates were resistant to fluoroquinolones by disc diffusion method. Sequencing results of these amplified genes did not show any reported mutation in the protein framework, suggesting the involvement of some other mechanisms in development of drug resistance. Among plasmid-mediated *qnrABSD* and *aac(6')-1b-cr*, only *qnrS* gene showed amplification in 20 (21.05%) isolates. All these isolates were resistant to quinolones by disc diffusion method.

For chloramphenicol resistance, *catA* and *catP* were detected in 32 (33.68%) and 24 (25.26%) isolates, respectively. In 8 (8.42%) phenotypically resistant isolates, both genes were present, while 12 (12.63%) isolates harbouring either of the 2 genes were sensitive, by disc diffusion method. The *aadA1* and *strAB* genes, responsible for aminoglycoside resistance, were found in 64 (67.36%) and 40 (42.10%) isolates, respectively. No phenotypic resistance was seen in 10 (10.52%) isolates amplifying these genes, while both the genes were observed in 20 (21.05%) isolates also resistant, by disc diffusion method.

In case of sulphonamides, 28 (29.47%) isolates amplified the *sul1* gene and 48 (50.52%) the *sul2* gene. These isolates had phenotypic resistance as well. Both these genes were amplified in 12 (12.63%) isolates. Tetracycline resistance was observed phenotypically in 89 (93.68%) isolates; *tetA* and *tetB* genes were detected in 12 (12.63%) and 51 (53.68%) isolates, respectively. All isolates but 4 (4.21%) showed phenotypic resistance. Simultaneous presence of both the genes was observed in 8 (8.42%) isolates. None of these isolates was sensitive to tetracyclines by disc diffusion method. All the PCR results were reproducible.

Prevalence of integrons

Class 1 integrons were identified in 30 (31.57%) isolates of which 12 were *S. flexneri*, 12 *S. dysenteriae*, 4 *S. sonnei*, and 2 *S. boydii.* Species-wise occurrence was 25.53%, 42.85%, 33.33%, and 25.00%, respectively. Class 2 integrons were detected in 40 (42.10%) isolates: 16 were *S. flexneri*, 15 *S. dysenteriae*, 5 *S. sonnei*, and 4 *S. boydii*. Species-wise relative occurrence was 34.04%, 53.57%, 41.66%, and 50%, respectively. All the isolates were found negative for class 3 integrons.

Ten isolates were positive for both class 1 and 2 integrons: 4 (8.51%) were *S. flexneri,* 5 (17.85%) *S. dysenteriae*, and 1 (8.33%) *S. sonnei*. The amplicons were confirmed by restriction analysis. The 893 bp PCR product of the *intI1* gene was restricted into the desired 662 and 231 bp fragments using *Nsb*I endonuclease restriction enzyme and expected fragments of 606 and 287 bp by using *Eco*521. The 467 bp PCR product of *intI2* gene was restricted into the desired 387 and 80 bp fragments using *Eco*521 endonuclease restriction enzyme.

Discussion

This study on shigellosis was focused on hospitalized diarrhoeal patients prior to antimicrobial therapy. Incidence of shigellosis was found to be 3.8%, which is less than that observed in the previous study (4.8%) conducted in Karachi and its slums (Zafar et al. 2009). In our report the most prevalent species was *S. flexneri*, followed by *S. dysenteriae*, *S. sonnei*, and *S. boydii*. The prevalence pattern identified during 2002–2004 in Karachi was *S. flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae* in descending order (Zafar et al. 2009), whereas in northern Pakistan, *S. dysenteriae* was dominant compared with other species (Ahmed et al. 2003).

The drug resistance patterns show great geographical variation (Phantouamath et al. 2005). Initially the drugs that were considered efficacious have now become impotent in the face of emerging resistance owing to their misuse (Ahmed et al. 2006). The impact can be more serious in countries like Pakistan owing to the acute nature of disease, antimicrobial resistance spread in other enteric pathogens, and nonavailability of efficient primary healthcare facilities to the general public (Sabir and Zafar 2005). None of the present local isolate was found pansensitive. Most of the isolates (92 out of 95 isolates) in our study were found to be multidrug resistant. Resistance to the first-line drugs ampicillin (96.84%), trimethoprim–sulphamethoxazole (96.84%), and tetracycline (93.68%) was alarmingly higher than that reported from 8 Asian countries, with percentage prevalence 53%, 81%, and 74%, respectively (Kuo et al. 2008). Prevalence of the penicillin resistance gene bla_{TEM} (78.94%) in 75 isolates was higher, contrary to other reports (Peirano et al. 2005). Among sulphonamide and tetracycline resistance genes *sul2* (50.52%) and *tetB* (53.68%) were more prevalent, consistent with a previous study by Peirano et al. (2005).

Fluoroquinolones are one of the most important groups of antibiotics used clinically. The resistance to nalidixic acid was found very high (66.31%), as compared with the previous report of 39% from Karachi, Pakistan, and 31.3% from Nepal (Wilson et al. 2006). However, ciprofloxacin resistance (12.63%) is similar to the reports (10%) from Asia, Middle East, and USA (Kuo et al. 2008). To our knowledge, this is the first time plasmid-mediated quinolone-resistant local isolates have been detected in Pakistan. This is a major public health concern, since plasmid-mediated quinolone-resistant determinants are responsible for the spread of resistance among the *Enterobacteriacae* members (Folster et al. 2011). Twenty isolates (21.05%) were positive for *qnrS*. Five of these isolates also showed simultaneous amplification of *gyrA*. However, 4 isolates were positive both for *gyrB* and *qnrS*. Similarly, amplification of *qnrS* has also been reported from China (Pu et al. 2009). Contrary to the study carried out in the United States where *qnrB*, *aac(6')-1b-cr*, and *qnrS* were detected in the majority of the ciprofloxacin-resistant cases with a history of recent travel to India, China, and Asia (Folster et al. 2011), no amplification of *qnrB* and *aac (6')-1b-cr* was obtained in the present study isolates. The increased resistance may be due to the liberal use of these drugs by quack doctors, easy access to drugs without any prescription, and lastly the microorganism's ability to acquire resistance genes (Navia et al. 2005).

Emerging resistance to cephalosporins in the present isolates, by the disc diffusion method, has been reported earlier (Vinh et al. 2009). The observation of 20% resistance to ceftriaxone in this study is an alarming increase from the 2.4% reported earlier by Khan et al. (2009). The $bla_{CTX\text{-}M}$ gene related to cephalosporins resistance was detected in 12 (12.63%) isolates, while reports showing 37.23% isolates from southern Vietnam and 8.1% isolates from China have been published (Nhu et al. 2010; Xiong et al. 2007).

Among aminoglycosides, gentamicin was found to be most effective, which is in agreement with other studies (Bercion et al. 2008), but the prevalence of relevant genes *aadA1* (67.36%) and *strAB* (42.10%) is in contradiction from previous reports from India (*aadA1* 20% and *strAB* 73.33%) (Pazhani et al. 2008).

Resistance to chloramphenicol (72.63%) observed in local isolates is higher as compared with earlier studies (Peirano et al. 2005). The *catA* prevalence (33.68%) noted in the present study is less than previous reports of 67.7% from Brazil and 55% from India (Pazhani et al. 2008; Peirano et al. 2005).

Integrons play a major role in horizontal transfer of drug resistance genes. Class 1 integrons have been reported in 40%–70% of Gram-negative clinical isolates (Gillings et al. 2008). Interestingly, in *Shigella* species, class 2 integrons have been reported in all isolates (Jin et al. 2010). The present study also showed a preponderance of class 2 integrons (40 (42.10%)) over class 1 integrons (30 (31.57%)), but it was not as dominating as reported above.

Both class 1 and 2 integrons were simultaneously present in 10 (10.52%) isolates: 4 isolates were *S. flexneri*, 5 *S. dysenteriae*, and 1 *S. sonnei*. This dual occurrence has been reported earlier in one isolate from Brazil (Peirano et al. 2005) and 7 isolates from Korea (Jin et al. 2010). This finding highlights the increasing sophistication in the armory of these pathogens.

It is concluded that multidrug resistance is a major emerging problem in shigellosis in Pakistan. The simultaneous presence of both class 1 and 2 integrons in 10 (10.52%) of local *Shigella* isolates, rarely reported globally and for the first time in Pakistan, indicates the gravity of this situation. *Shigella flexneri* was found to be the most prevalent and resistant species. Resistance to ceftriaxone is unique and alarming, as no previous study from Asia has shown such a high resistance of *Shigella* species to this drug. The present study also highlights the rapidly increasing resistance to ciprofloxacin, as compared with reports from other Asian and Middle-Eastern countries.

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References

Ahmed, K., Shakoori, F.R., and Shakoori, A.R. 2003. Aetiology of shigellosis in northern Pakistan. J. Health Popul. Nutr. **21**(1): 32–39. PMID:12751672.

Ahmed, S.F., Riddle, M.S., Wierzba, T.F., Messih, I.A., Monteville, M.R., Sanders, J.W., and Klena, J.D. 2006. Epidemiology and genetic characterization of *Shigella flexneri* strains isolated from three paediatric populations in Egypt (2000–2004). Epidemiol. Infect. **134**(6): 1237–1248. doi:10.1017/S095026880600642X. PMID:16690004.

Bercion, R., Njuimo, S.P., Boudjeka, P.M., and Manirakiza, A. 2008. Distribution and antibiotic susceptibility of *Shigella* isolates in Bangui, Central African Republic. Trop. Med. Int. Health, **13**(4): 468–471. doi:10.1111/j.1365-3156.2008.02023.x. PMID: 18282240.

Chau, T.T., Campbell, J.I., Galindo, C.M., Van Minh Hoang, N., Diep, T.S., Nga, T.T., et al. 2007. Antimicrobial drug resistance of *Salmonella enterica* serovar Typhi in asia and molecular mechanism of reduced susceptibility to the fluoroquinolones.

Antimicrob. Agents Chemother. **51**(12): 4315–4323. doi:10.1128/AAC.00294-07. PMID:17908946.

Chu, C., Chiu, C.-H., Wu, W.-Y., Chu, C.-H., Liu, T.-P., and Ou, J.T. 2001. Large drug resistance virulence plasmids of clinical isolates of *Salmonella enterica* serovar Choleraesuis. Antimicrob. Agents Chemother. **45**(8): 2299–2303. doi:10.1128/AAC.45.8.2299-2303.2001. PMID:11451688.

Folster, J.P., Pecic, G., Bowen, A., Rickert, R., Carattoli, A., and Whichard, J.M. 2011. Decreased susceptibility to ciprofloxacin among *Shigella* isolates in the United States, 2006 to 2009. Antimicrob. Agents Chemother. **55**(4): 1758–1760. doi:10.1128/AAC.01463-10. PMID:21220535.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., and Stokes, H.W. 2008. The evolution of class 1 integrons and the rise of antibiotic resistance. J. Bacteriol. **190**(14): 5095–5100. doi:10.1128/JB.00152-08. PMID:18487337.

Hens, D.K., Niyogi, S.K., and Kumar, R. 2005. Epidemic strain *Shigella dysenteriae* Type 1 Dt66 encodes several drug resistances by chromosome. Arch. Med. Res. **36**(4): 399–403. doi:10.1016/j.arcmed.2005.03.006. PMID:15950082.

Jacoby, G.A., Walsh, K.E., Mills, D.M., Walker, V.J., Oh, H., Robicsek, A., and Hooper, D.C. 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob. Agents Chemother. **50**(4): 1178–1182. doi:10.1128/AAC.50.4.1178-1182.2006. PMID:16569827.

Jin, Y.H., Oh, Y.H., Jung, J.H., Kim, S.J., Kim, J.A., Han, K.Y., et al. 2010. Antimicrobial resistance patterns and characterization of integrons of *Shigella sonnei* isolates in Seoul, 1999–2008. J. Microbiol. **48**(2): 236–242. doi:10.1007/s12275-010-9220-z. PMID:20437157.

Khan, E., Jabeen, K., Ejaz, M., Siddiqui, J., Shezad, M.F., and Zafar, A. 2009. Trends in antimicrobial resistance in Shigella species in Karachi, Pakistan. J. Infect. Dev. Ctries. **3**(10): 798–802. doi:10.3855/jidc.500. PMID:20009283.

Kuo, C.Y., Su, L.H., Perera, J., Carlos, C., Tan, B.H., Kumarasinghe, G., et al. 2008. Antimicrobial susceptibility of *Shigella* isolates in eight Asian countries, 2001–2004. J. Microbiol. Immunol. Infect. **41**(2): 107–111. PMID:18473096.

National Committee for Clinical Laboratory Standards. 2004. Performance standards for antimicrobial susceptibility testing; 14th informational supplement. Document M100-S14. National Committee for Clinical Laboratory Standards, Wayne, Penn., USA.

Navia, M.M., Gascon, J., and Vila, J. 2005. Analysis of the mechanisms of resistance to several antimicrobial agents in *Shigella* spp. causing travellers' diarrhoea. Clin. Microbiol. Infect. **11**(12): 1044–1047. doi:10.1111/j.1469-0691.2005.01277.x. PMID:16307563.

Nhu, N.T.K., Vinh, H., Nga, T.V.T., Stabler, R., Duy, P.T., Vien, L.T.M., et al. 2010. The sudden dominance of $bla_{CTX\text{-}M}$ harbouring plasmids in *Shigella* spp. circulating in southern Vietnam. PLoS Negl. Trop. Dis. **4**(6): e702. doi:10.1371/journal.pntd.0000702. PMID:20544028.

Pazhani, G.P., Niyogi, S.K., Singh, A.K., Sen, B., Taneja, N., Kundu, M., et al. 2008. Molecular characterization of multidrug-resistant *Shigella* species isolated from epidemic and endemic cases of shigellosis in India. J. Med. Microbiol. **57**(7): 856–863. doi:10.1099/jmm.0.2008/000521-0. PMID:18566144.

Peirano, G., Agerso, Y., Aarestrup, F.M., and dos Prazeres Rodrigues, D. 2005. Occurrence of integrons and resistance genes among sulphonamide-resistant *Shigella* spp. from Brazil. J. Antimicrob. Chemother. **55**(3): 301–305. doi:10.1093/jac/dki012. PMID:15681578.

Peng, J., Yang, J., and Jin, Q. 2009. The molecular evolutionary history of *Shigella* spp., and enteroinvasive *Escherichia coli*. Infect. Genet. Evol. **9**(1): 147–152. doi:10.1016/j.meegid.2008.10.003. PMID:19000785.

Phantouamath, B., Sithivong, N., Insisiengmay, S., Ichinose, Y., Higa, N., Song, T., and Iwanaga, M. 2005. Pathogenicity of *Shigella* in healthy carriers: a study in Vientiane, Lao People's Democratic Republic. Jpn. J. Infect. Dis. **58**(4): 232–234. PMID:16116257.

Pu, X.Y., Pan, J.C., Wang, H.Q., Zhang, W., Huang, Z.C., and Gu, Y.M. 2009. Characterization of fluoroquinolone-resistant *Shigella flexneri* in Hangzhou area of China. J. Antimicrob. Chemother. **63**(5): 917–920. doi:10.1093/jac/dkp087. PMID:19297378.

Robicsek, A., Strahilevitz, J., Sahm, D.F., Jacoby, G.A., and Hooper, D.C. 2006. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob. Agents Chemother. **50**(8): 2872–2874. doi:10.1128/AAC.01647-05. PMID:16870791.

Sabir, N., and Zafar, A. 2005. Cephalosporin resistant *Shigella flexneri* from a clinical isolate — a rare finding. J. Pak. Med. Assoc. **55**(12): 560–561. PMID:16438280.

Saeed, M.A., Haque, A., Ali, A., Mohsin, M., Bashir, S., Tariq, A., et al. 2009. A profile of drug resistance genes and integrons in *E. coli* causing surgical wound infections in the Faisalabad region of Pakistan. J. Antibiot. (Tokyo), **62**(6): 319–323. doi:10.1038/ja.2009.37. PMID:19444298.

Sharma, A., Singh, S.K., and Bajpai, D. 2010. Phenotypic and genotypic characterization of *Shigella* spp. with reference to its virulence genes and antibiogram analysis from river Narmada. Microbiol. Res. **165**(1): 33–42. doi:10.1016/j.micres.2008.02.002. PMID:19501495.

Sundström, L., Radström, P., Swedberg, G., and Sköld, O. 1988. Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of *dhfrV* and sulI and a recombination active locus of Tn*21*. Mol. Gen. Genet. **213**(2–3): 191–201. doi:10.1007/BF00339581. PMID:3054482.

Talukder, K.A., Islam, Z., Dutta, D.K., Islam, M.A., Khajanchi, B.K., Azmi, I.J., et al. 2006. Antibiotic resistance and genetic diversity of *Shigella sonnei* isolated from patients with diarrhoea between 1999 and 2003 in Bangladesh. J. Med. Microbiol. **55**(9): 1257–1263. doi:10.1099/jmm.0.46641-0. PMID:16914657.

Tamang, M.D., Oh, J.Y., Seol, S.Y., Kang, H.Y., Lee, J.C., Lee, Y.C., et al. 2007. Emergence of multidrug-resistant *Salmonella enterica* serovar Typhi associated with a class 1 integron carrying the *dfrA7* gene cassette in Nepal. Int. J. Antimicrob. Agents, **30**(4): 330–335. doi:10.1016/j.ijantimicag.2007.05.009. PMID:17629465.

Vinh, H., Baker, S., Campbell, J., Hoang, N.V., Loan, H.T., Chinh, M.T., et al. 2009. Rapid emergence of third generation cephalosporin resistant *Shigella* spp. in southern Vietnam. J. Med. Microbiol. **58**(2): 281–283. doi:10.1099/jmm.0.002949-0. PMID:19141753.

Whichard, J.M., Gay, K., Stevenson, J.E., Joyce, K.J., Cooper, K.L., Omondi, M., et al. 2007. Human *Salmonella* and concurrent decreased susceptibility to quinolones and extended-spectrum cephalosporins. Emerg. Infect. Dis. **13**(11): 1681–1688. doi:10.3201/eid1311.061438. PMID:18217551.

Wilson, G., Easow, J.M., Mukhopadhyay, C., and Shivananda, P.G. 2006. Isolation & antimicrobial susceptibility of *Shigella* from patients with acute gastroenteritis in western Nepal. Indian J. Med. Res. **123**(2): 145–150. PMID:16575113.

Xiong, Z., Li, T., Xu, Y., and Li, J. 2007. Detection of CTX-M-14 extended-spectrum β-lactamase in *Shigella sonnei* isolates from China. J. Infect. **55**(5): e125–e128. doi:10.1016/j.jinf.2007.07.017. PMID:17767959.

Zafar, A., Sabir, N., and Bhutta, Z.A. 2005. Frequency of isolation of shigella serogroups/serotypes and their antimicrobial susceptibility pattern in children from slum areas in Karachi. J. Pak. Med. Assoc. **55**(5): 184–188. PMID:15960281.

Zafar, A., Hasan, R., Nizami, S.Q., von Seidlein, L., Soofi, S., Ahsan, T., et al. 2009. Frequency of isolation of various subtypes and antimicrobial resistance of *Shigella* from urban slums of Karachi, Pakistan. Int. J. Infect. Dis. **13**(6): 668–672. doi:10.1016/j.ijid.2008.10.005. PMID:19135399.

Zong, Z., Partridge, S.R., Thomas, L., and Iredell, J.R. 2008. Dominance of $bla_{\text{CTX-M}}$ within an Australian extended-spectrum β-lactamase gene pool. Antimicrob. Agents Chemother. **52**(11): 4198–4202. doi:10.1128/AAC.00107-08. PMID:18725449.